Coinhibitory Pathways in Immunotherapy for Cancer

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Abstract

The immune system is capable of recognizing tumors and eliminates many early malignant cells. However, tumors evolve to evade immune attack, and the tumor microenvironment is immunosuppressive. Immune responses are regulated by a number of immunological checkpoints that promote protective immunity and maintain tolerance. T cell coinhibitory pathways restrict the strength and duration of immune responses, thereby limiting immune-mediated tissue damage, controlling resolution of inflammation, and maintaining tolerance to prevent autoimmunity. Tumors exploit these coinhibitory pathways to evade immune eradication. Blockade of the PD-1 and CTLA-4 checkpoints is proving to be an effective and durable cancer immunotherapy in a subset of patients with a variety of tumor types, and additional combinations are further improving response rates. In this review we discuss the immunoregulatory functions of coinhibitory pathways and their translation to effective immunotherapies for cancer.

INTRODUCTION

The critical role of coinhibitory pathways in preventing antitumor immunity is demonstrated by the remarkable efficacy of checkpoint blockade as a cancer immunotherapy, in which antibodies are used to block pathways that inhibit T cell responses to tumors. Seminal work demonstrating that blockade of the cytotoxic T lymphocyte antigen 4 (CTLA-4) inhibitory receptor promotes antitumor immune responses in mouse tumor models (1) led to the development (2) of an anti-CTLA-4 monoclonal antibody (ipilimumab) as a cancer therapy and its approval by the US Food and Drug Administration (FDA). Blockade of another checkpoint, the PD-1/PD-L1 inhibitory pathway, has led to striking clinical trial results (3, 4)—with 20–50% response rates across a range of cancers—and FDA approval of anti-PD-1 monoclonal antibodies for advanced melanoma, squamous and nonsquamous non–small cell lung cancer (NSCLC), and renal cell carcinoma. The broad effects and unprecedented durability of checkpoint blockade contrast with personalized cancer therapies that target a single oncogenic mutation but are limited by rapid development of resistance. The success of CTLA-4 and PD-1 checkpoint blockade has revitalized the field of cancer immunology and changed the approach to cancer therapy. Strong preclinical (5, 6) and clinical data demonstrate that combining PD-1- and CTLA-4-blocking antibodies with each other (7) or with other therapies can increase therapeutic efficacy and the percentage of responders; this combination was recently approved by the FDA for patients with BRAF V600 wild-type, unresectable, or metastatic melanoma. An important goal is to increase the proportion of patients who have objective and durable responses to checkpoint blockade by developing combination therapy approaches.

In this review we focus on advances in our understanding of checkpoint blockade and combination therapy strategies. We first will describe the current understanding of the mechanisms underlying checkpoint blockade, with a focus on the two most clinically relevant pathways thus far, the CTLA-4 and PD-1 pathways. Next, we review other inhibitory pathways that are attractive targets for immunotherapy and that have or soon will enter the clinic. We then discuss combination therapeutic strategies that are being examined preclinically and clinically. Finally, we consider important questions that need to be addressed to design more effective combination therapies involving checkpoint blockade.

IMMUNOEDITING

Belief in the immune system's importance in cancer pathogenesis has waxed and waned over the years. The idea that the immune system might limit tumor development (immunosurveillance) arose from the concept that the immune system can discriminate self from nonself and that tumors can be perceived as nonself or altered self. When athymic (nude) mice did not show an increased incidence of spontaneous or carcinogen-induced tumors compared to wild-type littermates, the idea of immune surveillance fell into disfavor. With improved mouse models of immunodeficiency (nude mice retain many aspects of innate immune function) and the discovery that the immune system not only can control development but also can shape the immunogenicity of tumor cells (8–10), cancer immunoediting emerged as a more nuanced concept that integrates the complex roles of immunity in cancer. In one context, the immune system provides surveillance against cancer by generating adaptive immune responses against cancer antigens; but, paradoxically, these may select for less-immunogenic tumor cells. In a second context, persistent inflammation at the site of a nascent tumor may promote disease progression through a mechanism in which tumors resemble wounds that do not heal (11).

The adaptive antitumor immune response can efficiently recognize neoantigens resulting from tumor-specific somatic mutations, antigens derived from oncogenic viruses, and antigens whose

expression is shared with tissues at immune-privileged sites (e.g., cancer testis antigens) (12). When cancer antigens yield peptides capable of binding to an individual's HLA alleles (neoepitopes), they can elicit $CD4^+$ T cell and $CD8^+$ T cell responses (13–15), as evidenced by the presence and prognostic significance of immune infiltrates in human tumors (16, 17). Clinically, host antitumor responses are evident in rare spontaneous tumor regressions (18), paraneoplastic syndromes, and autoimmune diseases such as scleroderma where somatic tumor mutations may serve as targets that trigger immune-mediated damage to normal tissues (19). The higher incidence of malignancies in immunosuppressed patients (20) and the pronounced and sustained responses to immunotherapies (21, 22) are further evidence of the clinical significance of antitumor immunity.

Antitumor immune responses are capable of controlling or eradicating the growth of malignant cells over prolonged periods of time; yet, tumors can evolve escape mechanisms under this selective pressure, ultimately selecting for malignant cells that thwart immune surveillance and manifest as cancer (23, 24). Escape mechanisms include tumor cell–intrinsic effects such as loss of tumor-antigen expression and induction of antiapoptotic pathways, rendering tumors resistant to cytotoxic immunity. Recent investigations have substantiated the concept of cancer immunoediting by demonstrating that genomic tumor alterations and neoantigen load are linked to immune responses (25, 26). Alternatively, escape may result from the establishment of an immunosuppressive state in the tumor microenvironment, with the production of indoleamine 2,3-dioxygenase (IDO), vascular endothelial growth factor (VEGF), and TGF-β; the recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs); and the promotion of the expression of coinhibitory molecules such as CTLA-4, PD-1, and PD-L1. Genomic studies in humans have emphasized the clinical significance of upregulation of immunosuppressive factors and increased expression of T cell coinhibitory molecules in cancer progression (27). The key role of these pathways in immune escape is the mechanistic basis of the clinical efficacy of therapeutic strategies that target immune checkpoint molecules.

COINHIBITORY PATHWAYS AND REGULATION OF TOLERANCE

The development of blocking antibodies to CTLA-4 and PD-1 as cancer therapies was based on insights into T cell activation. Our understanding of T cell costimulation has evolved from the two-signal model for T cell activation, which was proposed as a paradigm for activation of naive T cells but did not envision inhibitory signals. The fatal inflammatory phenotype of CTLA-4-deficient mice revealed the critical inhibitory function of CTLA-4 (28, 29) and demonstrated that T cell costimulatory receptors could deliver inhibitory, as well as stimulatory, second signals that modulate T cell receptor (TCR)-mediated T cell activation. The prefix co- in coinhibitory and costimulatory refers to how these second signals integrate with and modify the primary TCR signal. The striking phenotype of CTLA-4-deficient mice was also the first indication that coinhibitory receptors could regulate T cell tolerance. Many additional coinhibitory pathways are important to the induction and maintenance of T cell tolerance (**Figure 1**).

Coinhibitory pathways can regulate T cell tolerance in multiple ways. Coinhibitory receptors can control tolerance by restraining the initial activation of naive self-reactive T cells and/or responses of potentially pathogenic self-reactive effector T cells. In addition, coinhibitory pathways can regulate thymic-derived Tregs (tTregs) and Tregs generated at peripheral sites (pTregs) and contribute to functional specialization of Tregs. Some coinhibitory receptors mediate tTreg functions, whereas others inhibit tTreg subsets. For example, CTLA-4 limits Treg expansion but is a key mediator of Treg suppressive capacity (30). In contrast, PD-1 restrains the differentiation and function of T follicular regulatory cells, a specialized Treg subset that inhibits the germinal center reaction and antibody production (31).

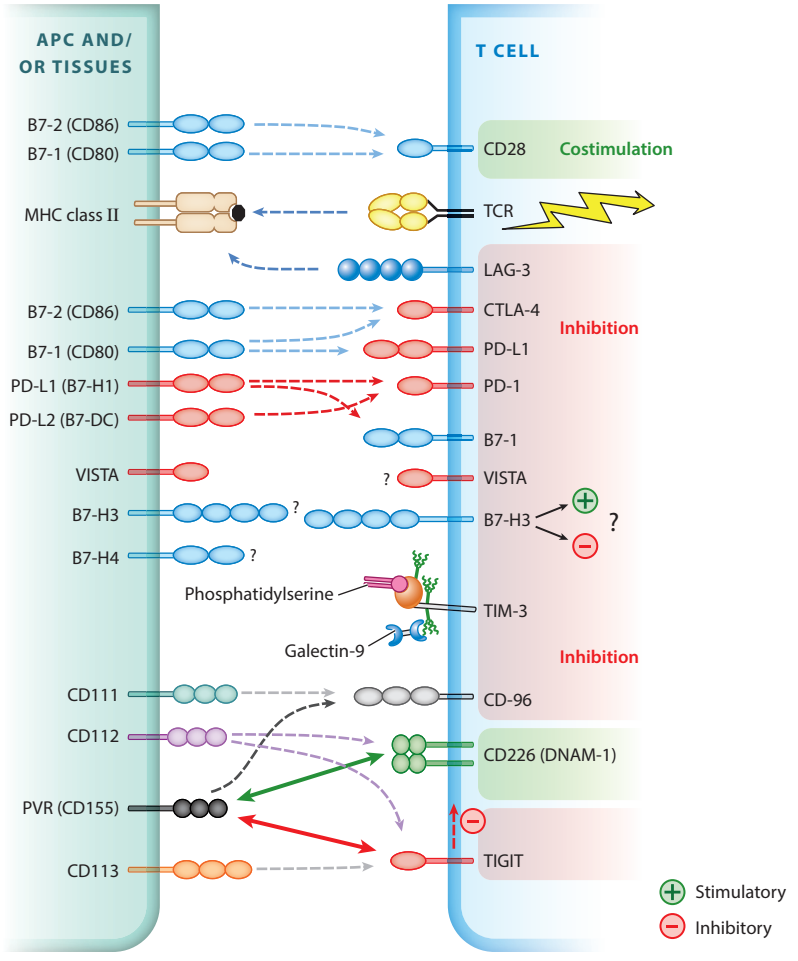


Figure 1

Overview of coinhibitory pathways. Upon T cell activation (mediated by TCR recognition of antigens presented on APCs and costimulation through the B7/CD28 interaction), many different pathways may inhibit the T cell response. The ligand interactions and precise mechanisms remain unknown for pathways such as VISTA, B7-H3, and B7-H4. Reminiscent of CD28 and CTLA-4, several receptors can have multiple binding partners. Some receptors are also expressed on NK cells (LAG-3, PD-1, TIM-3, CD96, TIGIT), and certain ligands are expressed on APCs and nonhematopoietic cells and in tumor tissues (PD-L1, PD-L2, B7-H3, VISTA, CD155, CD112). Abbreviations: APC, antigen-presenting cell; TIM-3, T cell–immunoglobulin–mucin domain 3; VISTA, V-domain immunoglobulin-containing suppressor of T cell activation.

Ligands for coinhibitory receptors can be expressed on nonhematopoietic cells, as well as antigen-presenting cells (APCs). Coinhibitory ligands on nonhematopoietic cells can mediate tissue tolerance, protecting target organs from attack by self-reactive T cells and immune-mediated damage (32). Although coinhibitory ligands are mainly thought to exert their effects by engaging coinhibitory receptors on T cells, the interactions between coinhibitory receptors and ligands can be bidirectional (33, 34). Despite coexpression on hematopoietic and nonhematopoietic cells, coinhibitory ligands are distinctly expressed in different tissue microenvironments, providing a

means for selective roles in tissue tolerance. Temporal as well as spatial differences in ligand expression may contribute to distinct immunoregulatory functions.

The large number of coinhibitory pathways raises questions about which pathways have overlapping and unique roles in T cell tolerance. Coinhibitory pathways may function at different stages of tolerance (induction versus maintenance) and different sites (lymphoid organs or target tissues). CTLA-4 exerts major effects during initial T cell priming in lymphoid organs because CD80 (B7-1) and CD86 (B7-2) are expressed mainly in lymphoid tissues. PD-1 can regulate primed T cells in tissues where its ligands are expressed (such as islet cells in pancreatic tissue), controlling maintenance of tolerance.

Tumors and a number of microbes that cause chronic infections appear to have exploited these tolerance mechanisms to evade eradication by the immune system. Blockade of coinhibitory pathways that regulate peripheral T cell tolerance has emerged as a promising strategy for cancer therapy but also carries risk of autoimmune adverse events, given the roles of these pathways in T cell tolerance. Combination therapies that target two coinhibitory receptors show enhanced antitumor efficacy but increase the potential for autoimmune sequelae, given the synergistic roles of some coinhibitory pathways, as discussed below.

CYTOTOXIC T LYMPHOCYTE ANTIGEN 4

Mechanism of Action

Anti-CTLA-4 antibodies were the first immune checkpoint blockade strategy to be clinically validated. The inhibitory receptor CTLA-4 is a key negative regulator of peripheral T cell responses (reviewed in 35 and 36). CTLA-4 is a homolog of the T cell costimulatory receptor CD28 and binds to the same ligands, CD80 and CD86 (37, 38), but with higher affinity (39). CTLA-4 is inducibly expressed upon activation of naive T cells ($CD4^+FoxP3^-$ and $CD8^+$) (40). CTLA-4 is constitutively expressed on $FoxP3^+$ regulatory T cells ($CD4^+FoxP3^+$) because CTLA-4 is a direct transcriptional target of Foxp3 (41). The critical role for CTLA-4 inhibitory signals in tolerance is demonstrated by the fatal T cell–mediated multiorgan inflammation that rapidly develops in CTLA-4 knockout mice, resembling systemic autoimmune disease (28, 29). CTLA-4 knockout mice die within two to four weeks of age even when rederived into germfree environments, suggesting the importance of self-antigens in driving the inflammatory phenotype. CTLA-4 blockade can exacerbate autoimmunity in mouse models of lupus, multiple sclerosis, and type 1 diabetes (42). Polymorphisms in the *ctla-4* gene are associated with human autoimmune diseases, including type 1 diabetes and Graves' disease (43). There are multiple splice variants of CTLA-4, and polymorphisms in the soluble form of CTLA-4 have been implicated in human autoimmune diseases. Heterozygous CTLA-4 mutations (which cause reduced CTLA-4 mRNA and protein expression) in humans can manifest as severe immune dysregulation, resulting in defective Treg function, hyperproliferative T cells, lymphocytic infiltrates in nonlymphoid organs, and autoantibodies (44, 45). A similar clinical syndrome has been linked to mutations in the gene encoding for the lipopolysaccharide-responsive and beige-like anchor (LRBA) protein, which result in LRBA deficiency and impaired CTLA-4 surface expression on effector T cells and Tregs owing to alterations in endosomal trafficking (46). Taken together, these findings underscore the critical role for CTLA-4 in controlling self-reactive T cells and T cell homeostasis.

CTLA-4 exerts its critical immunoregulatory functions by controlling $CD4^+FoxP3^-$ and $CD8^+$ T cells, as well as Tregs. CTLA-4 terminates responses of activated T cells and mediates Treg suppressive function (30). Although the critical roles for CTLA-4 in regulating the balance between T cell activation and tolerance are well established, many mechanistic questions

remain about how CTLA-4 mediates its inhibitory functions. CTLA-4 is believed to inhibit T cell responses in two major ways (**Figure 2*a***). (*a*) CTLA-4 can inhibit T cell activation intrinsically within CTLA-4 expressing cells, either by outcompeting CD28 for B7 ligand binding or by recruiting phosphatases to the cytoplasmic domain of CTLA-4, resulting in reduced TCR and CD28 signaling. Kong et al. (47) have shown that the kinase PKC-η constitutively binds to the CTLA-4 cytoplasmic domain in Tregs and that this interaction is an important mediator of CTLA-4 function in these cells. (*b*) In addition, CTLA-4 on one T cell may inhibit activation of other T cells in a cell-extrinsic fashion by reducing CD80 and CD86 expression on APCs, either indirectly by causing their downregulation (through cytokines such as IL-10) (30) or directly by removing them from APCs (transendocytosis), thereby reducing their availability for CD28 engagement (48). In addition, CTLA-4 may signal into dendritic cells by binding to CD80/CD86 and inducing the tryptophan-degrading enzyme IDO, which leads to suppression of T cell responses (33).

CTLA-4 as a Therapeutic Target

Seminal studies demonstrating that anti-CTLA-4 blocking antibodies could promote antitumor immune responses, regression of established tumors, and long-lived immunity in mouse solid and hematologic tumor models (1, 6, 49) led to the clinical development of anti-CTLA-4 blocking antibodies for cancer therapy. In murine models, anti-CTLA-4 monotherapy led to tumor regressions more frequently in immunogenic tumors and in settings of a lower tumor burden. Anti-CTLA-4 blocking antibodies result in an increased ratio of $CD4^+$ and $CD8^+$ effector T cells to $FoxP3^+$ Tregs in tumor infiltrates. Effector T cells and Tregs are both targeted with CTLA-4 antibodies, suggesting multiple mechanisms of action. $CD8^+$ T cells appear to always be required for therapeutic benefit of anti-CTLA-4 antibodies, whereas $CD4^+$ T cells are required in some, but not all, mouse tumor models. This likely reflects the critical role of cytotoxic $CD8^+$ T cells in antitumor immunity through the direct killing of tumor cells. Recent work suggests that the therapeutic effect of anti-CTLA-4 antibodies may be due to not only blocking CTLA-4 interaction with its ligands, but also depletion of intratumoral Tregs (the highest expressers of CTLA-4) through Fc receptor–mediated, antibody-dependent cellular cytotoxicity (49). In melanoma models, T cell–dependent depigmentation developed in a substantial proportion of surviving mice, indicating the delicate balance between tolerance and autoimmunity and the potential for immune-related adverse events related to anti-CTLA-4 therapy.

The clinical evaluation of the anti-CTLA-4 monoclonal antibodies ipilimumab and tremelimumab initiated a new era in immunotherapy. In a landmark study, Hodi and colleagues (2) demonstrated that ipilimumab mediates a statistically significant overall survival (OS) advantage in patients with previously treated metastatic melanoma. Patients who received ipilimumab alone or gp100 peptide vaccine in combination with ipilimumab had a median survival of 10.1 months compared to 6.4 months for patients receiving the gp100 peptide vaccine alone (2). A second phase 3 trial comparing dacarbazine alone to ipilimumab plus dacarbazine demonstrated significantly higher survival rates for this combination therapy at 1 year (47.3% versus 36.3%) and at 2 years (28.5% versus 17.9%) among patients with previously untreated metastatic melanoma (50). Based on these studies, ipilimumab was approved for metastatic melanoma in the United States and Europe in 2011. Despite early efficacy signals (51, 52), when compared directly to chemotherapy, tremelimumab failed to achieve a significant survival advantage as a first-line agent for metastatic melanoma (53), perhaps owing to the use of an Fc that does not mediate antibody-dependent cell cytotoxicity and Treg depletion or to other aspects of the clinical trial design (49, 54).

CTLA-4 blockade is also being explored in other solid tumors and has yielded efficacy signals as a single agent, albeit with more modest responses. A phase 2 trial of ipilimumab for metastatic

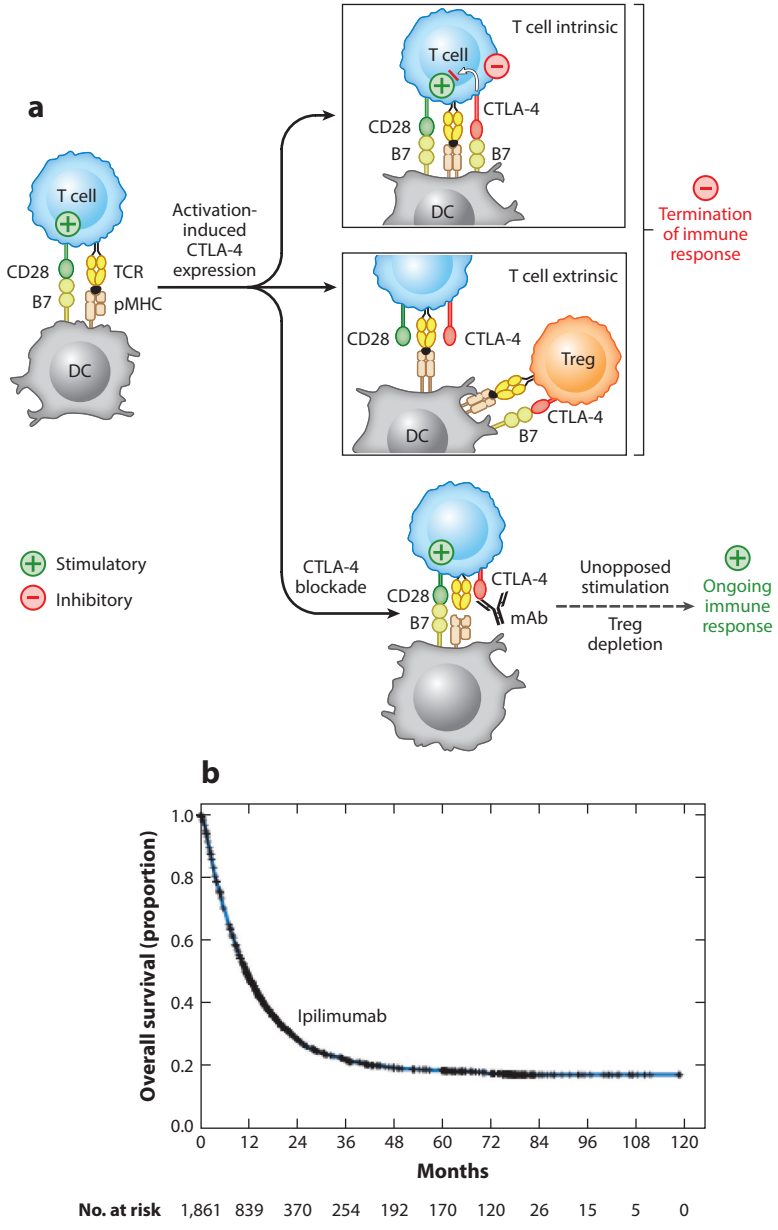


Figure 2

Mechanisms of the CTLA-4 pathway and effect of CTLA-4 blockade. (*a*) CTLA-4 is induced by T cell activation and can inhibit the immune response in a T cell–intrinsic fashion by intracellular signals that inhibit TCR and CD28 signaling or in a T cell–extrinsic fashion by reducing expression of B7 (CD80 and CD86) on APCs. Anti-CTLA-4 monoclonal antibody prevents the interaction between CTLA-4 and B7 and may deplete Tregs in the tumor microenvironment, thereby allowing CD28 signaling without CTLA-4 opposition and promoting an ongoing immune response. (*b*) A meta-analysis of patients with advanced melanoma who received ipilimumab and were followed up for 3 to 10 years demonstrated approximately 20% long-term overall survival, underscoring the durability of tumor control by immunotherapy (67). Abbreviations: APC, antigen-presenting cell; mAb, monoclonal antibody; TCR, T cell receptor.

renal cell carcinoma reported partial responses in 12.5% of patients (55), whereas a pilot trial in metastatic hormone-refractory prostate cancer found prostate-specific antigen (PSA) declined ≥50% in 2 of 14 patients (56). Nonetheless, a phase 3 randomized trial of ipilimumab versus placebo after radiation therapy in patients with metastatic, castration-resistant prostate carcinoma (mCRPC) failed to show a survival advantage (57). A randomized phase 2 trial of ipilimumab combined with paclitaxel and carboplatin chemotherapy showed a modest increase in progression-free survival (PFS) compared to chemotherapy alone in patients with advanced NSCLC (58). A small trial of CTLA-4 blockade after vaccination in patients with advanced ovarian cancer provided preliminary evidence of antitumor activity (59). Tremelimumab yielded partial-response rates of 17.6% in hepatocellular carcinoma (60), 2.2% in colorectal cancer, and 7% in mesothelioma (61, 62). Results from several ongoing or recently completed trials evaluating ipilimumab in multiple tumor types are expected to shed further light on the therapeutic potential of CTLA-4 blockade.

The clinical experience with anti-CTLA-4 blockade established several unique principles distinguishing checkpoint blockade from conventional therapies. A distinct set of immune-related adverse events (irAEs) was observed in up to 60% of patients, manifesting as inflammatory pathology in several organ systems, most notably the gastrointestinal tract, skin, and endocrine glands, underscoring the importance of CTLA-4 in the maintenance of peripheral tolerance. Interestingly, CTLA-4 was unexpectedly found to be expressed in the pituitary gland, suggesting that inflammation and endocrine dysfunction (hypophysitis) may involve a direct targeting of this tissue with the therapeutic antibody (63). Although such irAEs can be severe and even fatal, in the majority of patients they are reversible with corticosteroids and anti-inflammatory therapies, and the development of algorithms for prompt recognition and intervention has facilitated the successful management of these toxicities in immune checkpoint blockade. Further, it became clear that traditional measures of treatment response [such as the Response Evaluation Criteria in Solid Tumors (RECIST)], which are based on the presence and progression or regression of lesions following chemotherapy or radiation, were inadequate or even misleading in assessing cancer immunotherapy response. Active immune infiltrates in the tumor sometimes led to what appeared as initial disease progression in pre-existing masses or the development of new, previously undetected lesions (64). Consequently, immune-related response criteria were established to appropriately evaluate the unique response patterns of ipilimumab and other immunotherapies (65, 66). The most striking effect of CTLA-4 blockade is the ability to induce long-lasting tumor regressions. In a meta-analysis of 1,861 patients with unresectable or metastatic melanoma who received ipilimumab in phase 2 or 3 trials with follow-up as long as 10 years, the 3-year survival rates ranged from 20% for previously treated patients to 26% for treatment-naive patients. The survival curve reached a plateau of 21% around year 3 and remained stable for the duration of available follow-up (67) (**Figure 2*b***).

PROGRAMMED DEATH-1

Mechanism of Action

Programmed death-1 (PD-1) is a transmembrane protein receptor that functions as a major negative immune regulator (68), controlling T cell activation, T cell exhaustion, T cell tolerance, and resolution of inflammation. PD-1 appears within 24 hours of T cell activation and declines with the clearance of antigen (69–71). NFATc1, IRF9, and Notch promote PD-1 transcription, whereas T-bet acts as a transcriptional repressor of PD-1 (72–74).

When T cells are repetitively stimulated by antigen (as with chronic infection or cancer), the level of PD-1 expression remains high and T cells undergo epigenetic modifications and changes

in transcription factor expression, leading to differentiation into a state termed exhaustion. In addition to expressing PD-1, exhausted T cells can express multiple inhibitory receptors, making them susceptible to inhibition by multiple checkpoint pathways (75).

PD-1 can also be expressed on natural killer (NK) cells and B cells, limiting their effector functions (76–78), on macrophages, inhibiting innate immunity during sepsis, and on Tregs and T follicular regulatory cells, modulating their induction and function (79). Estrogen can stimulate PD-1 expression on T cells and APCs (80).

PD-1 has two ligands, programmed death-ligand 1 (PD-L1; also known as CD274 and B7-H1) (81, 82) and programmed death-ligand 2 (PD-L2, also known as CD273 and B7-DC) (83). Both are expressed on APCs as well as other hematopoietic and nonhematopoietic cell types (32). PD-L1 is more broadly expressed than PD-L2 (84). PD-L1 is widely expressed on hematopoietic and nonhematopoietic cells (including epithelial cells, vascular endothelial cells, and stromal cells) and is induced by proinflammatory cytokines (including type I and type II interferons, TNF-α, and VEGF). PD-L2 is expressed mainly on dendritic cells and macrophages and induced by many of the same cytokines as PD-L1, but IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are the most potent stimuli for PD-L2 expression. The induction of PD-1 ligands by proinflammatory signals in tissues may serve as a negative feedback mechanism that downregulates effector T cell activity, thereby protecting tissues from excessive immune damage or tumors from immune attack (84–86).

Adding to the complexity of the PD-1 pathway, PD-L1 can serve as a receptor for CD80 expressed on T cells, delivering yet another inhibitory signal (87, 88). RGMb (repulsive guidance molecule b) is a second receptor that binds to PD-L2 but not PD-L1, and it plays an important role in pulmonary tolerance (89). Further work is needed to understand how these newer pathways regulate antitumor immunity.

Although PD-1 was discovered in a T cell hybridoma undergoing TCR activation–induced cell death (90) (hence the name programmed death-1), it does not directly activate caspases and a cell death pathway. PD-L1 or PD-L2 binding to PD-1 results in tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of the tyrosine phosphatase SHP-2, leading to reduced phosphorylation of TCR signaling molecules (84), attenuated signals downstream of TCR stimulation, and decreased T cell activation and cytokine production (**Figure 3*a***). Signaling through PD-1 inhibits phosphatidylinositol 3-kinase activity (91), resulting in reduced activation of Akt and reduced expression of transcription factors important for effector function [Gata3, Tbx21 (T-bet), and Eomes] (92). PD-1 signals can stimulate expression of proteins that impair T cell proliferation and cytokine production [e.g., Batf (93)] and reduce antiapoptotic gene expression while increasing proapoptotic gene expression [e.g., Bcl2l1 (Bcl-xl) and Bcl2l11 (Bim), respectively] (94), thereby decreasing T cell survival. PD-1 signaling also can decrease the production of cytotoxic molecules by T cells, reducing their killing capacity. In addition, PD-1 signals alter T cell motility and length of engagement with dendritic cells and target cells (95–97). PD-1 signaling alters T cell metabolism, reprogramming it by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation (91, 98). Finally, the PD-1 pathway can promote the induction of regulatory T cells from naive or Th cells (pTregs), which suppress effector T cell functions (79). Thus, PD-1 signaling can modulate T cells in multiple ways that synergize to suppress immune responses (81, 83, 99).

PD-1 inhibitory signals control peripheral tolerance in several ways. PD-1:PD-L1 interactions regulate both the induction and maintenance of peripheral T cell tolerance. PD-1:PD-L1 interactions inhibit initial activation of self-reactive $CD4^+$ and $CD8^+$ T cells, and self-reactive effector $CD4^+$ and $CD8^+$ T cell responses. The PD-1 pathway restrains self-reactive T cells in target organs, maintaining tolerance in tissues and protecting them from immunopathology. Mice lacking PD-1 or its ligands do not spontaneously develop autoimmune disease. However,

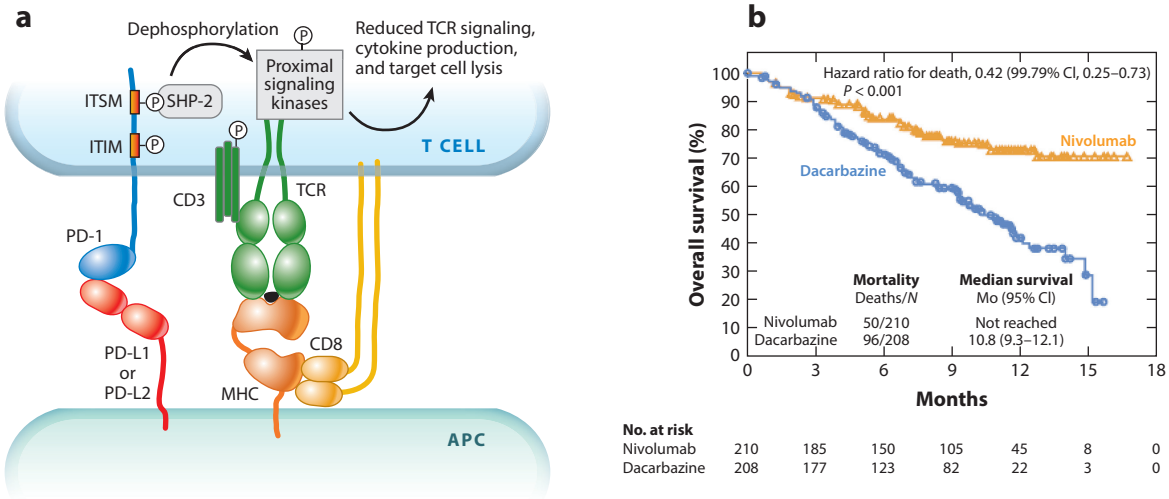


Figure 3

Mechanisms of the PD-1 pathway and effect of PD-1/PD-L1 blockade. (*a*) PD-1 is inducibly expressed on T cells. Engagement of PD-1 by PD-L1 or PD-L2 results in reduced phosphorylation of TCR signaling molecules, thereby limiting T cell effector functions. (*b*) A phase 3 study in patients with untreated BRAF mutation–negative melanoma showed a significant benefit with respect to overall survival for PD-1 blockade compared to chemotherapy (110).

PD-1 deficiency or blockade can accelerate and exacerbate autoimmunity. The milder phenotype of PD-1 knockout mice compared to CTLA-4 knockout mice predicts that PD-1 blockade may show a more favorable side effect profile compared to CTLA-4 blockade.

In the tumor microenvironment, tumor cells can express PD-L1 and/or PD-L2, as do many other cells (e.g., fibroblasts, endothelial cells, immune cells). Multiple, reinforcing mechanisms drive PD-L1 and PD-L2 expression on tumor cells, including upregulation by cytokines, chromosomal copy gain (amplification of chromosome 9p24, which contains PD-L1 and PD-L2), and Epstein Barr Virus LMP protein-driven expression. Moreover, oncogenic lesions such as lung carcinoma cells with activated epidermal growth factor receptor signaling can also drive PD-L1 expression (100). In multiple preclinical models PD-1/PD-L1 blockade enhanced antitumor T cell cytotoxicity, proinflammatory cytokine production, and proliferation, thereby promoting tumor destruction. Based on the kinetics of PD-1 expression in later phases of T cell activation and PD-L1 expression on tumor cells, the putative mechanism of PD-1 blockade is to release the negative regulation of T cells within the tumor microenvironment rather than at the priming step in lymphoid organs. PD-1 pathway blockade also can alter metabolism in the tumor microenvironment. Changes in metabolism of T cells and tumor cells regulate the antitumor immune response. Highly glycolytic tumor cells reduce glucose levels in the tumor microenvironment, thereby reducing the ability of tumor-infiltrating lymphocytes (TILs) to mount an antitumor immune response. PD-L1 on the tumor may promote Akt/mTOR activation and glycolysis in tumor cells (101). PD-1 blockade increased concentrations of glucose in the tumor microenvironment, and rates of glycolysis and T cells produced higher levels of IFN-γ (101).

PD-1 is expressed more frequently on tumor-specific TILs than on the bulk TIL population in the tumor microenvironment. TILs from patients who respond to PD-1 blockade are more clonal prior to therapy than those from patients who do not respond, suggesting a nascent antigen-specific

response that is restrained. TIL clonality is often used as a measure for antigen-specific T cell responses, because T cells proliferate in response to their cognate antigen (e.g., clonal expansion). A higher mutational burden is associated with a better response to PD-1 blockade. Nonsynonymous mutations likely give rise to neoantigens and induce tumor-specific T cells (102). TILs in tumors from patients given PD-1 blockade show increased proliferation and cytokine production as well as better killing capacity.

PD-1 as a Therapeutic Target

Several trials of PD-1 monoclonal antibodies have been conducted in advanced melanoma and lung cancer, leading to FDA approval in 2014 of nivolumab and pembrolizumab for advanced refractory melanoma and in 2015 of nivolumab for advanced refractory squamous and nonsquamous NSCLC and renal cell carcinoma. Additional trials in multiple tumor types have now been reported, and several generalizations can be made based on the results of these trials: (*a*) PD-1/PD-L1 therapy works in a substantial number of tumor types. (*b*) Certain tumor types, such as prostate and colon, have low response rates. (*c*) In many but not all responding tumor types, response to PD-1 pathway blockade is more frequent when PD-L1 is expressed in the tumor microenvironment (on either tumor cells or nontumor cells, or both); however, responses to PD-1 blockade can occur even when PD-L1 expression is not observed, and PD-L1 expression is not needed for response to combined PD-1 and CTLA-4 therapy. (*d*) Responses can be durable. (*e*) The safety profile is tolerable, and severe irAEs have been observed in approximately 10% of patients treated with PD-1 blockade, compared to 15–30% of patients treated with anti-CTLA-4 blocking antibodies. Adverse events following PD-1 and PD-L1 monoclonal antibody treatment have been reviewed and amelioration algorithms proposed (103). (*f*) Response rates are generally higher in tumor types with higher mutational burdens. (*g*) An irAE does not preclude beneficial disease response. (*h*) If disease relapses after drug discontinuation, the drug may be readministered in some instances and disease again controlled (104). (*i*) PD-1/PD-L1 therapy is a reasonably safe and effective foundation therapy, and combination with multiple other therapies may improve response rates. Some pivotal clinical trials that have led to these conclusions are discussed below.

Remarkably, the first evaluation study of an anti-PD-1 monoclonal antibody (nivolumab) demonstrated clinical effects in patients with melanoma, renal cell carcinoma, NSCLC, and colorectal cancer (105). This was followed by a dose-escalation trial administering multiple doses of nivolumab to 296 patients with advanced malignancies. Objective response rates (ORRs) of 28%, 27%, and 18% were seen in melanoma, renal cell cancer, and NSCLC patients, respectively (4). Responses lasted at least one year in the majority of patients. Immunohistochemical analysis suggested that PD-L1 expression on the tumor correlated with response. At the same time, a PD-L1 monoclonal antibody (BMS-936559) was reported to give durable tumor regressions, yielding ORRs ranging from 6% to 17% in renal cell carcinoma, NSCLC, ovarian cancer, and melanoma (3). These findings stimulated rapid development of PD-1 and PD-L1 monoclonal antibodies by multiple pharmaceutical companies (reviewed in 106) and initiation of phase 2 and 3 trials in multiple tumor types.

A phase 2 trial randomizing between pembrolizumab (anti-PD-1) and investigator-choice chemotherapy in patients with melanoma refractory to ipilimumab (and if applicable, BRAF inhibition) demonstrated a significantly higher 6-month progression-free survival (PFS) rate of 38% in the pembrolizumab group compared to 16% in the chemotherapy group, establishing the utility of PD-1 blockade in ipilimumab-refractory melanoma (107). Furthermore, a randomized comparison of PD-1 and CTLA-4 blockers in advanced melanoma clearly demonstrated the superiority of pembrolizumab over ipilimumab, with response rates on the order of 30% for pembrolizumab,

compared to 11.9% for ipilimumab. Additionally, the rate of irAEs was higher with ipilimumab (19.9%) compared to pembrolizumab administered at intervals of 2 (13.3%) or 3 (10.1%) weeks (108). When patients with advanced melanoma were randomized to receive pembrolizumab every 2 weeks or every 3 weeks, the former group had the highest ORRs (52%); further supporting PD-1 blockade as a salvage strategy, there was no significant difference in response between patients who had previously received ipilimumab and those who had not (109). The efficacy of PD-1 blockade in the up-front therapy of metastatic melanoma was established in a phase 3 evaluation of nivolumab compared to dacarbazine chemotherapy in previously untreated metastatic melanoma without a BRAF mutation. Here nivolumab was associated with significant improvements in OS at 1 year (72.9% in the nivolumab group versus 42.1% with dacarbazine), and ORR was 40% with nivolumab and 13.9% with dacarbazine (110) (**Figure 3*b***).

Nivolumab was FDA approved for refractory metastatic squamous cell NSCLC in 2015, based on results of a phase 3 comparison between nivolumab and docetaxel chemotherapy. In this trial, nivolumab (3 mg/kg every 2 weeks) resulted in significantly prolonged median OS (9.2 months versus 6.0 months), a higher OS rate at 1 year (42% versus 24%), a better response rate (20% versus 9%), and a low rate of higher-grade irAEs. Interestingly, PD-L1 expression in this study was neither prognostic nor predictive of benefit (111). Evidence for activity in relapsed nonsquamous NSCLC also has been presented, indicating that PD-1 blockade may be broadly active in lung cancer. Mutational burden may be associated with the likelihood of response in these smoking-related tumors, raising the possibility that neoantigens or other characteristics of tumor cells subject to DNA damage play critical roles in the therapeutic effects (112).

The efficacy of PD-1 pathway blockade is being established in an increasingly wide range of malignancies. A phase 3 study of nivolumab in 821 patients with metastatic renal cell carcinoma confirmed antitumor activity in this disease, with an ORR of 25% and a median PFS of 4.6 months (113). Overall survival was longer (25.0 versus 19.6 months), and the rate of grade 3 or 4 adverse events was lower with nivolumab than with everolimus (19% versus 37%), suggesting that nivolumab may prove superior to the standard of care for patients with recurrent renal cell carcinoma. An initial study of pidilizumab (CT-011, anti-PD-1) in several advanced hematologic malignancies showed clinical benefit in 33% of patients, with one complete response in a patient with non-Hodgkin lymphoma (114). The rationale to further explore PD-1 blockade in Hodgkin lymphoma (HL) was driven by preclinical evidence. The genes encoding PD-L1 and PD-L2 are key targets of a recurrent genetic abnormality (chromosome 9p24.1 amplification) in HL, driving PD-L1 expression on malignant Reed-Sternberg cells directly and through a JAK-STAT-mediated mechanism, thereby contributing to the extensive but ineffective immune-cell infiltrate characteristic of HL (115). A phase 1 study of 23 heavily pretreated patients with relapsed or refractory HL yielded a dramatic response rate of 87%, including 17% complete response, 70% partial response, and 13% stable disease; PFS at 24 weeks was 86% (116). Nivolumab in this setting had an acceptable safety profile, with 22% of patients experiencing some significant drug-related adverse events, though these were typically mild. Furthermore, the authors confirmed PD-L1 and PD-L2 expression and gene amplification in all available samples and low-level PD-1 expression on tumor-infiltrating T cells (116).

Atezolizumab (MPDL3280A, anti-PD-L1) has been explored in metastatic urothelial bladder cancer, given the poor outcomes and tolerance of chemotherapy associated with this entity. This study incorporated PD-L1 analysis of tumors and tumor-infiltrating immune cells by immunohistochemistry and demonstrated that expression is not required for responses but is associated with higher ORRs. Overall there was noteworthy clinical activity with 43% ORRs in patients whose tumors (or cellular infiltrates) expressed PD-L1, leading to FDA breakthrough status of the drug in urothelial bladder cancer and further clinical investigation (117).

Initial evidence of antitumor activity has also been observed in squamous cell head and neck, nasopharyngeal, Merkel cell, ovarian, and hepatocellular carcinomas, suggesting that viral proteins (HPV, EBV, HCV, HBV) may be critical determinants of clinical response (118). A variety of malignancies with DNA repair defects, such as microsatellite-instability colorectal cancer, respond well to PD-1 therapy, suggesting that mutational burden can predict clinical response (119). Activity has also been reported in mesothelioma and in gastric, triple negative breast, anal, and biliary cancers. Many clinical trials are underway to better define the antitumor activity of PD-1 or PD-L1 blockade in these diseases and other cancers.

COMBINATION OF CTLA-4 AND PD-1 PATHWAY BLOCKADE

CTLA-4 and PD-1 are nonredundant pathways for the inhibition of T cell activation and function. CTLA-4 blockade affects initial T cell priming in secondary lymphoid organs and also mediates depletion of Tregs via mechanisms of antibody-dependent, cell-mediated cytotoxicity in the tumor microenvironment (120). PD-1 blockade not only affects T cells later during activation in lymphoid organs but also affects T cell responses in tissues and tumors where both ligands can be expressed, enabling PD-1 pathway blockade to relieve suppression in the tumor microenvironment. CTLA-4 blockade is thought to drive tumor-specific T cells into the tumor microenvironment where T cell–mediated IFN-γ production upregulates PD-L1 expression in tumor tissues as well as on immune cells, suggesting that blockade of the PD-1 axis may be more effective in the setting of CTLA-4 blockade. The benefit of combined pathway blockade was supported by preclinical studies that demonstrated increased efficacy when PD-1 and CTLA-4 blockade were combined in a mouse model of MC38 colon adenocarcinoma (6) and when they were combined in a B16 mouse melanoma model to augment immune response to tumor vaccines expressing either GM-CSF or Flt-3 ligand (5).

The clinical combination has been rigorously evaluated in melanoma. An initial dose-escalation trial in advanced melanoma combined nivolumab and ipilimumab in either a concurrent or a sequenced regimen. The concurrent regimen was superior and associated with high ORRs (40% for all patients) but also a higher incidence of serious irAEs, although events were qualitatively similar to those observed with ipilimumab alone and mostly reversible (7). A double-blind randomized study in which patients with untreated melanoma received ipilimumab either with nivolumab or with placebo demonstrated the superiority of combination therapy over ipilimumab plus placebo, with an ORR of 61% compared to 11% in BRAF wild-type melanoma and an ORR of 52% versus 10% in BRAF mutant tumors. A complete response rate of 22% was observed with the ipilimumab-nivolumab combination, whereas ipilimumab monotherapy mediated no complete responses (21). A double-blind, randomized evaluation comparing nivolumab alone, ipilimumab alone, and the combination found that in patients with previously untreated melanoma, nivolumab alone or in combination with ipilimumab resulted in significantly longer PFS and higher ORRs, although the benefit of adding ipilimumab to nivolumab was most notable in PD-L1-negative tumors. The high rate of irAEs in the combination group, 55%, suggests that assessment of PD-L1 status may be a good guide for therapeutic decisions with respect to the risk-benefit ratio of combination therapy (121), although PD-L1 expression itself may vary during the course of therapy, depending on the mix of cytokines produced as the immune response develops (36).

OTHER COINHIBITORY PATHWAYS AS TARGETS FOR CANCER IMMUNOTHERAPY

The success of anti-CTLA-4 and anti-PD-1 cancer immunotherapy has led to the search for other coinhibitory pathways that can be targeted. There is a large number of coinhibitory molecules,

including LAG-3, TIM-3 (T cell–immunoglobulin–mucin domain 3), TIGIT, VISTA (V-domain immunoglobulin-containing suppressor of T cell activation), CD244, CD160, HHLA2, BTNL2, B7-H3, and B7-H4 (**Figure 1**). Many coinhibitory receptors are coexpressed with PD-1 on dysfunctional T cells in tumors. There are subsets of dysfunctional TILs, and TILs expressing multiple coinhibitory receptors are more dysfunctional than TILs expressing PD-1 alone (75, 123). These findings stimulated studies to test the potential for combination therapy of anti-PD-1 or anti-PD-L1 with other coinhibitory molecules, with the goal of increasing the proportion of patients who have objective and durable responses to checkpoint blockade. We review here several coinhibitory pathways that have been demonstrated to synergize with PD-1 pathway blockade.

Lymphocyte Activation Gene-3

LAG-3 is a CD4 homolog that binds MHC class II molecules (its only known ligand) with higher affinity than CD4 (124). LAG-3 is expressed on activated $CD4^+$ and $CD8^+$ T cells, thymic and peripherally induced Tregs, NK cells, NKT cells, B cells, and plasmacytoid dendritic cells (125).

LAG-3 negatively regulates $CD4^+$ and $CD8^+$ T cell proliferation, function, and homeostasis (126, 127). The inhibitory function of LAG-3 depends on signaling through its cytoplasmic domain KIEELE motif (128), but downstream signaling pathways underlying LAG-3 function are not yet clear. LAG-3 inhibitory signals regulate peripheral T cell tolerance by controlling self-reactive effector cells and Tregs (129–131). LAG-3 deficiency or antibody blockade can markedly accelerate NOD diabetes onset (132). LAG-3 limits the onset and progression of diabetes in NOD mice by inhibiting self-reactive $CD4^+$ and $CD8^+$ T cell infiltration and expansion in the islets but without affecting their cytokine profiles. LAG-3 also is required for maximal Treg function, as transfection of LAG-3 into T cells can confer regulatory functions (129). LAG-3 blockade or deficiency impairs the responsiveness of conventional T cells to suppression by Tregs and can impair generation of peripherally induced Tregs (133).

Whereas mice deficient in LAG-3 alone do not develop spontaneous autoimmunity, mice lacking both LAG-3 and PD-1 develop lethal, systemic autoimmunity (134), highlighting the synergy between these two pathways in controlling T cell tolerance. T cells in tumors and chronic viral infection often coexpress PD-1 and LAG-3, and combined blockade of PD-1 and LAG-3 in tumor models or the lymphocytic choriomeningitis virus (LCMV) chronic infection model has a greater therapeutic benefit than blockade of either alone (75, 135). Notably, LAG-3 and PD-1 coblockade can induce almost complete remission in ~80% of mice with preexisting MC38 or SA1N tumors, in contrast to only ~15% with the single blockers. Studies of TILs from patients with ovarian cancer showed that NY-ESO-1 antigen-specific $LAG3^+/PD\text{-}1^+$ $CD8^+$ T cells were impaired in their ability to respond to antigen stimulation but that combined LAG-3 and PD-1 blockade could restore T cell responsiveness to a greater extent than a single-agent blockade. Together, these data suggest that in tumors where LAG-3 and PD-1 are coexpressed on TILs, dual therapy may increase response rates and/or effectiveness of immunotherapy (136). The anti-LAG3 antibody BMS986016 has entered a phase 1 cancer clinical trial and includes a nivolumab (anti-PD-1) combination arm (NCT01968109). A LAG3-Fc fusion protein, IMP321 (Immutep), also is in clinical development and has been shown to increase tumor-reactive T cell responses in a phase I clinical trial (137).

T Cell Immunoglobulin and ITIM Domain

The coinhibitory receptor TIGIT (also called Vsig9, Vstm3, or WUCAM) is an immunoglobulin superfamily member (34, 138–140) in a pathway with stimulatory and inhibitory receptors and

multiple ligands (which are nectin and nectin-like proteins) that bind to these receptors (reviewed in 141). TIGIT and the costimulatory receptor CD226 (DNAM-1) share the ligands CD155 (PVR, NECL5) and CD112 (PVRL2, nectin-2), but TIGIT is their high-affinity receptor. TIGIT also binds to CD113 (PVRL3, nectin-3), and CD155 also binds to the inhibitory receptor CD96 (TACTILE). Mouse, but not human, CD96 binds to CD111 (nectin-1, PRVRL1).

TIGIT is induced upon activation of naive T cells and expressed by subsets of FoxP3$^+$ Tregs, activated and memory CD4$^+$ and CD8$^+$ T cells, and NK cells. CD155 and CD112 are both expressed on APCs, and on many tumor cell types (141). CD155 also is expressed on a variety of nonhematopoietic cell types. CD155 on tumor cells may promote tumorigenicity by contributing to loss of contact inhibition. CD155 expression is correlated with cancer invasiveness and metastasis (141).

TIGIT can exert immunosuppressive effects by several mechanisms. TIGIT can induce IL-10 and inhibit IL-12 production in dendritic cells by engaging CD155 on dendritic cells and thereby inhibit Th1 responses (34). TIGIT also can exert cell-intrinsic inhibitory effects in T cells (142). In addition, TIGIT can interact with CD226 in *cis* and prevent CD226 homodimerization (143).

TIGIT inhibits CD4$^+$ and CD8$^+$ T cell priming and regulates T cell tolerance by controlling self-reactive T cell activation and Tregs (142, 144). Mice lacking TIGIT do not develop spontaneous autoimmunity, but TIGIT blockade or deficiency can exacerbate autoimmune disease in mouse models of experimental autoimmune encephalomyelitis (EAE). Agonistic TIGIT monoclonal antibodies and TIGIT Fc can suppress EAE and CD4$^+$ T cell–dependent DTH responses. TIGIT$^+$ Tregs suppress proinflammatory Th1 and Th17 cells but not Th2 responses, and ligation of TIGIT on Tregs induces fibrinogen-like protein 2 (Fgl2), which promotes Treg-mediated suppression of Th1 and Th17 cells, while preventing suppression of Th2 cytokine production (144).

TIGIT also inhibits NK cell cytotoxicity. TIGIT has a cytoplasmic tail with an ITIM motif and an immunoglobulin tail tyrosine (ITT)-like motif (141). The ITT-like motif plays a critical role in TIGIT inhibitory signaling in NK cells as TIGIT binding to CD155 induces phosphorylation of the ITT motif and recruitment of SHIP1 to limit NF-κB signaling. The role of TIGIT in controlling NK cell–mediated antitumor immunity remains to be determined, but TIGIT deficiency did not affect B16 melanoma metastasis (a model where NK cells promote antitumor immunity).

The role of TIGIT in regulating dysfunctional CD8$^+$ T cells in tumors and chronic infection has now been demonstrated. TIGIT is expressed on CD8$^+$ TILs in multiple human tumor types and is coexpressed with PD-1 on the majority of TILs isolated from metastatic melanoma. TIGIT is also expressed on a subpopulation of TILs in mouse tumors. Studies in CT26 and EMT6 mouse cancer models show that coblockade of TIGIT and PD-L1 can enhance function of CD8$^+$ TILs and result in substantial tumor regression and improved survival, in contrast to minimal effects of blockade of TIGIT or PD-L1 alone (143). Coblockade boosted cytokine production by CD8$^+$ TILs. Similarly, in the LCMV chronic viral infection model, coblockade of TIGIT and PD-L1 synergized to reverse CD8$^+$ T cell exhaustion and enhance viral control to a greater extent than PD-L1 blockade alone (143). Likewise, TIGIT and PD-1 coblockade increased proliferation, cytokine production, and degranulation of tumor-antigen-specific circulating CD8$^+$ T cells and CD8$^+$ TILs from melanoma patients in vitro, compared to either single blockade alone. Interestingly, PD-1$^+$TIM-3$^+$CD8$^+$ TILs produced less TNF and IL-2 compared to TIGIT$^+$ PD-1$^+$, TIGIT$^+$PD-1$^-$, and TIGIT$^-$PD-1$^+$ TILS (145), suggesting that there are multiple subsets of dysfunctional TILs that vary in level of dysfunction. These findings suggest functional synergies between PD-1 and TIGIT in limiting tumor immunity and support exploration of combined TIGIT and PD-1 blockade for cancer immunotherapy.

V-Domain Immunoglobulin-Containing Suppressor of T Cell Activation

VISTA, also known as Differentiation of embryonic stems cells 1 (Dies1); platelet receptor Gi24; PD-1 homolog (PD-1H); and Death domain 1 alpha (DD1α), is an Ig superfamily member that shares homology with PD-L1 in its extracellular domain (146, 147). Mouse VISTA is mainly expressed on hematopoietic cells, including T cells, NK cells, macrophages, and dendritic cells, but not on B cells, and is most highly expressed on myeloid cells. Human VISTA protein is expressed on $CD4^+$ and $CD8^+$ T cells, subsets of $CD11b^{hi}$ monocytes, lymphoid and myeloid dendritic cell subsets, and neutrophils (148). The cell surface receptor for VISTA is not yet known, but VISTA interacts with Alk3, a component of the BMP4 receptor complex, and controls BMP4 signaling in mouse embryonic stem cells (149). Recent work has demonstrated that homophilic interactions of VISTA contribute to myeloid cell phagocytosis of apoptotic cells (150).

VISTA has inhibitory functions on APCs and T cells that regulate T cell activation and tolerance. Immobilized VISTA-Ig inhibited proliferation and cytokine production by resting and antigen-experienced mouse and human $CD4^+$ and $CD8^+$ T cells in vitro and also induced pTregs. T cells from VISTA-deficient mice produced increased IFN-γ (but not TNF-α or granzyme) following immunization with antigen (151). VISTA-deficient mice developed spontaneous T cell activation and multiorgan chronic inflammation at ~7 months of age, but without overt autoimmunity (151). However, VISTA deficiency can enhance development of EAE, and VISTA on T cells and hematopoietic cells contributes to exacerbated EAE. Anti-VISTA monoclonal antibody also intensified EAE. Synergies between VISTA and PD-1 in regulating T cell tolerance are demonstrated by studies of VISTA/PD-1 and VISTA/PD-L1 double knockout mice, which developed increased frequencies of activated T cells and inflammation compared to single knockout mice, but no spontaneous autoimmunity (152). These findings suggest that combined VISTA and PD-1 blockade may have therapeutic benefits associated with limited adverse events. Combined PD-1/VISTA deficiency accelerated onset and course of EAE to a greater extent than lack of either molecule alone.

In mouse tumor models, VISTA is highly expressed on myeloid cells and $FoxP3^+$ Tregs (146, 153). Anti-VISTA antibody increased $CD8^+$ T cell infiltration, proliferation, and effector function in tumors and reduced tumor growth in multiple mouse models (153). Anti-VISTA monoclonal antibody also decreased the number of MDSCs and increased activated dendritic cells within the tumor microenvironment in some tumor models. Anti-VISTA could synergize with a peptide-based cancer vaccine to reduce growth of established tumors. VISTA deficiency did not impair growth of B16 OVA melanoma, but tumor growth was significantly reduced in VISTA-deficient mice given a peptide-based vaccine compared to controls. Mice lacking VISTA also had markedly improved survival compared to wild-type mice given GL261 glioma and low-dose radiotherapy. In addition, anti-VISTA and anti-PD-L1 synergized to promote tumor regression and tumor-specific $CD8^+$ T cell function in the CT26 model and in mice given GM-CSF-secreting B16 melanoma vaccines and radiotherapy. These studies point to VISTA as an attractive target for combination therapies.

T Cell–Immunoglobulin–Mucin Domain 3

TIM-3 is a receptor within the TIM family of proteins (154). The important inhibitory functions of the TIM-3 pathway became apparent from studies using antagonists of the protein and mice deficient in the TIM-3 gene in transplantation, autoimmunity, and antigen challenge models (155). TIM-3 promotes T cell tolerance, as demonstrated by the defects in tolerance induction in TIM-3-deficient mice and the exacerbated EAE elicited by anti-TIM-3 monoclonal antibody

(156). In chronic viral infection models, coexpression of TIM-3 and PD-1 identified highly dysfunctional $CD8^+$ T cells (157). The identification of $TIM\text{-}3^+/PD\text{-}1^+$ $CD8^+$ TILs as more exhausted T cells suggested the importance of TIM-3 in cancer. In tumor models, all $TIM\text{-}3^+$ infiltrating T cells also expressed PD-1 (123). Work in syngeneic mouse tumor challenge models demonstrated modest utility of TIM-3 monoclonal antibody as a single agent but excellent antitumor efficacy in combination with PD-1 blockade (123) or an agonistic anti-4-1BB (158).

Multiple ligands for TIM-3 have been reported, including phosphatidylserine (PS), galectin-9 (GAL-9), HMGB1, and CEACAM-1 (155, 159, 160). The blocking characteristics of therapeutic TIM-3 monoclonal antibodies have not been thoroughly described, and their mechanism of action is not yet understood. TIM-3 is expressed by multiple human T cell populations (including Th1 cells, $CD8^+$ T cells, and Tregs), NK cells, NKT cells, as well as APCs such as dendritic cells and macrophages. TIM-3 on NK cells also has an active signaling role in IFN-γ production (161). Therefore, TIM-3 pathway blockade may affect cell types in addition to T cells.

Because TIM-3 is capable of mediating PS-mediated recognition and phagocytosis, its mechanism of action may differ greatly from those of other checkpoint receptors. One hypothesis is that blocking TIM-3 is efficacious because this alters immune system recognition and response to dead and dying tumor cells (162). TIM-3 cross-linking by GAL-9 may also regulate T cell responses to tumor cells (163). GAL-9 binds to specific sugar residues on multiple glycoproteins, such as 4-1BB and TIM-3, and may act as a general enhancer of glycoprotein receptor signaling by increasing cross-linking after the PS ligand is engaged (164). TIM-3 is also expressed on acute myelogenous leukemia (AML) stem cells and some other myeloid tumors but not hematopoietic stem cells (165, 166). Production of GAL-9 in AML may drive an autocrine stimulatory loop through TIM-3 (167). An anti-TIM-3 monoclonal antibody with cytotoxic activity eliminated AML stem cells in a xenograft model. Thus, either antagonistic or cytotoxic TIM-3 monoclonal antibodies may have therapeutic value, and the optimal time and setting for applying anti-TIM-3 therapy is under investigation. Preclinical data suggest that TIL coexpression of TIM-3 with PD-1 and/or LAG-3 may indicate that dual therapies are warranted (123). A number of TIM-3 antagonists are in development, and one has entered clinical trials as a single agent and in combination with PD-1.

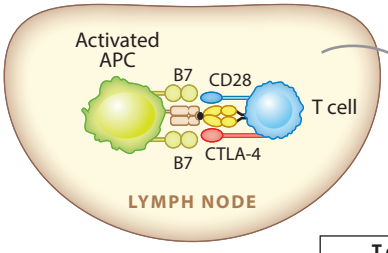
COMBINATION WITH OTHER MODALITIES

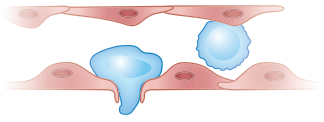
The clinical success of coinhibitory pathway blockade to date demonstrates the potential to induce effective and durable antitumor immune responses. However, curative responses are only observed in a subset of patients. Efforts to broaden the clinical benefit seek to complement checkpoint blockade with other therapeutic strategies. The key to rational design of combination therapies is an understanding of the mechanisms by which checkpoint blockade succeeds in some patients but fails in others, the identification of biomarkers predictive of response, and a clear understanding of how such therapeutics affect the immune system qualitatively, temporally, and spatially (**Figure 4**).

Antitumor immunity develops through a series of stepwise events, termed the cancer-immunity cycle (168). Blockade of coinhibitory pathways appears to be most beneficial when tumor immunity has developed but is being suppressed by compensatory mechanisms, as evidenced by a high expression level of immune-related genes, PD-1-expressing T cell infiltrates in the tumor, and PD-L1 expression on tumor cells (169–171). This suggests two broad mechanisms as the cause of checkpoint blockade failure: insufficient numbers of tumor-specific T cells in the microenvironment or additional mechanisms of intratumoral T cell suppression that have not been sufficiently

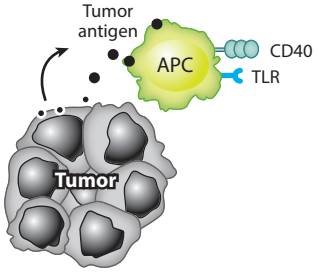
overcome. Combination therapies offer the opportunity to enhance different steps in the cancer-immunity cycle in order to establish an active immune microenvironment in which coinhibitory pathway blockade can unleash successful antitumor killing. Whereas some combination strategies may yield broad benefit, it is likely that optimal combinations are tumor specific or even patient specific, underscoring the need for reliable biomarkers.

T cell priming and activation	
Deficit	Therapeutic approach
Insufficient priming/ activation of naive T cells	Block multiple checkpoints (CTLA-4, PD-1, LAG-3, TIM-3) Activate stimulatory pathways (CD137, OX-40, CD27, ICOS, GITR) Administer stimulatory cytokines (IL-2, IL-12)



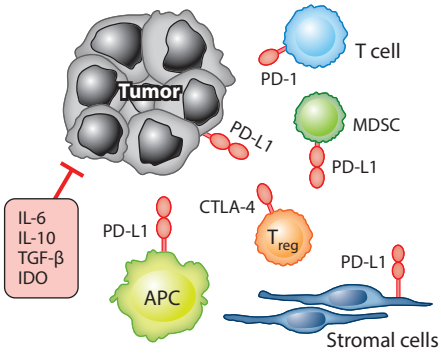


T cell trafficking and tumor infiltration	
Deficit	Therapeutic approach
Insufficient trafficking into tumor	Anti-VEGF Local or intravenous delivery of adoptively transferred T cells Cytokines



Checkpoint blockade

Cancer antigen release, uptake and processing	
Deficit	Therapeutic approach
Non-immunogenic cell death or insufficient neoantigens	Oncolytic viruses Chemotherapy Radiation therapy Cryotherapy Targeted therapy Epigenetic modifiers Blockade of phosphatidylserine
Insufficient antigen processing/DC maturation	Vaccines TLR agonists/STING GM-CSF IFN-α CD40 agonists



Recognition and killing of cancer cells	
Deficit	Therapeutic approach
Failure to overcome T cell suppression	Block multiple checkpoints (PD-1, PD-L1, LAG-3, TIM-3, CTLA-4) Activate stimulatory pathways (CD137, OX40, CD27, ICOS, GITR) Deplete/target immunosuppressive cells (Tregs, MDSCs, M2s) Target other suppressive mechanisms in microenvironment (IDO, TGF-β)
Unleashing intrinsic T cell immunity is insufficient	Induce/provide other antitumor immune cells (CARs, TCR-engineered T cells, NK cells)
Tumor burden/growth exceeds capacity for immune clearance	Reduction of tumor burden (surgery, radiation, chemotherapy, targeted therapy)

Enhancing Cancer Antigen Release, Uptake, Processing, and Maturation of Antigen-Presenting Cells

The presence of cancer antigens, uptake by APCs, and appropriate APC maturation signals mediated by the binding of damage-associated molecular patterns (DAMPs) to pattern-recognition receptors (PRRs) are required for successful antigen presentation. A high mutational load and neoepitope generation are associated with improved clinical benefit from CTLA-4 or PD-1 blockade (112, 172), supporting the goal of augmenting immunogenic tumor cell death and release of neoantigens. Radiation therapy kills tumor cells by direct cytotoxic effects, which in turn activate the DNA-damage response and release cancer antigens, proinflammatory cytokines, and DAMPs, thereby promoting cross presentation of cancer antigens (173). Bone-directed radiotherapy followed by ipilimumab or placebo for metastatic castration-resistant prostate cancer showed a benefit of ipilimumab on subgroup analysis but failed to reach statistical significance for the primary endpoint of OS (57). However, abscopal effects, by which localized radiation therapy in combination with systemic checkpoint blockade results in immune-mediated regression of nonirradiated distal metastases, have been demonstrated in mouse models (174, 175) and in the clinic (176–178).

Systemic chemotherapy not only can broadly promote proimmunogenic effects by mediating cytotoxic cell death and inducing a proinflammatory milieu, but it also can offer agent-specific modulatory effects, such as Treg depletion with cyclophosphamide or reduction of MDSCs mediated by gemcitabine, taxanes, and 5-FU (179). The addition of ipilimumab to dacarbazine resulted in improved OS compared to dacarbazine alone in patients with previously untreated metastatic melanoma, albeit with higher rates of adverse events (50), which were also observed in phase 2 studies in NSCLC combining ipilimumab with paclitaxel and carboplatin, which yielded more modest PFS benefits (58, 180).

To complement the specific, but often short-lived, effectiveness of inhibition of molecular pathways involved in tumor growth with the more broad and durable effects of immunotherapy, combination therapy with tyrosine kinase inhibitors (TKIs) has been a subject of active investigation (181). TKIs mediate both cytotoxic and proimmunogenic effects. In a model of gastrointestinal stromal tumor, imatinib, which inhibits the bcr-abl tyrosine kinase, reduced IDO expression by tumor cells, decreased Tregs, and increased numbers of infiltrating $CD8^+$ T cells (182). Given their activity in BRAF-mutant melanoma, immune effects of RAF inhibitors have been an important focus. RAF inhibitors increase expression levels of melanoma-associated antigens, inhibit recruitment of myeloid suppressor cells, and result in favorable ratios of $CD8^+$ T cells to Tregs in tumor infiltrates (183). They also promote T cell activation and proliferation, consistent with the paradoxical activation of the MAPK pathway, and this effect was potentiated by CTLA-4 blockade (184). Unfortunately, the clinical combination of the BRAF inhibitor vemurafenib with ipilimumab after a brief period of vemurafenib monotherapy resulted in high rates

Figure 4

Combination of coinhibitory pathway blockade with other therapeutic modalities. Deficits at important steps within the cancer-immunity cycle may prevent an active tumor immune infiltrate that can be unleashed by checkpoint blockade. Rational selection of combination therapies should be based on an emerging understanding of how other modalities can overcome such deficits to enhance development of antitumor immunity and synergize with checkpoint inhibitors to broaden their clinical benefit. Abbreviations: APC, antigen-presenting cell; CAR, chimeric-antigen receptor T cell; CTLA-4, cytotoxic T lymphocyte antigen 4; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid-derived suppressor cell; MΦ, macrophage; NK, natural killer; pMHC, major histocompatibility complex class I peptide complex; STING, stimulator of interferon genes; TCR, T cell receptor; TIM-3, T cell–immunoglobulin–mucin domain 3; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

of hepatotoxicity, perhaps reflecting enhanced T cell activation, leading to early termination of the phase 1 trial (185). Conversely, patients who received vemurafenib after ipilimumab or PD-1 blockade experienced pronounced drug hypersensitivity reactions (186, 187). High rates of renal toxicity were observed when sunitinib was combined with tremelimumab in metastatic renal cell carcinoma (188), whereas combination of nivolumab with sunitinib or pazopanib appeared to be well tolerated in early studies (189). Combination of tremelimumab with aromatase inhibition for breast cancer or with androgen deprivation for prostate cancer was also well tolerated, with evidence of biologic activity (190, 191).

Local delivery of oncolytic viruses is an alternative strategy to promote immunogenic tumor lysis characterized by antiviral and antitumor immune responses with increased $CD8^+$ T cell infiltration and PD-L1 upregulation (192–197). Localized intratumoral therapy with an oncolytic Newcastle disease virus in conjunction with systemic CTLA-4 blockade effectively eliminated distant established tumors in a B16 melanoma model (198). Clinical combination with ipilimumab has thus far yielded promising preliminary results in a phase 1b study of an oncolytic herpes virus encoding GM-CSF (199).

Studies have demonstrated that activation of the stimulator of interferon genes (STING) pathway in tumor-resident APCs is required for cross presentation of cancer antigens and induction of $CD8^+$ antitumor T cell responses (200, 201). Cytosolic sensing of tumor cell–derived DNA in the APC catalyzes the generation of cyclic dinucleotides that bind to STING and activate transcriptional programs, resulting in type I interferon production that activates APCs in both an autocrine and a paracrine fashion. In preclinical models, STING agonists were able to induce systemic immune responses capable of rejecting distant metastases (202) and augmented immunogenic effects of radiation (203). STING agonists may be particularly synergistic, given that the STING pathway was required for therapeutic effects of checkpoint blockade (201) and combination therapy with whole-tumor vaccine (STINGVAX) resulted in high PD-L1 expression and $CD8^+$ T cell infiltration (204). Alternative approaches to increase antigen presentation, such as the TLR9 agonist CpG or IFN-α2b, have been well tolerated in combination with CTLA-4 blockade, with evidence of some clinical benefit (205, 206).

Tumor vaccines have been developed in an effort to stimulate clinically relevant antitumor immunity for years. The rationale for vaccine-induced T cell priming in conjunction with blockade of pathways that inhibit such T cell responses is sound. In seminal preclinical studies, the efficacy of CTLA-4 blockade was greatly enhanced when combined with whole-tumor vaccines producing GM-CSF (207, 208). Clinically, such combination was explored in prostate cancer and pancreatic cancer, with some evidence of biologic activity and a side effect profile comparable to that of ipilimumab alone (209–211). A prospective randomized trial of systemic GM-CSF with ipilimumab in melanoma revealed an improved one-year survival rate and, notably, a reduced frequency of serious toxicities (212). Results from additional retrospective studies are consistent with these findings (213). In preclinical approaches with dendritic cell–based vaccines, CTLA-4 blockade enhanced efficacy compared to either therapy alone (214, 215). When combined with appropriate adjuvants, long-peptide vaccines have induced clinical responses and T cell immunity (216, 217). Early clinical studies combining peptide vaccines emulsified with the immunostimulatory montanide adjuvant and CTLA-4 blockade in melanoma resulted in tolerable autoimmune adverse events associated with lower rates of relapse and antigen-specific immune responses (218). However, the combination of ipilimumab with gp100 montanide-based peptide vaccine in a phase 3 trial was not superior to ipilimumab alone (2). Personalized vaccines, based on sequencing tumor and healthy tissues and synthesizing peptides predicted to result in neoepitopes, are a promising approach for combination in the future (219).

Naive T Cell Priming and Activation in Lymph Nodes

Following antigen uptake and APC maturation, APCs migrate to the lymph nodes or tertiary lymphoid structures, where they present cancer antigens to antigen-specific T cells. Successful priming occurs only when this interaction is accompanied by a CD28 costimulatory signal. The duration and magnitude of T cell priming is further regulated by the balance between the coinhibitory signals described herein, and costimulatory signals through 4-1BB, OX40, GITR, ICOS, and CD40 receptors. These costimulatory signals can be mimicked by agonistic monoclonal antibodies. Promising single-agent results with agonistic monoclonal antibodies have triggered early clinical studies in combination with checkpoint blockade. CD40 monoclonal antibodies have yielded objective and durable tumor responses in a minority of patients with pancreatic carcinoma, melanoma, or non-Hodgkin lymphoma (122, 220–222). Anti-CD40 treatment was shown to induce PD-L1 upregulation on monocytes, and combination with PD-1/PD-L1 axis blockade was highly synergistic in reduction of tumor burden in mice (223). Results from a completed phase 1 trial combining anti-CD40 with tremelimumab in metastatic melanoma are pending (trial NCT01103635; **https://clinicaltrials.gov**).

Agonistic anti-OX40 antibodies used as a single agent resulted in regressions of metastatic lesions in 12 of 30 treated patients with advanced malignancies (224), leading to ongoing single-agent studies. Anti-4-1BB (CD137) agonistic antibodies produced evidence of clinical activity in a number of advanced malignancies, although limited by liver toxicity particularly at higher doses (225–227). With appropriate management algorithms and dosing considerations, the partial remissions observed may justify pursuing 4-1BB agonistic combination therapy further, especially as mouse models suggest synergy of 4-1BB or OX40 agonists when combined with CTLA-4 (228) or PD-1 blockade (229, 230).

T Cell Trafficking and Infiltration into Tumors

Upon successful priming, T cells need to reach the tumor site in order to mediate antitumor immunity. However, tumors have developed strategies to suppress the recruitment of T cells to the tumor site (231). Disruption of chemokines that attract T cells (232) and inhibition of adhesion molecule expression on vascular endothelium through angiogenic growth factors, such as VEGF (233), play a role in preventing successful transmigration through the tumor endothelial barrier. The combination of ipilimumab with the VEGF inhibitor bevacizumab was therefore evaluated in metastatic melanoma. The combination was well tolerated, yielded a disease-control rate of 67.4%, and demonstrated activated vascular endothelium with extensive $CD8^+$ lymphocyte and macrophage infiltrates on on-treatment tumor biopsies, supporting further investigation (234). Based on these results, VEGF blockade is now also being explored in combination with anti-PD-L1 and anti-PD-1 antibodies.

OVERCOMING IMMUNE INHIBITION IN THE TUMOR MICROENVIRONMENT

Even when tumor-specific T cells have infiltrated the tumor microenvironment, additional mechanisms of suppression may prevent successful release of antitumor immunity through checkpoint blockade. Many cancers express IDO, which dampens antitumor immunity by depleting tryptophan, an amino acid required for T cell survival and effector functions, and by promoting the generation of Tregs (235–237). IDO expression has been associated with poor prognosis and mediates resistance to CTLA-4 blockade (238). Therapeutic agents interfering with the IDO pathway

have entered clinical testing in conjunction with chemotherapy (239), and in combination with ipilimumab, where preliminary data indicate clinical activity (240).

In addition to tumor cells, Tregs, MDSCs, and certain macrophage subsets can exert immunosuppressive effects in the tumor microenvironment, providing the rationale for depletion or inhibition of these populations (241, 242). Murine models suggest that depletion of MDSCs with a CSF1 receptor antibody or epigenetic modulation of MDSCs with azacitidine or entinostat enhances the effects of PD-1 and CTLA-4 blockade (243), and these approaches are now being applied in the clinic. The prognostic importance of intratumoral ratios of effector T cells to Tregs has been established in many studies. Depletion of $CD25^+$ Tregs enhanced efficacy of CTLA-4 blockade in B16 melanoma models (244), and clinical studies of anti-CCR4 monoclonal antibodies, which allow for selective Treg depletion, have shown clinical activity with 14% complete response rates in patients with T cell lymphoma (245).

In some instances, limited efficacy of coinhibitory pathway blockade may simply be a function of excessive or rapidly growing tumor burden, and careful timing of surgery, radiotherapy, targeted therapy, or chemotherapy may allow checkpoint blockade to succeed. Lastly, striking advances have been made with the adoptive transfer of TCR-engineered (246) and especially chimeric-antigen receptor T cells (22). Intravenous or local administration of such cells may circumvent trafficking issues, and adoptive T cell therapy may benefit from combination with PD-1 blockade in the microenvironment in some bulky hematologic or solid tumors.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

The clinical success of checkpoint blockade has revitalized cancer immunology and immunotherapy. Further work is needed to increase the efficacy of checkpoint blockade and to develop effective combination therapies with the goal of achieving durable antitumor responses in patients who do not benefit from monotherapy with one checkpoint blocker. To achieve these goals, many questions need to be addressed:

1. What are the mechanisms of response and resistance to checkpoint blockade? Increasing evidence suggests that tumors that respond to checkpoint blockade have an ongoing but ineffective immune response to tumor-derived neoepitopes. A better understanding of the similarities and differences between coinhibitory pathways, as well as mechanisms of synergy between coinhibitory pathways, is needed to optimize design of combination therapies. Insights may come from determining whether synergies between coinhibitory pathways affect similar or different molecular pathways.
2. What are mechanisms of durability of checkpoint blockade? The long-lasting effects of checkpoint blockade are remarkable. However, relatively little is known about mechanisms of durability and the necessary length of therapy. Further work is needed to understand how checkpoint blockade affects the generation, function, and maintenance of memory T cell subsets. Current studies following up on patients in early clinical trials have the potential to begin to answer the question of durability.
3. Are there biomarkers that can predict response to checkpoint blockade? It is imperative to develop biomarkers that help stratify patients and predict whether a patient is likely to respond to monotherapy, should receive combination therapy, or should receive other therapies entirely. Further investigations are needed to determine the predictive value of the degree/type of immune infiltrate in the tumor, mutational burden (neoepitopes), expression of coinhibitory molecules (e.g., PD-L1) by tumor cells or tumor immune cell infiltrates, and the utility of analyzing circulating tumor cells in the blood and tumor DNA in plasma for probing a tumor's microenvironment. Technological advances are needed for studying

clonal heterogeneity of both immune cells and tumor cells during tumor evolution. Understanding why tumors regress or progress may aid in the development of biomarkers of response as well as immunotherapeutic strategies.

4. How can we develop effective combination therapies with checkpoint blockers that increase therapeutic benefits and minimize adverse events? Tumors use multiple means to evade immune eradication, and a better understanding of the mechanisms contributing to suppression within the tumor microenvironment is needed. Some nonimmunologic therapies can affect the immune response to tumors. Further knowledge about how these therapies (radiation, chemotherapy, epigenetic modifiers, antiangiogenic agents, small molecules targeted at specific driver mutations) promote or inhibit immune responses should assist in designing rational combination therapies. Combination approaches with cancer vaccines, chimeric antigen receptor T cells, and other cellular therapies are exciting strategies for rendering tumors with little or no T cell infiltration responsive to checkpoint blockade.

This is an extraordinary time in cancer immunology and immunotherapy. The rapid advances in cancer immunotherapy provide a foundation with unprecedented potential for discovering and developing effective cancer therapies. Multidisciplinary collaborations among immunologists, engineers, cancer biologists, computational biologists, and clinicians will spur innovations built on this foundation and realize the tremendous potential of immunotherapy for people with cancer.

DISCLOSURE STATEMENT

G.J.F. and A.H.S. have patents/pending royalties on the PD-1 pathway from Bristol-Myers-Squibb, Roche, Merck, EMD-Serono, Boehringer-Ingelheim, AstraZeneca, and Novartis. G.J.F. has patents/pending royalties on TIM-3 from Novartis. G.J.F. has served on advisory boards for CoStim, Novartis, Roche, Eli Lilly, and Bristol-Myers-Squibb. A.H.S. has served on advisory boards for CoStim, Novartis, Surface Oncology, and Bristol-Myers-Squibb and has received research grants from Roche and Novartis. G.D. is an employee of Novartis.

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