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Perspective

Immune Checkpoint Blockade: A Common Denominator Approach to Cancer Therapy

Suzanne L. Topalian,[1,*] Charles G. Drake,[2,3] and Drew M. Pardoll[3]
[1]Department of Surgery
[2]The Brady Urological Institute
[3]Department of Oncology
Sidney Kimmel Comprehensive Cancer Center and Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA
*Correspondence: stopali1@jhmi.edu
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The immune system recognizes and is poised to eliminate cancer but is held in check by inhibitory receptors and ligands. These immune checkpoint pathways, which normally maintain self-tolerance and limit collateral tissue damage during anti-microbial immune responses, can be co-opted by cancer to evade immune destruction. Drugs interrupting immune checkpoints, such as anti-CTLA-4, anti-PD-1, anti-PD-L1, and others in early development, can unleash anti-tumor immunity and mediate durable cancer regressions. The complex biology of immune checkpoint pathways still contains many mysteries, and the full activity spectrum of checkpoint-blocking drugs, used alone or in combination, is currently the subject of intense study.

In the current era in oncology emphasizing personalized therapy, immune checkpoint blockade is distinguished by its "common denominator" approach. Although the vast somatic mutational diversity found in most human cancers creates challenges for therapies targeting individual mutations, it exposes a panoply of new antigens for potential immune recognition. However, cells of the adaptive and innate immune systems that recognize and are poised to attack cancer are held in check by molecular pathways that suppress their activation and effector functions. The seminal observation that blocking the prototypical immune checkpoint receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) could mediate tumor regression in murine models (Leach et al., 1996) led to the clinical development and approval of anti-CTLA-4 as a treatment for patients with advanced melanoma (Hodi et al., 2010). Subsequently, drugs blocking the distinct checkpoints Programmed Death 1 (PD-1) and its major ligand PD-L1 have shown great promise in treating many diverse cancer types, fueling the intensive examination of a growing cohort of unique checkpoint molecules as potential therapeutic targets. This has revealed new treatment options for patients and has revolutionized our approach to cancer therapy.

Biology of Immune Checkpoints: The Basics

The rapid-fire clinical successes from blocking CTLA-4 and PD-1, the first checkpoint receptors to be discovered, have opened prospects for extending the potential of cancer immunotherapy by inhibiting more recently discovered checkpoint ligands and receptors. It is clear that, despite some commonalities, CTLA-4 and PD-1 have distinct patterns of expression, signaling pathways, and mechanisms of action. Although discovered over 20 years ago, there are still many unanswered questions about their biology, particularly in the context of cancer.

The CD28/CTLA-4 System of Immune Modulation

The conventional wisdom underlying our vision of how CTLA-4 blockade mediates tumor regression is that it systemically activates T cells that are encountering antigens. CTLA-4 represents the paradigm for regulatory feedback inhibition. Its engagement down-modulates the amplitude of T cell responses, largely by inhibiting co-stimulation by CD28, with which it shares the ligands CD80 (B7.1) and CD86 (B7.2) (Figure 1; Lenschow et al., 1996). As a "master T cell co-stimulator," CD28 engagement amplifies TCR signaling when the T cell receptor (TCR) is also engaged by cognate peptide-major histocompatibility complex (MHC) (Schwartz, 1992). However, CTLA-4 has a much higher affinity for both CD80 and CD86 compared with CD28 (Linsley et al., 1994), so its expression on activated T cells dampens CD28 co-stimulation by out-competing CD28 binding and, possibly, also via depletion of CD80 and CD86 via "*trans*-endocytosis" (Qureshi et al., 2011). Because CD80 and CD86 are expressed on antigen-presenting cells (APCs; e.g., dendritic cells and monocytes) but not on non-hematologic tumor cells, CTLA-4's suppression of anti-tumor immunity has been viewed to reside primarily in secondary lymphoid organs where T cell activation occurs rather than within the tumor microenvironment (TME). Furthermore, CTLA-4 is predominantly expressed on CD4+ "helper" and not CD8+ "killer" T cells. Therefore, heightened CD8 responses in anti-CTLA-4-treated patients likely occur indirectly through increased activation of CD4+ cells. Of note, a few studies suggest that CTLA-4 can act as a direct inhibitory receptor of CD8 T cells (Fallarino et al., 1998; Chambers et al., 1998), although this role in down-modulating anti-tumor CD8 T cell responses remains to be directly demonstrated.

The specific signaling pathways by which CTLA-4 inhibits T cell activation are still under investigation, although activation of the phosphatases SHP2 and PP2A appears to be important in counteracting both tyrosine and serine/threonine kinase signals induced by TCR and CD28 (Rudd et al., 2009). CTLA-4 engagement also interferes with the "TCR stop signal," which maintains the immunological synapse long enough for extended or serial interactions between TCR and its peptide-MHC ligand (Schneider et al., 2006). Naive and resting memory T cells express CD28, but not CTLA-4, on the cell surface, allowing co-stimulation to dominate upon antigen recognition. However, CTLA-4 is rapidly mobilized to the cell surface from intracellular

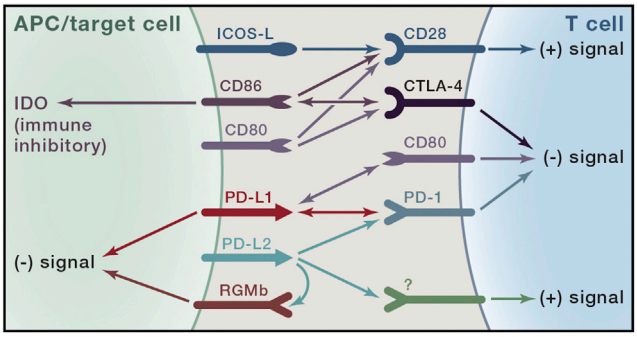

Figure 1. Complex Interactions between the CTLA-4/CD28 and PD-1 Families of Receptors and Ligands

Shown are the defined interactions between the co-inhibitory (checkpoint) receptors CTLA-4 and PD-1 and their ligands and related receptors. The two known ligands for CTLA-4 are CD80 (B7.1) and CD86 (B7.2). CD86 can "backward-signal" into APCs when engaged by CTLA-4, inducing the immune inhibitory enzyme IDO. CD80 and CD86 also bind the co-stimulatory receptor CD28 on T cells. Recently, another B7 family member, inducible costimulator ligand (ICOS-L), which was discovered as the ligand for the co-stimulatory receptor ICOS (not shown), has been reported to bind to CD28, leading to co-stimulation independent of CD80 or CD86. The two defined ligands for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC), bind to additional molecules. PD-L1 binds CD80 molecules expressed on activated T cells, mediating inhibition. Additionally, PD-L1 on APCs appears to provide inhibitory signals (backward signaling) when it is engaged by PD-1. PD-L2 binds another molecule, RGMb, which is expressed on macrophages and some epithelial cell types and appears to deliver an inhibitory immune signal through an as yet undefined mechanism. Although not identified, genetic evidence from *PD-1* knockout T cells and knockout mice suggests the existence of another receptor for PD-L2 that is co-stimulatory.

protein stores, allowing feedback inhibition to occur within an hour after antigen engagement. The central role of CTLA-4 in maintaining immune tolerance is dramatically demonstrated by the rapidly lethal systemic immune hyperactivation phenotype of *Ctla-4* knockout mice (Tivol et al., 1995; Waterhouse et al., 1995). In humans, anti-CTLA-4 treatment induces expression of activation markers on circulating T cells (Maker et al., 2005) and a high rate of inflammatory side effects (Phan et al., 2003). However, because melanoma patients appear to possess an unusually high proportion of tumor-reactive T cells, anti-tumor responses balance autoimmune toxicity and provide a clinical benefit to roughly 20% of patients with this disease (see below).

PD-1: Similarities to and Differences from CTLA-4

The PD-1 system of immune modulation bears similarities to CTLA-4 as well as key distinctions (Parry et al., 2005). Similar to CTLA-4, PD-1 is absent on resting naive and memory T cells and is expressed upon TCR engagement. However, in contrast to CTLA-4, PD-1 expression on the surface of activated T cells requires transcriptional activation and is therefore delayed (6–12 hr). Also in contrast to CTLA-4, PD-1 contains a conventional immunoreceptor tyrosine inhibitory motif (ITIM) as well as an immunoreceptor tyrosine switch motif (ITSM). PD-1's ITIM and ITSM bind the inhibitory phosphatase SHP-2. PD-1 engagement can also activate the inhibitory phosphatase PP2A. PD-1 engagement directly inhibits TCR-mediated effector functions and increases T cell migration within tissues, thereby limiting the time that a T cell has to survey the surface of interacting cells for the presence of cognate peptide-MHC complexes. Therefore, T cells may "pass over" target cells expressing lower levels of peptide-MHC complexes (Honda et al., 2014).

In contrast to CTLA-4, PD-1 blockade is viewed to work predominantly within the TME, where its ligands are commonly overexpressed by tumor cells as well as infiltrating leukocytes (Keir et al., 2008). This mechanism is thought to reflect its important physiologic role in restraining collateral tissue damage during T cell responses to infection. In addition, tumor-infiltrating lymphocytes (TILs) commonly express heightened levels of PD-1 and are thought to be "exhausted" because of chronic stimulation by tumor antigens, analogous to the exhausted phenotype seen in murine models of chronic viral infection, which is partially reversible by PD-1 pathway blockade (Barber et al., 2006).

Importantly, the phenotypes of murine knockouts of *PD-1* and its two known ligands are very mild, consisting of late-onset organ-specific inflammation, particularly when crossed to autoimmune-prone mouse strains (Nishimura et al., 1999, 2001). This contrasts sharply with the *Ctla-4* knockout phenotype and highlights the importance of the PD-1 pathway in restricting peripheral tissue inflammation. Furthermore, it is consistent with clinical observations that autoimmune side effects of anti-PD-1 drugs are generally milder and less frequent than with anti-CTLA-4.

Despite the conventional wisdom that CTLA-4 acts early in T cell activation in secondary lymphoid tissues whereas PD-1 inhibits execution of effector T cell responses in tissue and tumors, this distinction is not absolute. Beyond its role in dampening activation of effector T cells, CTLA-4 plays a major role in driving the suppressive function of T regulatory (Treg) cells (Wing et al., 2008; Peggs et al., 2009). Tregs, which broadly inhibit effector T cell responses, are typically concentrated in tumor tissues and are thought to locally inhibit anti-tumor immunity. Therefore, CTLA-4 blockade may affect intratumoral immune responses by inactivating tumor-infiltrating Treg cells. Recent evidence has demonstrated anti-tumor effects from CTLA-4 blockade even when S1P inhibitors block lymphocyte egress from lymph nodes (Spranger et al., 2014), indicating that this checkpoint exerts at least some effects directly in the TME as opposed to secondary lymphoid tissues. Conversely, PD-1 has been shown to play a role in early fate decisions of T cells recognizing antigens presented in the lymph node. In particular, PD-1 engagement limits the initial "burst size" of T cells upon antigen exposure and can partially convert T cell tolerance induction to effector differentiation (Goldberg et al., 2007).

Complex Receptor-Ligand Interactions in the PD-1 Pathway: Links and Analogies to the CD28/CTLA-4 System

The receptor-ligand interactions of the PD-1 system appear to be even more complex than the CD28/CTLA-4 system (Figure 1). The two ligands for PD-1 are PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), which share 37% sequence homology and arose via gene duplication (Dong et al., 1999, Latchman et al., 2001; Tseng et al., 2001). However, their regulation is highly divergent. PD-L1 is induced on activated hematopoietic cells and on epithelial cells by the inflammatory cytokine interferon (IFN)-γ, which is produced by some activated T and natural killer (NK) cells. PD-L2 has much more selective expression on activated dendritic cells and some macrophages. It is induced to a

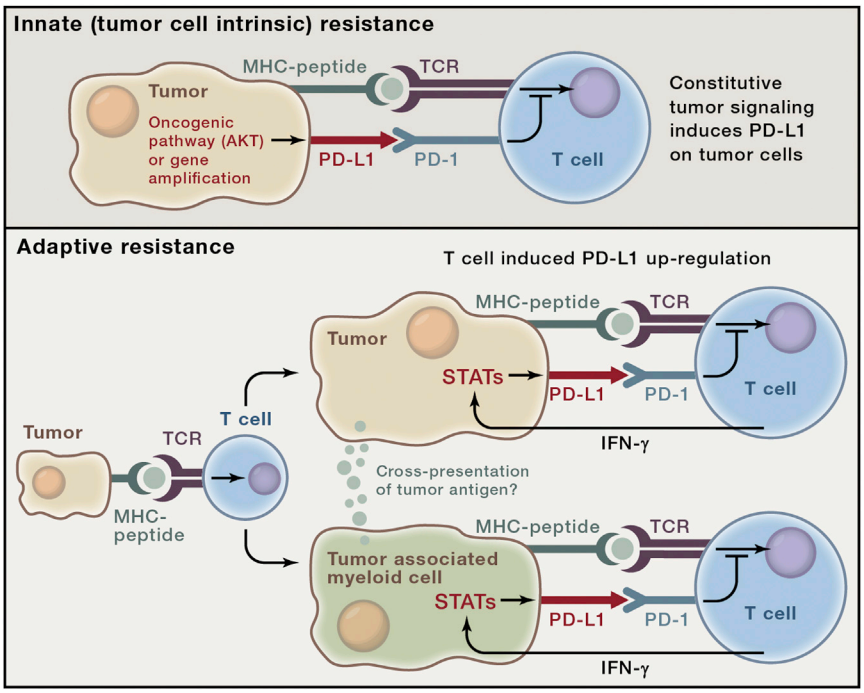

Figure 2. Two General Mechanisms for Expression of Checkpoint Ligands in the TME

The examples in this figure use the PD-1 ligand PD-L1 for illustrative purposes, although the concept likely applies to multiple checkpoint ligands. Top: innate immune resistance. In some tumors, constitutive oncogenic signaling, such as through activation of the AKT pathway or gene amplification, can upregulate PD-L1 expression on tumor cells independently of inflammatory signals in the TME. Bottom: adaptive immune resistance refers to PD-L1 induction in tumors as an adaptation to the sensing of an immune attack. In adaptive resistance, PD-L1 is not constitutively expressed but, rather, is induced by inflammatory signals, such as IFN-γ produced by T cells attempting to execute an active anti-tumor response. Expression of PD-L1 in a non-uniform distribution associated with lymphocyte infiltrates suggests adaptive induction in response to immune reactivity within the TME. Adaptive resistance can be generated by cytokine-induced PD-L1 expression on either tumor cells themselves or on leukocytes (macrophages, myeloid suppressor cells, dendritic cells, or even lymphocytes) in the TME. Inhibition of tumor-specific T cells by PD-L1- or PD-L2-expressing leukocytes may involve cross-presentation of tumor antigens so that PD-1-dependent inhibition is in *cis*. Adaptive resistance may be a common mechanism for the intratumoral expression of multiple immune checkpoint molecules.

much greater extent by interleukin 4 (IL-4) than by IFN-γ, further emphasizing differences in regulation of expression of PD-L1 and PD-L2.

Beyond their role as ligands for PD-1, PD-L1 and PD–L2 appear to have additional partners, indicating additional layers of immune modulation. An unexpected molecular interaction between PD-L1 and CD80 has been discovered (Butte et al., 2007; Park et al., 2010) whereby CD80 expressed on activated T cells (and possibly APCs) can function as a receptor rather than a ligand, delivering inhibitory signals when engaged by PD-L1. The relevance of this interaction in tumor immune resistance has not yet been determined. Recently, PD-L2 has been shown to bind to repulsive guidance molecule b (RGMb), which itself binds at least three other molecules in *cis* (neogenin and BMP receptors type I and II) (Xiao et al., 2014). This interaction appears to be inhibitory independent of PD-1, as demonstrated in a pulmonary tolerance model. Finally, evidence from murine models suggests that PD-L2 and, possibly, PD-L1 may bind to a co-stimulatory T cell receptor (Shin et al., 2003, 2005), an arrangement reminiscent of the CD80/CD86 ligand pair for the co-stimulatory CD28 and co-inhibitory CTLA-4 receptors. Understanding the roles of these various interactions in cancer is highly relevant for the development of immunomodulatory drugs and the discovery of biomarkers predictive of therapeutic response.

Mechanisms of PD-1 Ligand Induction: Implications for Cancer Immunotherapy

A key finding that encouraged the development of drugs blocking the PD-1 pathway for cancer immunotherapy was that PD-1 ligands are upregulated in many human cancers (Dong et al., 2002), whereas PD-1 is highly expressed on tumor-infiltrating lymphocytes (Ahmadzadeh et al., 2009; Sfanos et al., 2009). Indeed, PD-L1 appears to be the major ligand expressed in solid tumors, whereas PD-L2 (together with PD-L1) is highly expressed in certain subsets of B cell lymphomas (Ansell et al., 2015). Exploration of this phenomenon as a central process by which cancers resist elimination by endogenous tumor-specific T cells revealed two mechanisms for PD-1 ligand upregulation in cancer, known as intrinsic and adaptive immune resistance (Figure 2). These mechanisms are not mutually exclusive and may co-exist in the same TME. Intrinsic resistance refers to the constitutive expression of PD-L1 by tumor cells because of genetic alterations or activation of certain signaling pathways, such as the AKT pathway and STAT3, which are commonly activated in many cancers (Parsa et al., 2007; Marzec et al., 2008). Although PD-L1 induction by AKT and STAT3 signaling has been demonstrated in some tumor cell lines, the importance of this intrinsic pathway in PD-L1 expression by tumors in vivo remains to be determined. Genetic alterations in B cell lymphoma subtypes can drive expression of either or both PD-L1 and PD-L2. Primary mediastinal lymphomas commonly display gene fusions between MHC class II transactivator (CIITA) and PD-L1 or PD-L2, placing PD-1 ligands under the transcriptional control of the CIITA promoter, which is highly active in B cell lymphomas (Steidl et al., 2011). A significant subset of Hodgkin's lymphoma has amplification of chromosome 9p23-24, where *PD-L1* and *PD-L2* reside, resulting in overexpression of both ligands. Other cancers, such as a subset of Epstein-Barr virus-induced gastric cancers, also display gene amplification with consequent induction of PD-L1 and PD-L2.

The second mechanism, adaptive resistance, refers to the induction of PD-L1 expression on tumor cells in response to specific cytokines, in particular IFN-γ. Because IFN-γ is only produced by activated Th1-type helper CD4 cells, activated CD8

cells, and NK cells, this mechanism represents an adaptation of tumor cells upon "sensing" an inflammatory immune microenvironment that "threatens" the tumor. Indeed, human tumors show significant correlations between PD-L1 expression, levels of T cell infiltration, and IFN-γ in the TME (Taube et al., 2012; Spranger et al., 2013). Other inhibitory molecules in the TME, such as indoleamine 2′3′ dioxygenase (IDO), which inhibits immunity locally via conversion of tryptophan to kynurenines, are also induced by IFN-γ and coordinately upregulated with PD-L1. The concept of adaptive resistance does not solely apply to induction of PD-L1 on tumor cells. Early studies demonstrated that PD-L1 expression on myeloid cells, including dendritic cells, can significantly impair activation of tumor-specific T cells. Inhibition of T cell responses can be mediated by PD-L1+ suppressive myeloid cells or dendritic cells (DCs) in the TME as well as in tumor-draining lymph nodes (Curiel et al., 2003). In some tumors, such as microsatellite instability (MSI) colon cancer, myeloid rather than tumor cells are the major cell type expressing PD-L1 (Llosa et al., 2015). A recent report suggests that PD-L1 expression by infiltrating myeloid cells rather than tumor cells is more predictive of response to PD-1 pathway blockade (Herbst et al., 2014). The relative importance of PD-L1 expression on leukocytes in the TME, which would provide "third-party" inhibition, versus direct expression by the tumor cells, remains to be determined.

Implications of Adaptive Immune Resistance

The adaptive resistance mechanism of intratumoral PD-L1 induction, together with the broad therapeutic activity of PD-1 pathway blockade in human cancer, validates one of the most important tenets underlying cancer immunology and immunotherapy, namely, that many cancer patients contain a significant repertoire of tumor-specific T cells capable of killing their tumor save for the adaptive induction of immune checkpoints in the TME. It also implies that PD-L1 expression in the tumor represents a measure of the potential for a patient's immune system to recognize the tumor. One of the major unanswered questions is this: what are the dominant antigenic targets that T cells recognize when checkpoints are blocked? Circumstantial evidence supports the notion that neoantigens created by the multiple somatic mutations in cancers provide such targets. Indeed, a recent report has demonstrated that melanomas with higher mutational loads were more responsive to anti-CTLA-4 therapy (Snyder et al., 2014). Also, the tumor types that have been shown to respond to anti-PD-1/PD-L1 therapy tend to be those with higher median mutational loads (i.e., carcinogen-induced cancers such as melanoma, lung, bladder, and head and neck cancers). However, there has been much evidence over the past 20 years that shared self-antigens upregulated in tumors by epigenetic mechanisms (e.g., cancer-testis antigens) are also able to provide selective tumor targeting. The relative importance of mutation-dependent, tumor-specific neoantigens versus tumor-associated self-antigens as T cell targets remains to be determined.

Finally, the adaptive resistance mechanism has profound implications for developing synergistic combinatorial cancer immunotherapies. One of the most promising general approaches to immunotherapy utilizes positive drivers of anti-tumor immune responses, such as vaccines, intratumoral injection of immune activators, and co-stimulatory receptor agonists. These modalities with the potential to enhance anti-tumor responses would also be expected to enhance the adaptive induction of checkpoints like PD-1 ligands. This has, in fact, been demonstrated in animal models of vaccination (Fu et al., 2014). Therefore, positive drivers of anti-tumor immunity may be synergistic with PD-1 pathway inhibitors. Such approaches are just beginning to enter the clinic.

Clinical Impact of Drugs Blocking CTLA-4 and PD-1

Anti-CTLA-4

The anti-CTLA-4 monoclonal antibodies (mAbs) ipilimumab, a fully human IgG1 (Bristol-Myers Squibb), and tremelimumab, a fully human IgG2 (Pfizer, MedImmune), were the first immune checkpoint-blocking drugs to enter clinical testing in oncology. Although designed as CTLA-4-blocking mAbs, these drugs have recently been postulated to have unique functions endowed by their specific isotypes, with evidence suggesting that ipilimumab may deplete Tregs overexpressing CTLA-4 (Selby et al., 2013). In 2011, ipilimumab was approved in the United States and Europe as therapy for advanced unresectable melanoma based on results from two phase III trials showing significant extensions in overall survival (OS) (Hodi et al., 2010; Robert et al., 2011). Long-term follow-up in a pooled meta-analysis of 1,861 melanoma patients receiving ipilimumab in phase II or III trials revealed durable survival in approximately 20%, in some cases extending to 10 years (Schadendorf et al., 2015). Interestingly, this survival rate is approximately double the observed rate of tumor regressions measured by standard oncologic criteria (~10% complete responses [CRs] and partial responses [PRs]). Factors contributing to this phenomenon may include prolonged disease stabilization, unconventional "immune-related" response patterns, or a heightened responsiveness of ipilimumab-refractory patients to subsequent therapies. Although tremelimumab, a distinct CTLA-4-blocking mAb, showed promise in early-phase melanoma trials, it did not meet its designated endpoint when randomized against standard chemotherapy in a first-line phase III melanoma trial (Ribas et al., 2013).

Ipilimumab has so far shown only modest anti-tumor effects in non-melanoma cancers, and tremelimumab is still in early testing for these indications (reviewed in Weber, 2014). Kidney, lung, and prostate cancer have been the most intensively studied. In a phase II study of metastatic renal cell carcinoma (RCC, n = 61), a partial response rate of 10% was observed with ipilimumab monotherapy (Yang et al., 2007). In lung cancer, treatment-naive patients with non-small-cell lung cancer (NSCLC) (n = 204) or extensive disease small-cell lung cancer (ED-SCLC) (n = 130) received standard chemotherapy alone or combined with ipilimumab during initial ("concurrent") or later ("phased") chemotherapy cycles in a phase III trial (Lynch et al., 2012; Reck et al., 2013). For both diseases, a brief but statistically significant 1-month extension of progression-free survival measured by immune-related criteria (irPFS) was observed in patients receiving phased ipilimumab plus chemotherapy compared with chemotherapy alone. In NSCLC, there was also a significant 1-month extension of PFS measured by standard criteria in the phased ipilimumab arm. Although ipilimumab did not have a significant impact on OS in either NSCLC or ED-SCLC, a subset analysis appeared to show improved activity in patients with squamous NSCLC, providing the basis for an ongoing phase III trial of

Table 1. Drugs in Clinical Development that Block PD-1 or PD-L1

Target	Drug Name	Other Names	Source	Isotype and Characteristics	Clinical Testing Phase
PD-1	MEDI0680	AMP-514	MedImmune/ AstraZeneca	information not available	phase I
	nivolumab	Opdivo, BMS-936558, MDX-1106, ONO-4538	Bristol-Myers Squibb, Ono Pharmaceuticals	fully human IgG4[a]	approved, treatment-refractory unresectable melanoma (Japan, United States) and squamous NSCLC (United States)
	pembrolizumab	Keytruda, MK-3475, lambrolizumab	Merck	humanized IgG4	approved, treatment-refractory unresectable melanoma (United States)
	pidilizumab	CT-011	CureTech	humanized IgG1	phase I-II
PD-L1	BMS-936559	MDX-1105	Bristol-Myers Squibb	fully human IgG4[a]	phase I
	MEDI4736	none	MedImmune/ AstraZeneca	Fc-modified human IgG1[b]	phase I-III
	MPDL3280A	RG7446	Genentech/ Roche	Fc-modified human IgG1[b]	phase I-III
	MSB0010718C	none	EMD Serono	fully human IgG1[a]	phase I-II

[a]Fully human mAbs were produced in genetically engineered mice.
[b]Fc-modified mAbs were engineered to abrogate ADCC and complement-dependent cytotoxicity (CDC).

ipilimumab plus chemotherapy in this histology. Similarly, trials of ipilimumab in metastatic castration-resistant prostate cancer (mCRPC) have yielded weak but positive signals of activity. In phase I/II trials in which patients received ipilimumab alone or combined with systemic granulocyte-macrophage colony-stimulating factor or focal radiotherapy, prostate-specific antigen reductions of $\geq$50% were observed in some patients, and isolated examples of measurable tumor regression were reported (Fong et al., 2009; Slovin et al., 2013), supporting further study. In a phase III trial of ipilimumab versus placebo after bone-directed radiotherapy in 799 patients with docetaxel-refractory mCRPC, median OS was 11.2 versus 10.0 months, respectively ($p = 0.053$), failing to meet the trial's primary endpoint (Kwon et al., 2014). However, there was a statistically significant 1-month improvement in PFS and a suggestion that OS was prolonged in a subgroup of patients with favorable prognostic features. A separate phase III trial of ipilimumab in chemotherapy-naive patients with asymptomatic or minimally symptomatic mCRPC without visceral metastases has recently completed accrual.

Valuable clinical experience gained from studies of anti-CTLA-4 mAbs paved a path for accelerated development of other drugs in class by providing a framework for treatment strategy, toxicity management, and efficacy evaluation. New principles emerged that distinguished immune checkpoint blockade from traditional cancer therapies. First, a new category of side effects, so-called "immune-related adverse events" (irAEs), was recognized and characterized, leading to algorithm development for early detection and management. Drug-related irAEs were severe in 15%–30% of patients receiving anti-CTLA-4, sometimes resulting in fatalities. These irAEs were associated with inflammation in normal tissues such as the gut, skin, and endocrine glands and resembled phenotypes observed in human *CTLA-4* heterozygotes with reduced CTLA-4 expression (Topalian and Sharpe, 2014). Their occurrence in individuals with no prior history of autoimmunity validates the mechanism of action of anti-CTLA-4 in "releasing the brakes" on immune responses and underscores the precarious balance that normally exists between self-tolerance and autoimmunity. Second, a new category of clinical response termed "immune-related response" was recognized in which major and durable tumor regressions could occur after apparent initial disease progression on treatment (Wolchok et al., 2009). Tumor enlargement measured by conventional radiologic scans may result from drug-induced inflammation at tumor sites or could reflect actual tumor growth followed by delayed regression. Such phenomena pose challenges for the appropriate management of individual patients and the selection of informative endpoints for trials of immune checkpoint-blocking drugs.

Drugs Blocking the PD-1 Pathway

Information garnered from trials of anti-CTLA-4 agents fast-forwarded the development of drugs blocking PD-1 or its major ligand, PD-L1. As predicted by murine models, these drugs have heightened tumor selectivity and reduced toxicity compared with anti-CTLA-4, supporting their administration in an outpatient setting. Furthermore, although they are effective against advanced treatment-refractory melanoma, with recent regulatory approvals for two anti-PD-1 drugs in this setting, they also appear to have a much broader spectrum of anti-tumor activity than anti-CTLA-4. Reproducible and durable regressions of epithelial cancers (lung, head and neck, and bladder cancers, among others) have catapulted the launching of hundreds of ongoing clinical trials in diverse disease indications. Although several different anti-PD-1/PD-L1-blocking mAbs are currently in clinical testing (Table 1), the fact that anti-tumor activity has been observed with all of them highlights the PD-1 pathway as a dominant intratumoral immunosuppressive pathway and a key target in cancer therapy.

The first-in-human trial of nivolumab anti-PD-1 provided seminal evidence that this treatment approach could potentially impact diverse cancer types, including common epithelial cancers, with objective responses reported in patients with melanoma, kidney, and colorectal cancer (Brahmer et al., 2010). A transient tumor regression in one patient with NSCLC provided the impetus for investigating a larger NSCLC cohort

in a follow-up multi-dose trial of nivolumab in multiple cancer types (Topalian et al., 2012). Results from this trial showed notable objective response rates in patients with advanced treatment-refractory NSCLC (17%, n = 129), RCC (27%, n = 34), and melanoma (31%, n = 107). Importantly, responses were quite durable, with many persisting even after drug discontinuation, and long-term follow-up revealed OS of 9.9, 22.4, and 16.8 months, respectively (Topalian et al., 2014). These non-randomized data compared favorably to historical response rates in similar patient populations, spurring phase III testing of nivolumab in all three cancers. A recent phase III report showed the superiority of first-line nivolumab versus standard chemotherapy in patients with advanced melanoma (Robert et al., 2015). These findings have incentivized the aggressive clinical development of PD-1 pathway-blocking drugs by multiple pharmaceutical and biotechnology companies (Table 1), and the clinical activity of these drugs in melanoma, RCC, and NSCLC has been confirmed (Brahmer et al., 2012; Hamid et al., 2013; Herbst et al., 2014; Motzer et al., 2014). Nivolumab was recently approved by the Food and Drug Administration for chemotherapy-refractory squamous NSCLC. However, the full activity spectrum of PD-1 pathway-blocking drugs is not yet known, with recent evidence of efficacy in advanced chemotherapy-refractory bladder cancer (Powles et al., 2014), Hodgkin's lymphoma (Ansell et al., 2015), head and neck, gastric, triple-negative breast, and ovarian cancers.

Combination Therapies Based on PD-1 Pathway Blockade

Despite these promising results, the majority of patients treated with anti-PD-1/PD-L1 monotherapies do not achieve objective responses, and most tumor regressions are partial rather than complete. Animal models suggest that treatment combinations based on PD-1 pathway blockade may be synergistic, including anti-CTLA-4 or other checkpoint inhibitors, chemotherapy, tyrosine kinase inhibitors, focal irradiation, cancer vaccines, or immune agonist mAbs. Appropriate preclinical models are valuable in providing a basis for prioritizing clinical translation. A wide variety of treatment combinations are now under clinical development in diverse cancer types. Early and substantial tumor regressions observed with a combination of anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) in advanced melanoma have garnered attention, although associated irAEs were also amplified (Wolchok et al., 2013). Results from ongoing prospectively randomized trials will be needed to define the role of this treatment combination in melanoma and other cancers.

Biomarkers of Response

As mentioned earlier, studies of peripheral blood have yielded pharmacodynamic evidence of global T cell activation in patients receiving anti-CTLA-4 (Maker et al., 2006), although these changes do not appear to correlate with clinical outcomes. Peripheral T cell activation does not occur to the same degree in patients receiving anti-PD-1/PD-L1 (Brahmer et al., 2010), as might be anticipated because the TME is thought to be the main site of activity of this pathway. Accordingly, tumor tissue has become the focal point for exploring potential biomarkers of response to anti-PD-1 drugs. Early studies revealed a correlation between pretreatment tumor cell expression of the ligand PD-L1 by immunohistochemistry (IHC) and the likelihood of response to anti-PD-1 (Brahmer et al., 2010; Topalian et al., 2012). With the advent of several new automated PD-L1 IHC tests and interrogation of hundreds of patients with a variety of cancer types, a significant but not absolute relationship between PD-L1 expression in the TME and responsiveness to PD-1 pathway blockade has been confirmed. The potential importance of PD-L1 expression by infiltrating immune cells (Herbst et al., 2014), the presence and location of CD8+ tumor-infiltrating lymphocytes (Tumeh et al., 2014), and other factors (Taube et al., 2014) are currently under intense study individually and in combination to discern more sensitive and specific predictors of clinical outcomes.

On the Horizon: Targeting Novel Checkpoints

Although antibody blockers of CTLA-4 and PD-1 are the focus of clinical attention at this time, it is likely that blockade of additional checkpoints will result in even further clinical activity. This is because multiple checkpoints appear to be co-expressed with PD-L1 and PD-1 in tumors. We review here some of the most actively studied "next-generation" checkpoint molecules for which antibody blockers are already in the clinic or soon to be tested in clinical trials, many in combination with anti-PD-1 or anti-PD-L1.

Lymphocyte Activation Gene 3

Lymphocyte activation gene 3 (LAG-3, CD223) is an immune checkpoint molecule expressed on activated T cells (Huard et al., 1994), NK cells (Triebel et al., 1990), B cells (Kisielow et al., 2005), and plasmacytoid dendritic cells (Workman et al., 2009). Structurally, LAG-3 is highly homologous to the CD4 T cell co-receptor and lies proximal to the *CD4* gene on human chromosome 12, but, at the amino acid level, it is less than 20% homologous to CD4, indicating that the two genes likely diverged early in evolution (Dijkstra et al., 2006). The only known ligand for LAG-3 is MHC II (Huard et al., 1997), although its structural interactions with MHC II are more limited than those of CD4 (Fleury et al., 1991; Moebius et al., 1993). Early studies showed that LAG-3 was selectively upregulated on CD4+ Tregs (Huang et al., 2004). Here a LAG-3-blocking antibody mitigated Treg activity in vivo, and transfection of antigen-specific CD4 T cells with full length, but not truncated, LAG-3 could confer in vitro Treg function. More recent studies have also suggest that LAG-3 blockade (or genetic knockout) affects the ability of conventional T cells (Tconv) to be suppressed by Tregs (Sega et al., 2014; Durham et al., 2014). Additionally, LAG-3 has a CD8 T cell-intrinsic role because LAG-3 blocking antibodies were found to augment CD8 T cell function in vivo in the absence of CD4 T cells Grosso et al., 2007). As described above, exhausted or dysfunctional T cells can express multiple immune checkpoint molecules, and LAG-3 and PD-1 are commonly co-expressed in models of chronic infection Blackburn et al., 2009) as well as models of self-antigen recognition (Grosso et al., 2009). These studies have been extended to human tumors in that a significant fraction of antigen-specific CD8 T cells in patients with ovarian cancer and melanoma co-express LAG-3 and PD-1 (Matsuzaki et al., 2010; Baitsch et al., 2012). Evidence for synergistic immunosuppression mediated by LAG-3 and PD-1 comes from studies in which double-knockout mice were generated. Although neither *LAG-3* nor *PD-1* single knockout animals succumb to autoimmunity, combined knockout results in multi-organ lymphocytic infiltration,

and early death (Woo et al., 2012). Nearly identical results were obtained in models of autoimmunity (Okazaki et al., 2011), reinforcing the notion that LAG-3 and PD-1 are potentially synergistic in regulating T cell function. A role for dual blockade of LAG-3 and PD-1 in tumor immunity is suggested by studies in which most tumors implanted in *PD-1/LAG-3* double-knockout mice were rejected, whereas *PD-1* single-knockout mice showed delayed tumor growth. Similarly, combined antibody-mediated blockade of LAG-3 and PD-1 resulted in tumor rejection in several models without any short-term evidence of autoimmune side effects. An anti-LAG-3 blocking mAb has recently entered clinical testing in cancer (clinical trial NCT01968109) in a phase I trial that includes cohorts receiving anti-LAG-3 monotherapy or combination therapy with anti-PD-1.

Killer Inhibitory Receptors

NK cells are a population of innate immune cells with well documented roles in infectious and tumor immunity (Marcus et al., 2014). Like activated CD8 T cells, NK cells mediate target cell apoptosis via secretion of preformed granules containing perforin and granzymes. However, unlike CD8 T cells, NK cells do not recognize unique peptides in the context of classical MHC I molecules. Instead, NK function is controlled by the complex interplay of a series of activating receptors and killer inhibitory receptors (KIRs) and their ligands. In humans, KIR molecules are polymorphic and bind to certain MHC I alleles, and not all KIR/ligand pairs are equally capable of inhibiting NK cell function. Indeed, bone marrow transplants in which donor NK cells lack the ability to be inhibited by host KIR ligands have been shown to result in lower relapse rates and improved OS, supporting the importance of this cell type in cancer immunity (Benson and Caligiuri, 2014). The relative importance of NK cells in murine models of cancer immunotherapy has been documented by multiple studies but is especially highlighted by studies in which NK cell activation via IL-15 can eradicate fairly advanced tumors in the absence of CD8 T cells (Liu et al., 2012). So, in a sense, KIRs can be thought of as immune checkpoint molecules, and blocking KIRs on NK cells could be exploited to augment anti-tumor immunity. To that end, a fully human anti-KIR mAb has entered clinical testing. This antibody (initially IPH-2101, Innate Pharma; now lirilumab, Bristol-Myers Squibb) binds to the human KIR molecules KIR2DL-1, KIR2DL-2, and KIR2DL-3 as well as to KIR2DS-1 and KIR2DA-2, preventing their binding to HLA-C MHC I molecules (Romagné et al., 2009). A phase I trial of anti-KIR in acute myelogenous leukemia has been completed. Several studies in hematologic and solid cancers are ongoing, but of particular interest are trials in which lirilumab is being combined with anti-PD-1 (nivolumab, clinical trial NCT01714739) or with anti-CTLA-4 (ipilimumab, clinical trial NCT01750580). These trials are important in that each seeks to combine innate immune activation via anti-KIR with activation of the adaptive immune system, therefore offering the potential for additive or synergistic anti-tumor efficacy.

B7-H3

B7-H3 (CD276) was initially identified using a bioinformatics approach in which human genome databases were queried for sequences with homology to previously identified B7 family members (Chapoval et al., 2001). It is a type I transmembrane protein with single variable and constant immunoglobulin domains. B7-H3 mRNA is widely expressed in normal tissues (Sun et al., 2002), but protein expression is more restricted and is controlled by post-transcriptional mechanisms. The understanding of B7-H3 biology is complicated by the fact that it can be expressed on both immune and non-immune cells. On immune cells, B7-H3 appears to exert a stimulatory role: down-regulation of B7-H3 expression using anti-sense oligonucleotides inhibits T cell production of IFN-γ (Chapoval et al., 2001). Therefore, B7-H3 might be considered not as a classical immune checkpoint molecule but, rather, as a co-stimulatory receptor more analogous to CD28. Although this model is supported by numerous studies (Yi and Chen, 2009), several studies suggest an alternative model in which B7-H3 down-modulates T cell activation. These studies include the finding that B7-H3-blocking antibodies exacerbate disease in the experimental autoimmune encephalomyelitis (EAE) murine model as well as in several other models (Suh et al., 2003). In terms of cancer immunity, there is a similar lack of clarity in that the induction of expression of B7-H3 in tumor cell lines increases their immunogenicity and leads to more rapid rejection (Luo et al., 2004). But in many human tumors, expression of B7-H3 in situ has been associated with a poor outcome. This is especially notable in RCC and prostate cancer, where expression correlates with an increased risk of death (Crispen et al., 2008; Chavin et al., 2009). Based on the notion that B7-H3 protein is overexpressed in multiple tumor types, a mAb with enhanced antibody-dependent cellular cytotoxicity (ADCC) function has been developed (Loo et al., 2012) and has entered clinical trials (clinical trial NCT01391143). This agent is not being deployed as an immune checkpoint-blocking antibody. Rather, it is being tested as a traditional tumor-targeting antibody similar in concept to rituximab or trastuzumab.

T Cell Immunoglobulin and Mucin-3

T cell immunoglobulin and mucin-3 (TIM-3) is an immune checkpoint molecule expressed on activated human T cells, NK cells, and monocytes. *TIM-3* knockout mice, similar to *LAG-3* knockouts, do not develop overt autoimmunity (Sánchez-Fueyo et al., 2003), suggesting that TIM-3 and LAG-3 may have similarly subtle effects in modulating immune cell function. Consistent with this hypothesis, TIM-3 blockade accelerates the disease phenotype in murine models prone to developing autoimmunity, including non-obese diabetic (NOD) (Sánchez-Fueyo et al., 2003) and EAE models (Monney et al., 2002). Functionally, TIM-3 binds to galectin-9 (as well as several other ligands), as supported by data showing that administration of galectin-9 in vitro causes cell death of Th1 cells in a TIM-3-dependent manner (Zhu et al., 2005). Recent studies showed that TIM-3 is co-expressed with and binds to CECAM1 and that this interaction is important in TIM-3′s regulatory function (Huang et al., 2015). In other work, the role of the TIM-3 immune checkpoint was studied in several murine cancer models (Sakuishi et al., 2010), including the CT26 colon carcinoma, 4T1 mammary carcinoma, and B16 melanoma. Interestingly, TIM-3 was nearly universally co-expressed with PD-1 on the majority of TILs. Co-expression of both checkpoint molecules reflected a more exhausted phenotype, functionally defined by a T cell's reduced ability to proliferate and secrete IFN-γ, IL-2, and tumor necrosis factor α (TNF-α). Combined blockade was more effective in controlling tumor growth than blocking either checkpoint alone, confirming the notion that combined immune checkpoint blockade offers a potential treatment strategy for a wide variety

of cancers and that, besides CTLA-4 and LAG-3, other checkpoints might synergize with PD-1 to down-modulate T cell responses to tumors. Anti-human TIM-3 blocking antibodies have not yet entered the clinic but are under development.

V-Domain Ig-Containing Suppressor of T Cell Activation

V-domain Ig-containing suppressor of T cell activation (VISTA) is a relatively recently described negative regulator of T cell function (Wang et al., 2011). Unlike PD-1 and CTLA-4, VISTA is predominantly expressed on myeloid and granulocytic cells, with only weak T cell expression in mice and humans (Lines et al., 2014; Wang et al., 2011). Functionally, VISTA blockade attenuates tumor outgrowth, especially when combined with a cancer vaccine (Wang et al., 2011). In terms of human cancers, VISTA expression has been described in colorectal tumors; here expression appears to be confined to CD11b+ cells, whereas expression on CD8 T cells was not detected (Lines et al., 2014). These early studies are relatively limited in scope, and a more comprehensive analysis of VISTA expression in various human tumor types is warranted. In addition, the relative efficacy of VISTA blockade compared with PD-1 or CTLA-4 blockade awaits the development of suitable reagents, but, as is the case for the other checkpoint molecules discussed above, the notion that VISTA expression so far appears to be selective for the myeloid compartment of tumors suggests the possibility of clinical effects distinct from those mediated by currently available checkpoint blocking antibodies as well as the potential for additive or synergistic benefits.

T Cell ITIM Domain: TIGIT

Like B7-H3, TIGIT was initially identified through a genomic search for structures shared among regulatory receptors, including a conserved ITIM motif (Yu et al., 2009). Initial studies suggested that TIGIT functions by transmitting a negative signal to DCs, decreasing IL-12 secretion while simultaneously enhancing IL-10 levels. A more recent study, however, shows that TIGIT functions as an immune checkpoint, downregulating proliferation of both murine and human T cells (Johnston et al., 2014). The ligand for TIGIT is the poliovirus receptor (PVR), but PVR also binds to the T cell surface molecule CD226. In this way, TIGIT biology is perhaps reminiscent of the interaction between B7 molecules and CTLA-4/CD28. Binding of PVR to TIGIT mediates an inhibitory signal, whereas binding of PVR to CD226 transmits a positive costimulatory signal to T cells. Blocking TIGIT with a specific mAb showed efficacy in both viral and tumor models, including an additive anti-tumor effect when both PD-L1 and TIGIT were blocked simultaneously. The relevance of these data to human cancer awaits future clinical development, but it is worth noting that genomic profiling studies showed that CD8a expression correlates closely with TIGIT expression in tissue from lung cancer patients (Johnston et al., 2014).

IDO

Although not an immune checkpoint in the classical sense, several inhibitory pathways mediated by overexpression of IDO in various tumor types play an important role in downregulating anti-tumor immunity (Prendergast et al., 2014). As briefly mentioned above, IDO catabolizes the breakdown of tryptophan to kynurenine (and other metabolites). T cells require adequate tryptophan levels for survival and effector function, and, therefore, IDO-mediated tryptophan deficiency results in T cell tolerance and lack of effector function and promotes the differentiation of naive CD4 T cells into Tregs (Fallarino et al., 2006). In addition, IDO expression in a relatively small population of tumor-associated DC allows the suppression of effector T cell responses (Mellor and Munn, 2004). Both the IDO pathway inhibitor D-1MT and small-molecule enzymatic inhibitors of IDO1 (INCB024360 and NLG919) have entered clinical trials, and phase I data from a trial combining D-1MT (indoximod) with chemotherapy were published recently, demonstrating tolerability for the combination as well as evidence of anti-tumor activity (Soliman et al., 2014).

Conclusions

Recent years have seen a rapid expansion of our knowledge of immune regulation. Basic principles established in laboratory models of infection, autoimmunity, and transplantation have proved to be transportable to human cancer, supporting the development of drugs modulating anti-tumor immunity. The successful application of the immune checkpoint blockers anti-CTLA-4 in melanoma and anti-PD-1/PD-L1 in multiple cancer types has established immunotherapy as a viable treatment option for patients with advanced cancers and has opened the doors to developing a new generation of immune modulators that may be most effective when employed in treatment combinations. Armed with a new understanding and unprecedented opportunities, the field of immunotherapy is now standing on the threshold of great advances in the war against cancer.

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All authors contributed to the design and writing of this manuscript.

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