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REVIEW

Fundamental Mechanisms of Immune Checkpoint Blockade Therapy

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ABSTRACT

Immune checkpoint blockade is able to induce durable responses across multiple types of cancer, which has enabled the oncology community to begin to envision potentially curative therapeutic approaches. However, the remarkable responses to immunotherapies are currently limited to a minority of patients and indications, highlighting the need for more effective and novel approaches. Indeed, an extraordinary amount of preclinical and clinical investigation is exploring the therapeutic potential of negative and positive costimulatory molecules. Insights into the underlying biological mechanisms and functions of these molecules have, however, lagged significantly behind. Such understanding will be essential for the rational design of next-generation immunotherapies. Here, we review the current state of our understanding of T-cell costimulatory mechanisms and checkpoint blockade, primarily of CTLA4 and PD-1, and highlight conceptual gaps in knowledge.

Significance: This review provides an overview of immune checkpoint blockade therapy from a basic biology and immunologic perspective for the cancer research community. *Cancer Discov; 8(9); 1069–86.* *©2018 AACR.*

INTRODUCTION

Immune checkpoint blockade therapies are now FDA approved for the treatment of a broad range of tumor types (Table 1), with approval likely for additional indications in the near future. The realization of long-term durable responses in a subset of patients represents a transformative event. Since the 2011 FDA approval of ipilimumab (anti-CTLA4) for the treatment of metastatic melanoma, 5 additional checkpoint blockade therapies, all targeting the PD-1/PD-L1 axis, have been approved for the treatment of a broad range of tumor types. Additionally, ipilimumab plus nivolumab (anti–PD-1) combination therapy has been approved for the treatment of advanced melanoma with favorable outcomes compared with either monotherapy. However, as we look to the future and aspire to extend these remarkable responses to more patients and tumor types, many aspects of T-cell activation and the mechanisms of checkpoint blockade remain to be understood. Here, we review how the negative costimulatory molecules CTLA4 and PD-1 attenuate T-cell activation. We also discuss current dogma and recent conceptual advances related to the mechanisms of action of anti–PD-1 and anti-CTLA4 therapies in the context of antitumor immunity. These discussions highlight the importance of understanding the underlying fundamental biological phenomena for effective translational and clinical research. In the context of the current landscape of cancer immunotherapy, fully understanding how anti-CTLA4 and anti–PD-1 checkpoint blockade therapies work will be critical for effectively combining them with other immunotherapeutic, chemotherapeutic, and targeted approaches.

Immune checkpoint blockade removes inhibitory signals of T-cell activation, which enables tumor-reactive T cells to overcome regulatory mechanisms and mount an effective antitumor response (1–3). Such regulatory mechanisms normally maintain immune responses within a desired physiologic range and protect the host from autoimmunity. Immunologic tolerance is achieved through multiple distinct mechanisms that can be defined as central and peripheral. Central tolerance is mediated through clonal deletion of high-affinity self-reactive clones during negative selection in the thymus. However, because self-reactivity is selected for during positive selection in the thymus, additional mechanisms are required to restrain autoreactivity. Peripheral tolerance is mediated through a variety of mechanisms, including regulatory T cells (Treg), T-cell anergy, cell-extrinsic tolerogenic signals, and peripheral clonal deletion. The immune system exerts a strong selective pressure throughout tumor progression, leading to immune tumor editing (4). As a result, malignant tumors often co-opt immune suppressive and tolerance mechanisms to avoid immune destruction. Immune

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Table 1. Summary of the tumor types for which immune checkpoint blockade therapies are FDA-approved

Tumor type	Therapeutic agent	FDA approval year
Melanoma	Ipilimumab	2011
Melanoma	Nivolumab	2014
Melanoma	Pembrolizumab	2014
Non–small cell lung cancer	Nivolumab	2015
Non–small cell lung cancer	Pembrolizumab	2015
Melanoma (BRAF wild-type)	Ipilimumab + nivolumab	2015
Melanoma (adjuvant)	Ipilimumab	2015
Renal cell carcinoma	Nivolumab	2015
Hodgkin lymphoma	Nivolumab	2016
Urothelial carcinoma	Atezolizumab	2016
Head and neck squamous cell carcinoma	Nivolumab	2016
Head and neck squamous cell carcinoma	Pembrolizumab	2016
Melanoma (any BRAF status)	Ipilimumab + nivolumab	2016
Non–small cell lung cancer	Atezolizumab	2016
Hodgkin lymphoma	Pembrolizumab	2017
Merkel cell carcinoma	Avelumab	2017
Urothelial carcinoma	Avelumab	2017
Urothelial carcinoma	Durvalumab	2017
Urothelial carcinoma	Nivolumab	2017
Urothelial carcinoma	Pembrolizumab	2017
MSI-high or MMR-deficient solid tumors of any histology	Pembrolizumab	2017
MSI-high, MMR-deficient metastatic colorectal cancer	Nivolumab	2017
Pediatric melanoma	Ipilimumab	2017
Hepatocellular carcinoma	Nivolumab	2017
Gastric and gastroesophageal carcinoma	Pembrolizumab	2017
Non–small cell lung cancer	Durvalumab	2018
Renal cell carcinoma	Ipilimumab + nivolumab	2018

NOTE: A summary of the tumor indications, therapeutic agents, and year of FDA approval for immune checkpoint blockade therapies. FDA approval includes regular approval and accelerated approval granted as of May 2018. Ipilimumab is an anti-CTLA4 antibody. Nivolumab and pembrolizumab are anti–PD-1 antibodies. Atezolizumab, avelumab, and durvalumab are anti–PD-L1 antibodies. Tumor type reflects the indications for which treatment has been approved. Only the first FDA approval granted for each broad tissue type or indication for each therapeutic agent is noted. In cases where multiple therapies received approval for the same tumor type in the same year, agents are listed alphabetically.

Abbreviations: MSI, microsatellite instability; MMR, mismatch repair.

checkpoint blockade inhibits T cell–negative costimulation in order to unleash antitumor T-cell responses that recognize tumor antigens. Importantly, the development of immune checkpoint blockade therapies was predicated on basic research that identified key regulatory mechanisms of T-cell activation. However, there remains much to be understood about these mechanisms, further insight into which will be essential for the rational development of immunotherapeutic approaches. The current clinical landscape of cancer immunotherapy and mechanisms of resistance to immunotherapy have been recently reviewed (5–7). Here, we primarily discuss what is known about the regulatory mechanisms of CTLA4 and PD-1 and the therapeutic implications of these insights.

MECHANISMS OF CTLA4-MEDIATED NEGATIVE COSTIMULATION

CTLA4 expression and function is intrinsically linked with T-cell activation. CTLA4 is immediately upregulated following T-cell receptor (TCR) engagement (signal 1), with its expression peaking 2 to 3 days after activation (8, 9). CTLA4 dampens

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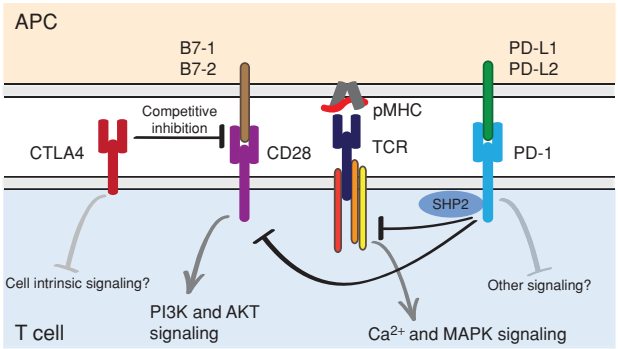

Figure 1. Molecular mechanisms of CTLA4 and PD-1 attenuation of T-cell activation. Schematic of the molecular interactions and downstream signaling induced by ligation of CTLA4 and PD-1 by their respective ligands. The possibility of additional downstream cell-intrinsic signaling mechanisms is highlighted for both CTLA4 and PD-1.

TCR signaling through competition with the costimulatory molecule CD28 for the B7 ligands B7-1 (CD80) and B7-2 (CD86), for which CTLA4 has higher avidity and affinity (refs. 10–12; Fig. 1). Because both B7-1 and B7-2 provide positive costimulatory signals through CD28 (refs. 13; signal 2), competitive inhibition of both molecules by CTLA4 is necessary to effectively attenuate T-cell activation. CD28 and CTLA4 also display rapid binding kinetics with B7-1 (12), which, coupled with differences in binding strengths, allows for swift competitive inhibition by CTLA4. In addition to upregulation of CTLA4 expression upon T-cell activation, CTLA4 contained in intracellular vesicles is rapidly trafficked to the immunologic synapse (14). The degree of CTLA4 recruitment to the immunologic synapse correlates directly with TCR signal strength. Once trafficked to the immunologic synapse, CTLA4 is stabilized by B7 ligand binding, allowing it to accumulate and effectively outcompete CD28 (15). Through this mechanism, CTLA4 attenuates positive costimulation by CD28 and thus limits CD28 downstream signaling, which is primarily mediated by PI3K and AKT (16, 17). This results in robust regulation of TCR signal amplitude and, thus, T-cell activity. Because CTLA4-negative costimulation is intrinsically linked to expression of B7 ligands and CD28-mediated positive costimulation, CTLA4 primarily functions to regulate T-cell activity at sites of T-cell priming (e.g., secondary lymphoid organs). In addition to this core function, CTLA4 also attenuates T-cell activation in peripheral tissues given that B7 ligands are constitutively expressed to varying degrees by antigen-presenting cells (APC) but can also be expressed by activated T cells. Because of its central role in regulating T-cell activation, negative costimulation by CTLA4 is critical for tolerance. Reflective of this, biallelic genetic deletion of *Ctla4* leads to massive lymphoproliferation that mice succumb to at 3 to 4 weeks of age (18–20).

In addition to the cell-intrinsic functions through which CTLA4 primarily attenuates T-cell activity, CTLA4 can modulate T-cell activation through several cell-extrinsic mechanisms. Indicative of cell-extrinsic regulatory mechanisms, the presence of CTLA4-competent T cells is sufficient to prevent lethal lymphoproliferation due to genetic deletion of *Ctla4* (21). The majority of cell-extrinsic suppressive function of CTLA4 is mediated through Tregs (22, 23). Specific loss of CTLA4 in Tregs is sufficient to induce aberrant T-cell activation and give rise to autoimmunity (24, 25). This indicates that Treg-derived CTLA4 is necessary to maintain immunologic tolerance, although it is unlikely that Treg-derived CTLA4 is sufficient to maintain T cell–mediated tolerance. In terms of a potential molecular mechanism, CTLA4 expressed by Treg cells may attenuate T-cell activation in a cell-extrinsic manner by limiting the availability of the B7 ligands B7-1 and B7-2 for CD28-mediated positive costimulation of nearby effector T cells. CTLA4 also has cell-extrinsic contributions within the effector compartment. CTLA4 expressed by effector T cells can compete for B7 ligands in *trans* (26). Additionally, it has been reported that CTLA4 can also act to limit the overall availability of B7 ligands through transendocytosis of B7 ligands from APCs (27). The degree to which these cell-extrinsic processes contribute to T-cell tolerance remains to be fully resolved, particularly in the context of tumor immunity.

Recent work by Sharpe and colleagues demonstrated that genetic loss of CTLA4 in Tregs in adulthood surprisingly confers resistance to experimental autoimmune encephalomyelitis (EAE; ref. 28). Conditional deletion of *Ctla4* in Tregs is necessary and sufficient to confer resistance to EAE, suggesting that unrestrained peripheral Treg expansion and/or increased Treg activation can prevent autoimmunity. A significant implication of this finding is that Treg depletion may counter expansion of Treg cells induced by CTLA4 blockade and thus lead to enhanced efficacy of anti-CTLA4 therapy. This observation also raises the possibility that CTLA4 has differential functions in conventional and regulatory T cells during development and in adulthood. Alternatively, the apparently discordant observations between global and conditional *Ctla4* knockout mice may be the result of the intrinsic difference in antigen affinity of conventional T cells and Tregs. Tregs are selected for higher-affinity TCR for self-peptide MHC complexes, and thus because CTLA4 expression correlates with TCR signal strength, have concomitantly higher CTLA4 (29, 30). This mechanism attenuates strong TCR signals, allowing medium-strength TCR signals to also result in robust T-cell activation. As a result, loss of CTLA4 may disproportionally affect T cells with high-affinity antigen receptors. These findings may support the strength of signal model proposed more than 20 years ago (31). Moving forward, it will be critical to precisely dissect the function of downstream signaling components and assess their relative functional contribution to CTLA4-mediated regulation of T-cell activity.

MECHANISMS OF PD-1–MEDIATED ATTENUATION OF T-CELL ACTIVITY

The primary biological functions of PD-1 are to maintain peripheral tolerance and to maintain T-cell responses within a desired physiologic range. Because the PD-1/PD-L1 regulatory system is induced by immune responses (discussed in greater detail below), this forms a negative feedback loop to attenuate local T-cell responses and minimize tissue damage. PD-1 regulates T-cell activation through interaction with PD-L1 and PD-L2 (refs. 32–34; Fig. 1). PD-1 is expressed upon activation of T and B lymphocytes (35). Because of

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the expression of its ligands, which are widely expressed in nonlymphoid tissues, PD-1 acts primarily to dampen T-cell activation in the periphery (36). PD-L1 expression, and to a lesser degree expression of PD-L2, is induced in response to inflammatory cytokines such as IFNγ (32, 33). Thus, PD-1 regulation of T-cell activity occurs in response to cytolytic and effector T-cell function [e.g., CD8 cytotoxic T lymphocyte and type 1 helper (Th1) CD4 T cells] in an inducible manner. Upon engagement with PD-L1 and PD-L2, PD-1 is thought to primarily transmit a negative costimulatory signal through the tyrosine phosphatase SHP2 to attenuate T-cell activation. The recruitment of SHP2 directly attenuates TCR signaling via dephosphorylation of proximal signaling elements (37). This molecular mechanism reflects a dichotomy in modes of regulation utilized by CTLA4 and PD-1 engagement (38). These data indicate that in contrast to CTLA4-mediated regulation, PD-1 directly regulates TCR signaling to attenuate T-cell activity. However, recent evidence indicates that CD28 is a primary target for PD-1–induced attenuation of T-cell signaling (39). These studies utilized a cell-free membrane reconstitution model to examine functional relationships during T-cell activation and reveal that PD-1 leads to preferential dephosphorylation of CD28 rather than the TCR, via recruitment of SHP2. This suggests that both CTLA4 and PD-1, at least in part, act through a similar molecular mechanism of attenuating CD28-mediated costimulation (Signal 2). Thus, modulation of CD28 signaling could represent a functional convergence point of CTLA4- and PD-1–mediated regulation. Interestingly, recent findings indicate that SHP2 is not essential for responses to anti–PD-1 therapy or induction of T-cell exhaustion *in vivo* (40). This is suggestive of functional redundancy in the signaling pathways downstream of PD-1. Such redundancy is most likely mediated through redundant phosphatases (e.g., SHP1) but alternatively could be mediated through wholly distinct mechanisms. It is critical to further define the immediate signaling events downstream of CTLA4 and PD-1 to distinguish shared and distinct molecular mechanisms of these T-cell regulatory pathways.

Functionally, PD-1 is essential for homeostatic maintenance of peripheral tolerance as evidenced by the autoimmune pathologies that arise upon genetic deletion of *Pdcd1* (encoding PD-1). As an example, genetic loss of *Pdcd1* leads to development of lupus-like autoimmune pathology in aged C57BL/6 mice and autoimmune dilated cardiomyopathy in BALB/c mice (41, 42). The strong murine strain dependency of the PD-1 knockout phenotype raises the possibility that the observed autoimmunity may be driven by recognition of strain-specific antigens in the absence of PD-1 inhibitory signaling; however, this remains to be definitively tested. It is a critical point that although PD-1 is often used as a marker of exhaustion, it is not sufficient to define a functionally exhausted population. PD-1 is a marker of activated T cells, of which exhausted T cells are a subset. Exhausted T cells are often defined by coexpression of PD-1, LAG3, and TIM3. However, an essential distinction is that exhausted T cells (phenotypically defined) are still functionally active, but harbor reduced capacity. Thus, for example, exhausted CD8 T cells are still able to contribute to antitumor immune responses, but are likely less potent on a per-cell basis.

T-cell exhaustion is an important mechanism that limits T-cell activity in the presence of chronic antigen stimulation and acts to preserve T-cell clones that would otherwise perish under such conditions due to activation-induced cell death. Consistent with this notion, persistent PD-1 signaling induces metabolic restriction, which is a functional driver of T-cell exhaustion (43). Upon ligation, PD-1 attenuates glycolysis but simultaneously promotes fatty-acid oxidation and lipid catabolism, thus inducing a switch in energy derivation (44). In contrast, ligation of CTLA4 attenuates glycolysis independent of regulation of lipid metabolism. Interestingly, this metabolic switch is involved in determining T-cell effector versus memory fate and is driven in part by mitochondrial regulation (45). Such changes are likely driven by the changes in gene expression and epigenetic regulation that are induced by continuous PD-1 engagement. Indeed, chronic antigen stimulation in viral systems leads to dramatic changes in gene regulation and stable epigenetic reprogramming of T cells (46, 47). Together, such transcriptional, epigenetic, and metabolic changes define the exhausted T-cell state. Recent evidence suggests that such epigenetic changes can prevent the rescue of an exhausted state by checkpoint blockade and attenuate tumor responses to therapy (48).

Emerging evidence has also identified new functional roles for the PD-1/PD-L1 signaling axis. For example, macrophage expression of PD-L1 may lead to active eviction of T cells from the tumor microenvironment (49). This suggests that in addition to regulation of T-cell activation and cytolytic capacity, PD-1 signaling may also regulate T-cell trafficking and migration. Furthermore, it has been reported that PD-1 may also have tumor cell–intrinsic function (50). Future studies are needed to determine the degree to which such "noncanonical" mechanisms contribute to therapeutic efficacy.

MECHANISMS OF NEGATIVE COSTIMULATION VERSUS MECHANISMS OF CHECKPOINT BLOCKADE

Insights into the normal biological roles and molecular mechanisms of costimulatory molecules undoubtedly inform our understanding of mechanisms of action of cancer therapeutics targeting these molecules. Differences will remain however, given the properties of specific therapeutics (e.g., antibody isotype, off-target recognition, kinetics) as well as the property of cancer being self-derived but ideally recognized as foreign by the immune system. Based on our understanding of how the molecules themselves act to attenuate T-cell activity, it is thought that anti-CTLA4 and anti–PD-1 primarily act at different stages of the cancer-immunity cycle (51). Conceptually, the current model posits that CTLA4 blockade primarily acts at sites of priming in which CD28-positive costimulation is involved (e.g., tumor-draining lymph nodes) whereas PD-1 blockade primarily acts in inflamed peripheral tissues (e.g., tumor; Fig. 2). Recent evidence, discussed below, raises the possibility that the mechanisms of action of CTLA4 and PD-1 blockade are not limited to only these tissue sites.

Many of the principles and lessons learned in viral systems are applicable to tumor immunity, as cancer is highly

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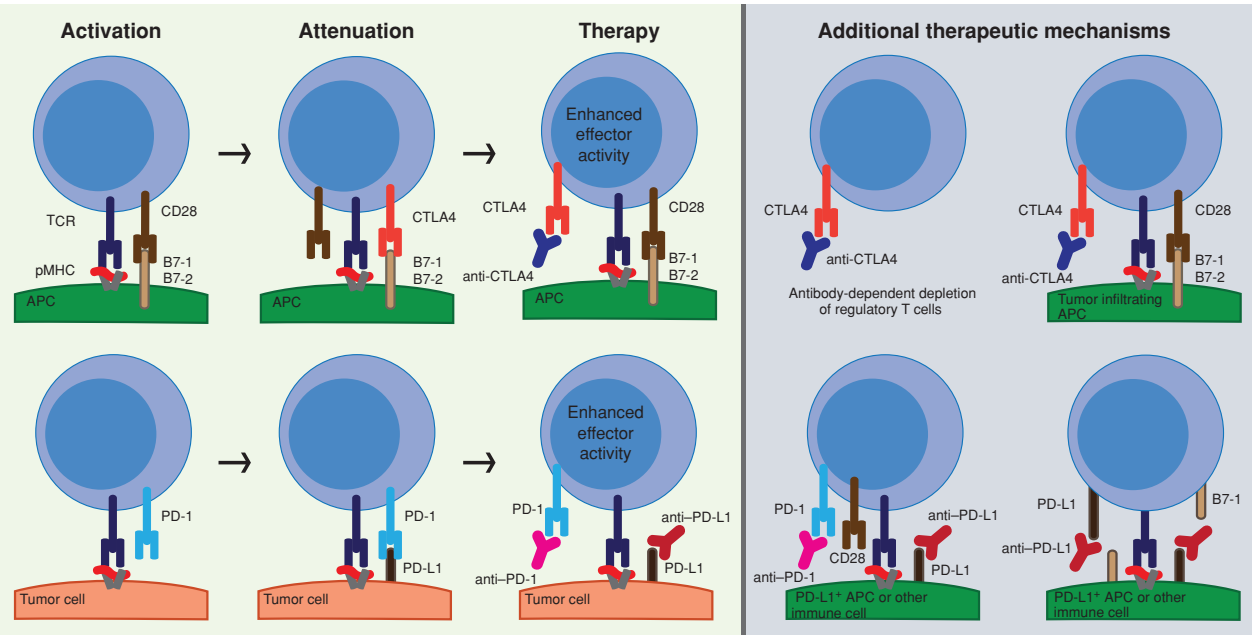

Figure 2. Schematic of the molecular mechanisms of action of CTLA4 and PD-1 blockade. The step-wise progression of T-cell activation, attenuation by normal regulatory mechanisms, and release of such negative regulation by therapeutic intervention using anti-CTLA4 or anti–PD-1 antibodies is outlined (left). In addition to cell-intrinsic enhancement of effector function, several additional mechanisms are thought to contribute to the efficacy of anti-CTLA4 and anti–PD-1 therapy (right). These include antibody-mediated depletion of Tregs, enhancement of T-cell positive costimulation within the tumor microenvironment, blockade of host-derived PD-L1 signals from nontumor cells in the microenvironment (as opposed to tumor cell–derived PD-L1), and blockade of interactions between PD-L1 and B7-1.

analogous to infectious disease contexts in which chronic antigen stimulation results in T-cell exhaustion (52). For example, blockade of PD-1 is sufficient to enhance the activity of exhausted T cells in the context of chronic viral infection, leading to viral clearance (53). Recent findings demonstrate that CD28 costimulation is necessary for responses to PD-1 blockade in the settings of both viral infection and tumor rejection (54). Together, these findings indicate that additional positive costimulation is required for therapeutic efficacy despite prior activation. This raises the possibility that PD-1 blockade acts not only in peripheral tissues (e.g., tumor) but also in sites of priming. The mechanisms of action of PD-1 and CTLA4 blockade and of the normal biological functions of these molecules are highly complex and clearly not fully understood. It is likely that subtle nuances in pertinent aspects of such mechanisms (e.g., timing, kinetics, target cell type, cognate antigen availability, anatomic location) will have profound impact on the final biological outcomes.

MECHANISMS OF ACTION OF CTLA4 BLOCKADE–INDUCED TUMOR REJECTION

CTLA4 blockade is thought to induce tumor rejection through a number of distinct mechanisms. The primary mechanism seems to be through direct blockade of CTLA4 competition for B7-1 and B7-2 costimulatory ligands, which allows for unrestrained CD28-mediated positive costimulation. Indeed, crystallographic structural analyses of the ipilimumab:CTLA4 complex reveal that the ipilimumab binding epitope overlaps with the B7 interaction domain, indicating that steric inhibition of B7 interactions underlies the primary mechanism of action of ipilimumab (55). Because tumor cells do not express B7 ligands, this action largely occurs in tumor-draining lymph nodes in which tumor antigens can be cross-presented by APCs to prime tumor-reactive T cells. It is also feasible that APCs within the tumor microenvironment may also cross-present tumor antigens to activate cognate tumor-reactive T cells. In either case, tumor cell death is required to release tumor cell antigens (e.g., neoantigens, tumor-associated antigens) that can be subsequently processed and presented by APCs. In the context of effective antigen presentation, CTLA4 blockade then enhances CD28 costimulation and thus activation. The extent to which APCs can directly process and cross-present tumor antigens within the tumor microenvironment to prime (or reprime) T cells *in situ* remains unclear. An interesting possibility is that antigen presentation may be taking place in tumor-associated tertiary lymphoid structures (TLS), the presence of which is generally associated with improved survival (56, 57). The role of TLS in antitumor immunity is complex and likely context-dependent however, as Treg populations within TLS have been shown to suppress antitumor T-cell responses (58). Understanding when and where antitumor T cells are primed and subsequently regulated (and thus potentially sensitive to checkpoint blockade therapy) remains a critical open question.

Emerging evidence suggests that anti-CTLA4 does not impose a generalized effect on all T cells. CTLA4 blockade leads to specific expansion of tumor neoantigen–specific CD8 T cells within the tumor microenvironment, but not secondary lymphoid organs (59). Consistent with this notion, anti-CTLA4 leads to expansion of specific tumor-infiltrating T-cell populations including a subset of phenotypically exhausted CD8 T cells and a PD-1^{+}ICOS^{+}TBET^{+} Th1-like CD4 effector

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T-cell population (60). This population appears to differ from canonical Th1 cells due to coexpression of ICOS and PD-1, which are markers of T follicular helper cells. Whether such cells reflect a distinct type of T-cell that emerges after therapy or, alternatively, an activated phenotype of a preexisting minor population remains to be fully determined. These findings are supported by clinical observations of expansion of ICOS$^+$ CD4 effector T cells following ipilimumab therapy in multiple tumor types (61–64) as well as following treatment with tremelimumab, another anti-CTLA4 antibody (65). Thus, the expansion of ICOS$^+$ CD4 effector T cells may be used as a pharmacodynamic marker of anti-CTLA4 therapy (66). Furthermore, the expansion of specific types of CD4 effector T cells raises the possibility that anti-CTLA4 not only enhances T-cell activation, but may also affect T-cell differentiation. The extent to which effects on T-cell lineage choices may contribute to the mechanisms and efficacy of immune checkpoint blockade therapies remains unclear. Notwithstanding, together these and other data discussed below indicate that CTLA4 blockade enhances antitumor immunity through modulation and expansion of particular T-cell populations.

In addition to these mechanisms of CTLA4 blockade–induced tumor rejection, depletion of Treg populations has also been identified as a mechanism of action of anti-CTLA4 therapy in murine tumor models (67–69). Treg depletion contributes partially to antitumor efficacy, as significant therapeutic benefit was still observed in Fc-gamma-RIV knockout C57BL/6 hosts (67). Interestingly, Treg depletion appears to be carried out with differential efficiency depending on the context. Treatment with depleting clones of anti-CTLA4 decreases intratumoral but not peripheral populations of Treg cells (68). This can be explained by the increased expression of CTLA4 by intratumoral Tregs, or alternatively by differences in the abundance and activity of Fc receptor–expressing cell populations in each context (e.g., tumor-associated macrophages). A recent report asserts that the efficacy of anti-CTLA4 is completely independent of its regulation of B7 ligand interactions and, instead, derives solely from antibody-mediated Treg depletion (70). This is a conclusion that is in conflict with numerous lines of prior evidence, and the experimental observations presented therein are insufficient to definitively arrive at this conclusion. For example, indirect readouts are used to infer molecular interactions and arrive at key observations such as the dispensability of B7 ligands for the therapeutic efficacy of anti-CTLA4. These conflicting findings may be explained by technical limitations of the biological systems utilized rather than biological independence of anti-CTLA4 and inhibition of B7 ligand interactions. Furthermore, this finding is in direct conflict with structural biology studies that revealed that ipilimumab binds CTLA4 precisely in the B7 interaction domain to mediate steric hindrance (55). Although other prior studies (discussed below in greater detail) do suggest that Treg depletion contributes to the mechanism of action of anti-CTLA4, a large body of work strongly implicates regulation of B7 ligand interactions as a critical mechanism. Notably, blockade of both effector and regulatory T-cell compartment–derived CTLA4 is required for effective tumor rejection (71). Notwithstanding, it is clear that detailed and careful determination of the relative contribution of each of the mechanisms of action of anti-CTLA4 therapies, particularly in the context of human immunity, is required.

The relative contribution of cell-intrinsic enhancement of effector function versus Treg depletion to the efficacy of ipilimumab in humans remains unclear. Ipilimumab was purposefully selected to be a blocking antibody based on the understanding that loss of CTLA4 would lead to enhanced T-cell activity. Thus, although ipilimumab is a fully human IgG1 antibody, it was not developed to be a depleting antibody. Consistent with this, there is not definitive evidence of Treg depletion in patients treated with ipilimumab. It is difficult to perform the studies required to resolve this issue of contention, as such studies are hindered by the necessity for paired pre- and post-therapy sampling, high tumor heterogeneity (in terms of immune infiltration), lack of clarity of how to best normalize quantification, and because FOXP3 is expressed in activated effector T cells in humans. It has been reported that ipilimumab can induce antibody-dependent cellular cytotoxicity (ADCC)–mediated killing of Tregs by nonclassic monocytes in *ex vivo* cultures (72). Moreover, recent evidence indicates that germline presence of a high-affinity polymorphism in the Fc receptor (CD16a-V158F) is associated with improved responses to ipilimumab (73). This suggests that Fc-mediated cell depletion functionally contributes in part to the mechanism of ipilimumab. In contrast, the similarity in response rates of two anti-CTLA4 antibodies (tremelimumab and ipilimumab) despite different antibody isotypes supports the notion that efficacy derives from enhancement of effector function rather than depletion. Tremelimumab is a fully human IgG2 antibody, whereas ipilimumab is a fully human IgG1 antibody, which is notable because IgG1 antibodies more effectively mediate ADCC than IgG2 antibodies based on their respective binding affinity for human Fc receptors (74). Although tremelimumab did not reach statistical significance in overall survival at the planned second interim analysis in the phase III clinical trial in metastatic melanoma, follow-up analyses suggest that responses to tremelimumab are roughly comparable to those of ipilimumab (75). Pooled analyses of the phase I and II clinical trials of tremelimumab reveal a 5-year survival rate of 20% (76), which is similar to the 21% 3-year survival rate observed in patients with metastatic melanoma treated with ipilimumab (77). These data support a model in which anti-CTLA4 both enhances cell-intrinsic effector function through blockade and induces Fc-mediated cellular depletion.

Modulation of the TCR repertoire may also contribute to the therapeutic effects of CTLA4 blockade. For example, ipilimumab treatment leads to a remodeling and broadening of the peripheral TCR repertoire (78, 79). Consistent with these findings, ipilimumab therapy broadens the functional reactivity of peripheral blood CD8 T cells for melanoma antigens (80). Interestingly, TCR repertoire broadening is also associated with immune-related adverse events (irAE) due to ipilimumab treatment (81), although it remains to be determined whether the underlying mechanisms and cognate antigens involved in therapeutic efficacy and irAEs are similar. Together, these observations suggest that TCR repertoire broadening due to blockade of CTLA4 has significant clinical relevance. Mechanistically, loss of CTLA4 may lower

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the threshold for TCR ligation required for effective T-cell activation given that CTLA4 normally acts to attenuate TCR signal strength. Upon blockade of CTLA4, antigens with low signal strength that are not normally sufficient to generate an effective T-cell response may be allowed to emerge. Such T-cell clones could recognize tumor-specific antigens (e.g., subdominant neoantigens) or tumor-associated antigens. In addition, the activity of high-affinity tumor-reactive clones would also be boosted by blockade of CTLA4 through this mechanism. Taken together, our understanding of the biology underlying CTLA4 indicates that its blockade acts to increase T-cell costimulation in multiple distinct ways, resulting in more robust activation of tumor-reactive T cells. Multiple lines of evidence indicate that tumor mutational burden (TMB) is associated with improved responses to checkpoint blockade (82–86). This supports a model in which neoantigens are a major driver of tumor immunogenicity. On the other hand, some tumor types such as renal cell carcinoma exhibit responsiveness to checkpoint blockade despite low mutational burden (87). It remains a possibility that low-TMB tumors that respond to checkpoint blockade harbor low numbers of highly immunogenic tumor-specific neoantigens. Alternatively, mechanistically distinct types of antitumor immune responses may underlie responses of low-TMB tumor types. Relatedly, the relative contribution of public antigens (shared; e.g., overexpressed genes) and private antigens (tumor-specific; e.g., neoantigens) to antitumor immune responses remains a key outstanding question.

MECHANISMS OF ACTION OF PD-1 BLOCKADE–INDUCED TUMOR REJECTION

PD-1 blockade is able to induce tumor rejection through reinvigoration of CD8 T cells, leading to both increased functional activity and frequency. Blockade of the PD-1 signaling axis prevents PD-1–mediated attenuation of proximal TCR signaling, allowing for restoration of activity of exhausted CD8 effectors. Thus, despite continued PD-L1 expression within the tumor microenvironment, exhausted T cells are able to be reinvigorated and mount an effective immune response. Clinical evidence supports a model in which blockade of the PD-1 signaling axis is most effective in tumors in which an endogenous T-cell response has already been elicited but is suppressed through PD-1 engagement by its ligands PD-L1 and PD-L2 (88, 89). However, the response of some PD-L1–negative tumors indicates that the presence of a preexisting immune response, as arbitrarily defined by the presence of tumor-infiltrating T cells, is not required for tumor rejection to be induced by PD-1 blockade. Recent evidence suggests that a subset of $CXCR5^+$ $PD\text{-}1^+$ CD8 T cells is responsible for immediate proliferative expansion following PD-1 blockade (90). Longitudinal profiling of peripheral blood from patients treated with anti–PD-1 therapies reveals expansion of $PD\text{-}1^+$ CD8 T cells with kinetics consistent with this notion (91). The antigen specificity of the T cells that mediate responses to checkpoint blockade therapy remains ill-defined. Recent evidence from a neoadjuvant trial of nivolumab in the context of non–small cell lung cancer supports the notion that anti–PD-1 therapy enhances neoantigen-specific T-cell responses (92). It is likely that only specific T-cell populations (defined by antigen specificity and/or phenotype) functionally mediate responses to checkpoint blockade therapy. Consistent with this notion, exhausted T cells display a distinct epigenetic profile, and this epigenetic reprogramming can limit T-cell reinvigoration (47, 48, 93, 94). These data suggest that PD-1 blockade may not be sufficient to functionally restore T cells once they reach a threshold level of exhaustion. Recent work reveals a high degree of phenotypic and functional heterogeneity within exhausted CD8 T cells (95). It will be conceptually critical to understand how functional heterogeneity of exhausted T cells affects the mechanisms of action and efficacy of specific checkpoint blockade therapies.

Despite much active investigation and interest in the field, the precise molecular and cellular events that mediate enhancement of antitumor immunity by PD-1 blockade remain not fully understood. Recent studies have revealed subtleties that are likely to have very important consequences for therapeutic efficacy and rational design of new strategies. For example, although PD-1 blockade primarily leads to the expansion of CD8 T cells, CD4 T cells are required for effective responses (96). Although this is not entirely surprising given the critical roles CD4 help plays during a wide range of processes including memory formation and antibody production, this emphasizes the complexity in defining mechanisms of action. In particular, this highlights the distinction between cellular processes that are modulated by therapy and cellular processes that are required for therapeutic efficacy.

It remains unclear what specific aspects of CD4 help are functionally required for clinical responses to checkpoint blockade. In addition to facilitating T-cell memory formation, it is tempting to speculate that CD4 helper T cells may also enhance antitumor immunity by increasing CD8 T-cell and antibody entry into peripheral tissue sites, as has been analogously observed in viral contexts (97, 98). In addition to ambiguities at the cellular level, emerging evidence has shed new insights into molecular mechanisms of PD-1 blockade. In addition to restoring T-cell activity through modulation of TCR signaling and gene expression, blockade of the PD-1 signaling axis is able to reverse the associated metabolic reprogramming to an extent, which in part mediates T-cell reinvigoration (43). Supportive of this finding, anti–PD-1 treatment has been shown to regulate metabolic function based on gene set enrichment analysis of tumor antigen–specific tumor-infiltrating lymphocytes (99). In contrast, CTLA4 blockade primarily leads to changes in genes associated with proliferation and cell cycle. In addition to preventing attenuation of T-cell activation, PD-1 blockade may also act through additional mechanisms that contribute partially to its therapeutic efficacy. For example, it has been reported that tumor cell–intrinsic PD-1 can promote melanoma growth (50).

In addition to direct blockade of PD-1, antibodies targeting PD-L1 are also sufficient to induce immune tumor rejection. Blockade of PD-L1 is thought to largely phenocopy the effect of PD-1 blockade given the dominance in expression of PD-L1. PD-L1 is induced by Th1 cytokines (e.g., IFNγ) whereas PD-L2 is induced by Th2 cytokines (100). This differential regulation may in part explain the efficacy of PD-L1 blockade because Th1-skewed responses would be more favorable for antitumor immune responses. In contrast to anti–PD-1 antibodies, blockade of PD-L1 may also derive part of its efficacy from ADCC. It was recently

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demonstrated that Fc receptor binding is important for the efficacy of anti–PD-L1, but not anti–PD-1, antibody therapy–induced tumor regression in murine tumor models (101). Another complicating aspect of the underlying biology is that in addition to canonical binding relationships described, B7-1 and PD-L1 also interact, leading to inhibition of T-cell activity (102). Significantly, these data suggest that anti–PD-1 and anti–PD-L1 therapies are not completely mechanistically equivalent. Recent evidence indicates that host-derived PD-L1 expression is required for the PD-L1 blockade–induced tumor rejection (103, 104). Other evidence indicates, however, that tumor-derived PD-L1 is sufficient to inhibit antitumor immunity via attenuation of CD8 T-cell cytotoxicity (105). How these apparently disparate findings can be integrated remains to be fully understood. Nonetheless, they raise the possibility that PD-L1 can inhibit T cell–mediated tumor cell killing through both cell-autonomous and nonautonomous mechanisms.

THERAPEUTIC COMBINATIONS

Despite the remarkable progress that has been achieved with monotherapies, there is a tremendous need to improve efficacy across tumor types. Understanding which aspects of the tumor microenvironment functionally limit responses to immune checkpoint blockade therapy is an active area of investigation. A primary mechanism is compensatory upregulation of additional immune checkpoint molecules, which limit the therapeutic efficacy of monotherapy approaches. For example, increased expression of PD-L1 (and engagement of PD-1) may in part explain why anti-CTLA4 monotherapy has not resulted in significantly enhanced response rates in tumor types other than melanoma. Consistent with this notion and our understanding that PD-1 and CTLA4 attenuate T-cell activation through distinct mechanisms, combination blockade of PD-1 and CTLA4 improves therapeutic efficacy compared with either monotherapy (106–108). These findings reflect the enhanced efficacy also observed in preclinical models (109). Notably, combination treatment is able to produce complete responses in 36% of patients, and over half of patients with melanoma achieve objective responses (107). Pooled analysis of the 3-year follow-up from the phase II and III clinical trials in melanoma reports a 57% 3-year overall survival in the ipilimumab plus nivolumab group (110). Based on the assumption that durability of responses to combination therapy reaching 3 years will at least equal or exceed that observed in response to ipilimumab monotherapy (77), it is conceivable that greater than half of patients with metastatic melanoma treated with the combination of ipilimumab and nivolumab may achieve long-term responses lasting 10 years or more. Nivolumab plus ipilimumab also improves overall survival versus standard-of-care sunitinib in advanced renal cell carcinoma (111), suggesting that combination therapy may have broad therapeutic efficacy.

Mechanistically, it remains unclear whether the enhanced efficacy of combination anti–PD-1 and anti-CTLA4 therapy is mediated by additive engagement of the cellular and molecular mechanisms of the respective monotherapies or, alternatively, through mechanisms distinct from the component monotherapies. Profiling of peripheral blood supports a model in which PD-1 and CTLA4 act through independent mechanisms, with combination inhibition of PD-1 and CTLA4 leading to distinct immune responses (112). Similar analyses have interestingly revealed immunologic changes in peripheral B cells associated with the development of immune-related adverse events (113). These observations provide additional support to the notion that combination therapy induces distinct cellular and molecular changes and highlight that these mechanisms may be either direct or indirect. Given that PD-1 and CTLA4 attenuate T-cell activity through separate molecular mechanisms and that blockade of these respective molecules regulate distinct cell populations (60), multiple possible mechanisms may underlie the enhanced efficacy of anti-CTLA4 and anti–PD-1 combination therapy (Fig. 3). Resolving this ambiguity and determining the precise cellular and molecular mechanisms of combination anti-CTLA4 plus anti–PD-1 therapy is critical.

The molecular and cellular mechanisms of anti-CTLA4 and anti–PD-1 monotherapies may provide insights, although it remains unclear the degree to which mechanisms of combination therapy directly reflect those of monotherapies. Given that both CTLA4 and PD-1 have cell-intrinsic regulatory activity, simultaneous blockade of both molecules may lead to functional convergence through enhancement of T-cell activity (whether by coregulation of CD28 or other signaling pathways involved in T-cell activation). Conceptually, this convergence may occur in non–mutually exclusive scenarios (Fig. 3). In the first scenario, CTLA4 and PD-1 are simultaneously targeted on the same cell, leading to an additive increase in CD28 costimulation and T-cell activity. In the second scenario, combination therapy targets T cells at different times with respect to activation and/or trafficking. This model is supported by the distinct kinetics of PD-1 and CTLA4 expression during T-cell activation. Through such a mechanism, combination therapy may broaden the duration and integrated strength of T-cell costimulation by CD28. Both of these scenarios are in part predicated on the assumption that anti–PD-1 and anti-CTLA4 target the same cell population. It remains conceptually unclear whether immune checkpoint blockade reestablishes positive costimulatory levels to normal maximal levels (Fig. 4A), expands the range of T-cell clones able to activate by lowering the costimulatory threshold (Fig. 4B), or, alternatively, whether checkpoint blockade is able to increase activity on a per-cell basis by enhancing costimulatory signals beyond normal physiologic levels (Fig. 4C). A fascinating possibility is that positive costimulation beyond physiologic levels may allow for the acquisition of enhanced cytolytic capabilities or novel properties not displayed by canonical T-cell populations.

In addition to dual engagement of convergent molecular pathways, engagement of distinct cellular biology by anti-CTLA4 and anti–PD-1 may also contribute to the enhanced efficacy of combination therapy. Indeed, anti-CTLA4 but not anti–PD-1 checkpoint blockade leads to the expansion of a tumor-infiltrating $ICOS^+$ Th1-like CD4 effector population (60). Consistent with additional reports (90, 91, 114, 115), this suggests that anti–PD-1 primarily acts through targeting of CD8 T-cell populations. This significant difference in the mechanisms of anti-CTLA4 and anti–PD-1 raises the possibility that the enhanced efficacy of combination therapy is due to engagement of multiple distinct populations. Thus,

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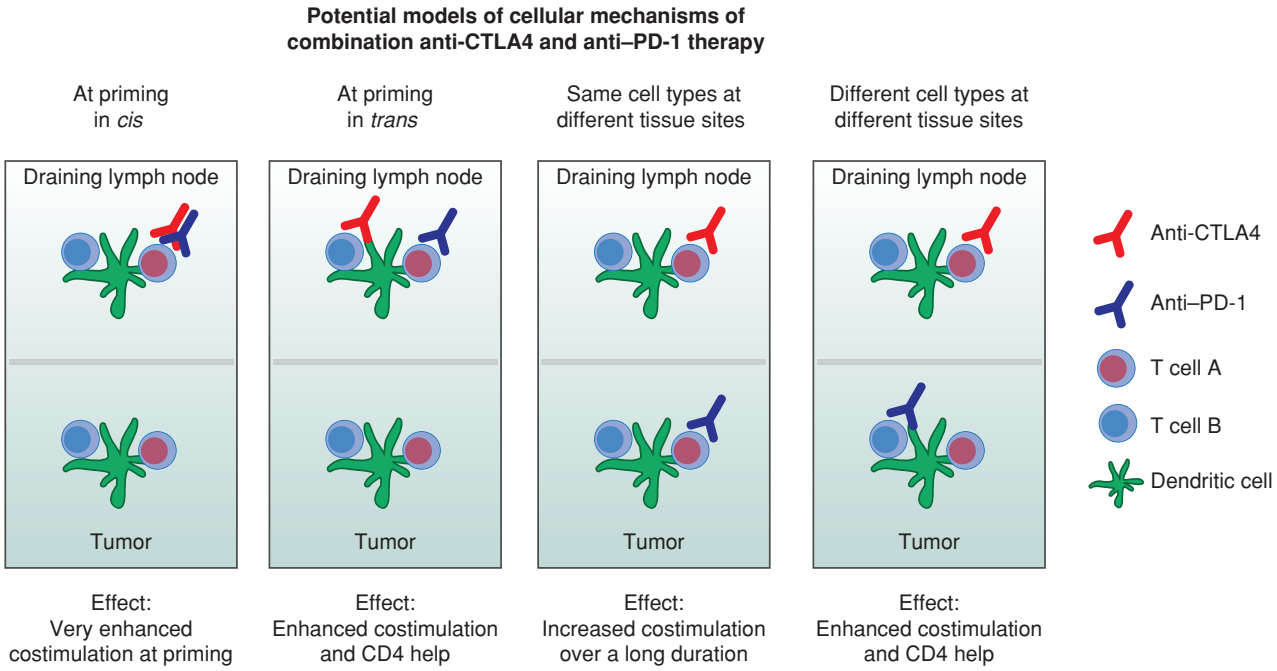

Figure 3. Potential cellular mechanisms that mediate tumor rejection in response to combination anti-CTLA4 and anti–PD-1 checkpoint blockade. Multiple non–mutually exclusive models of the cellular mechanisms underlying combination anti-CTLA4 plus anti–PD-1 therapy of action are proposed. Models described from left to right: (i) the same T cells may be targeted at the site of priming, leading to enhanced penetrance of effective blockade (i.e., a greater proportion of target cells receive sufficient signal to increase activation) and/or enhanced costimulatory signals beyond normal limits, (ii) different T-cell populations are targeted within the site of priming, potentially leading to synergistic effects through cell-extrinsic processes (e.g., providing CD4 help to CD8 effector T cells), (iii) the same T cells are targeted but with different spatiotemporal kinetics leading to perhaps prolonged costimulatory signaling, and (iv) different T-cell populations are targeted in different tissues (e.g., PD-1 blockade primarily acting on preexisting tumor-infiltrating CD8 T cells whereas CTLA4 acts on CD4 effector T cells in secondary lymphoid organs). T-cell subsets are denoted as "A" and "B" given that the precise populations that are directly targeted remain to be fully defined, particularly in the context of kinetics of therapy and different tissue sites. Conceptually, T-cell "A" and "B" could, for example, represent particular subsets of tumor-specific CD8 T cells and CD4 effector T cells, respectively. Potential effects are noted below each scenario; however, there is certain to be additional aggregate effects and differences between these models. Only secondary lymphoid organs (e.g., draining lymph node) and tumor are described, but other tissue sites may have functional contributions to this process as well.

multiple, non–mutually exclusive mechanisms may facilitate the enhanced efficacy of combination therapy (Fig. 3). Thus, combination therapy may utilize cellular and molecular mechanisms as yet unidentified, which are completely distinct from those that mediate monotherapy-induced tumor rejection. Distinguishing these possibilities will be significant in guiding whether insights into monotherapy mechanisms can be extrapolated to understand how respective combinations work.

The engagement of the CD4 effector compartment resulting in expansion of Th1-like CD4 effectors following anti-CTLA4 but not anti–PD-1 also provides some mechanistic rationale for the possibility that sequential treatment of anti-CTLA4 followed by anti–PD-1 therapy may be advantageous. An increase in CD4 help during the priming and early activation stage as a result of CTLA4 blockade would likely improve T-cell memory development as well as infiltration into peripheral tissues (e.g., tumor). However, upon entry into the tumor microenvironment, Th1 and CD8 effector T cells will induce PD-L1 expression on tumor cells and stromal cells, attenuating T-cell activity. Thus, sequential combination of first CTLA4 and then PD-1 blockade could potentially induce T-cell infiltration of immunologically barren tumors and allow them to maintain effective cytolytic activity within the tumor microenvironment. In contrast, results from a phase II open-label study suggest that nivolumab followed by ipilimumab has improved efficacy compared with the ipilimumab followed by nivolumab in advanced melanoma (116). These observations remain to be further validated, but nonetheless raise the question of whether sequential therapies can be designed based only on mechanisms of action of monotherapies and also the extent to which properties such as kinetics of response need to be considered. In addition, these observations raise the possibility that anti-CTLA4–induced CD4 help may not be required for rejection of already well-infiltrated tumors (e.g., melanoma). Nonetheless, sequential treatment may minimize the increase in adverse events that are associated with simultaneous combination treatment. On the other hand, simultaneous combination therapy has enhanced overall response rates in melanoma compared with monotherapies (107, 110), and a key unanswered question is whether sequenced therapy has similarly enhanced response rates and long-term efficacy compared with simultaneous combination therapy.

More broadly, the relative contribution of each of the several known molecular mechanisms of CTLA4 and PD-1 blockade to therapeutic efficacy remains unclear. Such differences may manifest in distinct requirements for the induction of effective immune responses in the context of each therapy. For example, several lines of evidence indicate that cross-priming mediated

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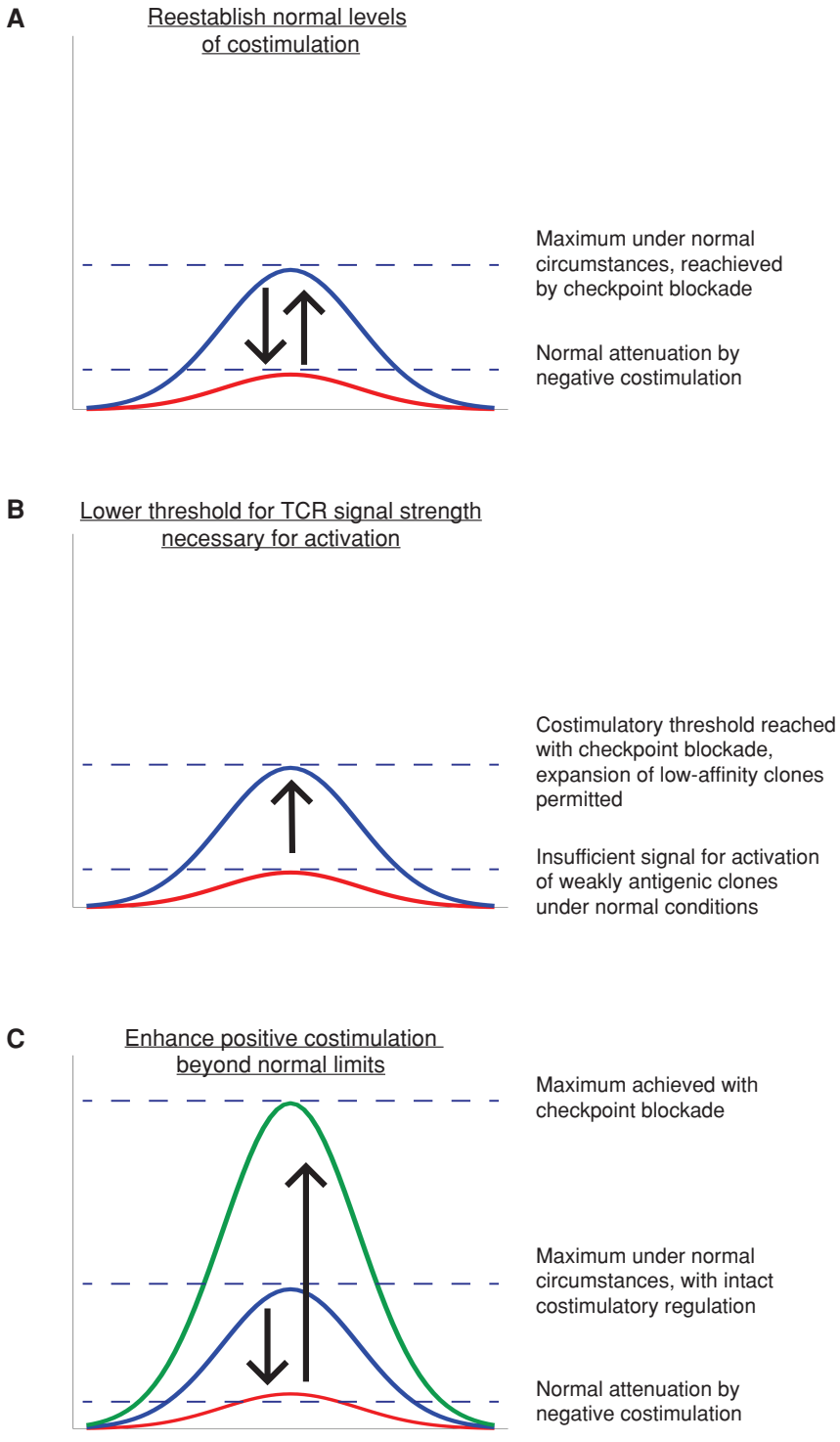

Figure 4. Potential models of how immune checkpoint blockade restores positive costimulation and modulates T-cell activity. Three non–mutually exclusive theoretical models of how checkpoint blockade regulates the strength of positive costimulation and enhances antitumor immunity. Distinguishing these models will have significant implications for the types and specificities of T cells that are functionally essential for therapeutic efficacy. **A,** In the first model, checkpoint blockade restores the positive costimulatory signaling to levels similar to those reached prior to attenuation (e.g., by PD-1 or CTLA4). Presumably, in this model, enhanced efficacy due to checkpoint blockade would be primarily derived from an increase in the number of activated and cytolytic T cells. **B,** In the second model, increased positive costimulation due to blockade of negative costimulatory molecules lowers the effective threshold required for TCR signal strength. This in effect would allow for activation and expansion of weaker T-cell clones (i.e., low-affinity, low-avidity), which are normally restrained. **C,** In the third model, checkpoint blockade leads to an increase in positive costimulatory signals beyond levels that are achieved in normal scenarios. In this model, enhanced efficacy could be derived from increased number of activated T cells and/or acquisition of new or enhanced functional properties due to super physiologic levels of costimulatory signaling.

by CD103$^+$ BATF3-dependent dendritic cells is required for effective antitumor immunity and responses to checkpoint blockade (117–119). Indeed, CD103$^+$ dendritic cell populations appear to be the primary cell population to efficiently take up tumor antigen and present it within the draining lymph node (120). Whether the same modes of antigen presentation are important for anti-CTLA4 and anti–PD-1 therapies is unclear. Mechanistic differences between these therapies may impose distinct requirements in terms of the cellular context and temporal dynamics of antigen presentation.

How immune checkpoint blockade mechanistically interacts with conventional therapies (e.g., surgery, chemotherapy, radiation, targeted therapies) and other immune-based therapies (e.g., chimeric antigen receptor T-cell therapy, other adoptive transfer

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approaches, cytokine therapy, personalized tumor vaccines) is a topic of clear relevance and active investigation. For example, radiation treatment and blockade of the PD-1/PD-L1 axis have been shown to have additive effects through nonredundant mechanisms (121, 122). Notably, abscopal responses have been observed following concurrent radiation and CTLA4 blockade, highlighting a potential mechanistic basis for synergistic efficacy (123, 124). Targeted inhibition of immunosuppressive myeloid populations in combination with immune checkpoint blockade therapy leads to enhanced efficacy (125). Additional clinical variables and patient characteristics may also prove to be significant modulators of response to immunotherapy. For example, recent work has elucidated a role for the gut microbiome in defining tumor responses to immunotherapies. Colonization by specific commensal bacteria strains modulates the efficacy of immune checkpoint blockade therapy in preclinical and clinical settings (126–129). This highlights how a diverse set of host properties, in addition to tumor characteristics, can contribute to sensitivity to immunotherapy.

BEYOND CTLA4 AND PD-1

T-cell costimulatory molecules as a functional category represent a large number of proteins belonging to multiple structurally defined superfamilies. The therapeutic potential of many of these targets is now being investigated preclinically and clinically. Among these molecules are LAG3, TIM3, TIGIT, VISTA, and ICOS from the immunoglobulin superfamily (IgSF) and OX40, GITR, 4-1BB, CD40, and CD27 from the tumor necrosis factor receptor superfamily (TNFRSF). However, our collective understanding of the fundamental biological roles of these molecules remains unsatisfactory and, in many cases, is being outpaced by clinical investigation. There are many additional costimulatory molecules of potential therapeutic value, including newly identified B7 ligand family members (130, 131) as well as, undoubtedly, additional as yet uncharacterized regulatory molecules (132). A summary of the current state of our understanding of the biology of these molecules is described in Table 2.

Deeper understanding of the basic biological roles of costimulatory molecules is critical for the rational development of new immune checkpoint blockade therapies. For example, even as therapies targeting other costimulatory molecules move forward in clinical trials, it remains unclear in several instances what the identity of the associated ligand(s) or receptor(s) is, or even whether the target is the receptor or ligand. More ubiquitously, in most cases the precise molecular mechanisms remain unresolved. In addition to cases in which the biology simply remains unknown, there has also been confusion caused by apparently discordant data within the field. Whether these findings reflect additional as yet unappreciated biological complexity or, alternatively, technical differences in experimental systems remains to be fully resolved.

For example, although major histocompatibility complex II (MHC-II) has been previously reported to be the ligand of the coinhibitory receptor LAG3 (133), LSECtin has also been reported to be an additional ligand (134). LSECtin is expressed by liver and tumor cells and may account for the biological role of LAG3 in CD8 and natural killer (NK) cells, as neither cell type interacts with MHC-II. Even more complexity has been observed in the context of the coinhibitory receptor TIM3, as four ligands have been reported to date: Galectin-9 (135), PtdSer (136), HMGB1 (137), and CEACAM1 (138). How ligand interactions are regulated, whether they affect each other's binding, and whether each ligand leads to unique downstream signaling events remains unclear. Furthermore, although TIM3 is thought of primarily as a marker of T-cell activation and exhaustion, TIM3 also functions to attenuate NK cell cytotoxicity (139). This observation is conceptually significant beyond its pertinence to TIM3 biology, as it suggests that other costimulatory molecules have biologically significant functions in multiple cell types. VISTA presents yet additional ambiguity, as studies have described it as both a ligand on APCs (with homology to PD-L1) with an unknown receptor (140) and as a receptor on T cells with an unknown ligand (141). Similarly, the biological roles of several B7 ligand family members, including their counterreceptors, remain undetermined. B7-H3 is believed to have both costimulatory and coinhibitory roles, possibly dependent on its expression context, whereas both its receptor and the molecular mechanisms of its posttranscriptional regulation remain unclear (142).

Our understanding of the biological functions of costimulatory molecules has been augmented by preclinical and clinical studies using immunomodulatory agents. For example, TIGIT and PD-1 are coexpressed by human melanoma infiltrating NY-ESO-1–specific CD8 T cells (143), consistent with preclinical findings that dual blockade can enhance tumor-infiltrating CD8 T-cell effector function and tumor rejection (144). These findings are consistent with prior observations that TIGIT is induced upon activation and regulates TCR activation pathways in a cell-intrinsic manner (145). These observations suggest that TIGIT and PD-1 blockade may act in *cis*, but through additive mechanisms enhancing T-cell activity. Mechanistic studies have also revealed potential combinatorial strategies to target other nonredundant aspects (e.g., tissue site of action, immune cell population, and biological process) of the cancer-immunity life cycle (51). Analysis of clinical samples reveals that VISTA is expressed primarily on M2 macrophages following ipilimumab treatment in the context of prostate cancer (146). In addition, VISTA and PD-1/PD-L1 have been shown to have nonredundant inhibitory effects on T cells (147). Engagement of innate immunity represents another aspect that can be leveraged to develop effective antitumor immunity. For example, treatment with CD40 agonistic antibodies enhances APC function and, together with chemotherapy, is able to induce effective T cell–dependent immune responses to immunologically "cold" tumors (148, 149). Such studies that identify potential therapies with nonredundant, and ideally synergistic, mechanisms of action will be critical in guiding rational design of combination therapies.

CONCLUDING REMARKS

Here, we have reviewed the current understanding of the biological functions of T-cell costimulatory molecules and the mechanisms through which blockade of these molecules can induce tumor rejection. We have largely focused on CTLA4 and PD-1 immune checkpoint blockade, as well as additional costimulatory molecules of therapeutic interest. Much remains to be understood in how CTLA4, PD-1, and other costimulatory molecules actually attenuate T-cell

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Table 2. Summary of the biological and molecular functions of T-cell costimulatory molecules

Molecule	Ligand(s)	Receptor expression pattern	Biological function	Molecular function	References
Coinhibitory					
CTLA4	B7-1 (CD80), B7-2 (CD86)	Activated T cells, Treg	Negative T-cell costimulation (primarily at priming); prevent tonic signaling and/or attenuate high-affinity clones	Competitive inhibition of CD28 costimulation (binding of B7-1 and B7-2)	(8, 10–12, 38, 157–161)
PD-1	PD-L1, PD-L2	Activated T cells, NK cells, NKT cells, B cells, macrophages, subsets of DC; as a result of inflammation	Negative T-cell costimulation (primarily in periphery); attenuate peripheral activity, preserve T-cell function in the context of chronic antigen	Attenuate proximal TCR signaling, attenuate CD28 signaling	(32–35, 38, 39, 53, 100, 162–165)
PD-L1	PD-1, B7-1 (CD80)	Inducible in DC, monocytes, macrophages, mast cells, T cells, B cells, NK cells	Attenuate T-cell activity in inflamed peripheral tissues	PD-1 ligation; cell-intrinsic mechanism unclear	(33, 34, 102)
LAG3	MHC-II, LSECtin	Activated CD4 and CD8 T cells, NK cells, Treg	Negative regulator of T-cell expansion; control T-cell homeostasis; DC activation	Competitive binding to MHC-II; proximal LSECtin mechanism unknown	(133, 134, 166–170)
TIM3	Galectin-9, PtdSer, HMGB1, CEACAM-1	Th1 CD4 and Tc1 CD8, Treg, DC, NK cells, monocytes	Negative regulation of Type 1 immunity; maintain peripheral tolerance	Negative regulation of proximal TCR components; differences between ligands unclear	(135–139, 171)
TIGIT	PVR (CD155), PVRL2 (CD112)	CD4 and CD8, Treg, TFH, NK cells	Negative regulation of T-cell activity; DC tolerization	Competitive inhibition of DNAM1 (CD226) costimulation (binding of PVR), binding of DNAM1 in *cis*; cell-intrinsic ITIM-negative signaling	(144, 145, 172–176)
VISTA	Counter-receptor unknown	T cells and activated Treg, myeloid cells, mature APC	Negative regulation of T-cell activity; suppression of CD4 T cells	Increase threshold for TCR signaling, induce FOXP3 synthesis; proximal signaling unknown	(140, 141, 146, 147, 177, 178)
Costimulatory					
ICOS	ICOSL	Activated T cells, B cells, ILC2	Positive costimulation; Type I and II immune responses; Treg maintenance; TFH differentiation	p50 PI3K recruitment (AKT signaling); enhance calcium signaling (PLCγ)	(179–186)
OX40	OX40L	Activated T cells, Treg, NK cells, NKT cells, neutrophils	Sustain and enhance CD4 T-cell responses; role in CD8 T cells and Tregs	Regulation of BCL2/XL (survival); enhance PI3K/AKT signaling	(187–193)
GITR	GITRL	Activated T cells, Treg, B cells, NK cells, macrophages	Inhibition of Tregs; costimulation of activated T cells, NK cell activation	Signal through TRAF5	(194–200)
4-1BB (CD137)	4-1BBL	Activated T cells, Treg, NK cells, monocytes, DC, B cells	Positive T-cell costimulation; DC activation	Signal through TRAF1, TRAF2	(201–205)
CD40	CD40L	APCs, B cells, monocytes, nonhematopoietic cells (e.g., fibroblasts, endothelial cells)	APC licensing	Signal through TRAF2, 3, 5, 6; TRAF-independent mechanisms?	(206–209)
CD27	CD70	CD4 and CD8 T cells, B cells, NK cells	Lymphocyte and NK cell costimulation; generation of T-cell memory	Signal through TRAF2, TRAF5	(210–214)

NOTE: A summary of the ligands, immunologic expression pattern, biological function, and molecular mechanisms is presented for selected costimulatory and coinhibitory receptors. Molecular functions (i.e., downstream signaling) reflect predominant currently known mechanisms, but additional mechanisms are likely to contribute significantly.

Abbreviations: NK, natural killer; NKT, natural killer T cell; TFH, T follicular helper; TRAF, tumor necrosis factor receptor–associated factors; DC, dendritic cell.

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activation at the molecular, cellular, and physiologic levels. Such mechanistic insight into the biological functions of these molecules will be critical for the development of new approaches and continued improvement of immunotherapeutic strategies. Moving forward, it is likely that combinatorial therapies, utilizing one or more immunotherapies, will become standard of care for a wide breadth of tumor types. Fundamental investigation and understanding of the underlying biology are likely to reveal additional potent biological variables that we have yet to appreciate.

A critical open question is the degree to which the manifestation of irAEs is functionally and mechanistically associated with therapeutic efficacy. If distinct mechanisms underlie these biological responses, a tantalizing possibility is that mechanisms underlying efficacy and irAEs may be able to be engaged separately. Understanding the etiology of irAEs will be even more important in the context of combination therapies, which, at least in the context of anti-CTLA4 and anti–PD-1, have higher rates of irAEs than monotherapies. The safety profile of combination ipilimumab plus nivolumab therapy has been previously reviewed (150). Although most irAEs associated with checkpoint blockade therapy do not reflect the induction of autoimmunity, emerging evidence indicates that autoimmune conditions such as type 1 diabetes and myocarditis can develop at very low frequencies. Fulminant myocarditis has been reported as a potential rare adverse event due to anti–PD-1 monotherapy and combination CTLA4 and PD-1 blockade (151, 152). The development of such rare autoimmune adverse events will become even more relevant as checkpoint blockade therapies are utilized in neoadjuvant (prior to surgery) and adjuvant (following surgery) clinical settings. Understanding how specific immune checkpoint blockade therapies modulate the T-cell repertoire and T-cell function will be essential for distinguishing mechanisms that underlie therapeutic efficacy and irAEs. Indeed, distinct immunologic profiles are associated with colitis induced by anti-CTLA4 and anti–PD-1 therapy (153). One of the key limitations that is currently hampering efforts to understand the manifestation of irAEs is the lack of appropriate preclinical animal models. The development of animal models that faithfully recapitulate irAEs is greatly needed to enable mechanistic investigation of immune checkpoint blockade–associated irAEs.

Of central importance to the mechanisms of action of immune checkpoint blockade therapy is understanding what properties define the antigens that are actually being recognized and mediating tumor rejection. It has been observed that the T-cell repertoire broadens following anti-CTLA4 therapy in patients with melanoma (78). Conversely, response to PD-1 may correlate with reduced intratumoral T-cell clonality (89). This apparent contradiction may reflect observations that tumor regression is often mediated by a small number of dominant neoepitopes (99, 154). Indeed, conservation of abundant clonotypes is associated with better clinical response to anti-CTLA4 therapy (79). Relatedly, PD-1 blockade agents (nivolumab and pembrolizumab) have remarkable efficacy in mismatch repair–deficient and microsatellite instability–high adult and pediatric tumors (83, 84, 155). In addition to these therapies receiving the first tumor tissue–agnostic FDA approval, this is significant because it provides an example of how mechanistic understanding can identify patient populations likely to benefit from immunotherapeutic approaches. It is important, however, to point out that neoantigen burden represents only one mechanism through which tumors can be recognized by the immune system. Based on the correlation between response rates to anti–PD-1 therapies and TMB across tumor types, it has been estimated that 55% of the variation in therapeutic efficacy can be explained by TMB (87). It is of critical importance to understand additional biological properties, tumor-intrinsic or host-derived, that are significant modulators of therapeutic response. For example, recent studies reveal that genomic lesions in a chromatin remodeling complex component are associated with response to checkpoint blockade (156), providing potential alternative mechanisms of immune recognition.

Immunotherapy has ushered cancer treatment into a new era. In order to translate the progress and success to additional tumor types and to increase the proportion of patients that attain durable responses, we must continue to strive to understand the underlying biological mechanisms. In this review we have highlighted known mechanisms of anti-CTLA4 and anti–PD-1 immune checkpoint inhibitors, but these are by no means complete. There are surely additional surprises awaiting us as we move forward, expanding our understanding of the immune system and its role throughout tumor progression.

Disclosure of Potential Conflicts of Interest

J.P. Allison has ownership interest (including stock, patents, etc.) in Jounce Therapeutics, Forty Seven, ImaginAb, Marker Therapeutics, Tvardi, Constellation, Neon Therapeutics, Apricity, BioAtla, and Polaris, and is a consultant/advisory board member for Jounce Therapeutics, Forty Seven, ImaginAb, Marker Therapeutics, Tvardi, Amgen, Oncolytics, Pieris, Neon Therapeutics, Apricity, BioAtla, and Polaris. No potential conflicts of interest were disclosed by the other authors.

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