Clinical Cancer Research

Molecular Pathways: Coexpression of Immune Checkpoint Molecules: Signaling Pathways and Implications for Cancer Immunotherapy

Christopher J. Nirschl and Charles G. Drake

Abstract

The expression of immune checkpoint molecules on T cells represents an important mechanism that the immune system uses to regulate responses to self-proteins. Checkpoint molecules include cytotoxic T lymphocyte antigen-4, programmed death-1, lymphocyte activation gene-3, T-cell immunoglobulin and mucin protein-3, and several others. Previous studies have identified individual roles for each of these molecules, but more recent data show that coexpression of checkpoint molecules occurs frequently on cancer-specific T cells as well as on pathogen-specific T cells in chronic infections. As the signaling pathways associated with each checkpoint molecule have not been fully elucidated, blocking multiple checkpoints with specific monoclonal antibodies results in improved outcomes in several chronic viral infections as well as in a wide array of preclinical models of cancer. Recent clinical data suggest similar effects in patients with metastatic melanoma. These findings support the concept that individual immune checkpoint molecules may function through nonoverlapping molecular mechanisms. Here, we review current data regarding immune checkpoint molecule signaling and coexpression, both in cancer and infectious disease, as well as the results of preclinical and clinical manipulations of checkpoint proteins. *Clin Cancer Res; 19(18); 4917–24. ©2013 AACR.*

Disclosure of Potential Conflicts of Interest

C.G. Drake has a commercial research grant from Bristol-Myers Squibb, has an ownership interest (including patents) in Amplimmune, and is a consultant/advisory board member for Bristol-Myers Squibb, Dendreon, and CoStim. No potential conflicts of interest were disclosed by the other author.

CME Staff Planners' Disclosures

The members of the planning committee have no real or apparent conflict of interest to disclose.

Learning Objectives

Upon completion of this activity, the participant should understand the molecular pathways used by individual immune checkpoint proteins. The participant should also have a better understanding of the preclinical and clinical rationale for combined immunologic checkpoint blockade and the development of current therapeutics aimed at blocking immune checkpoint proteins.

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Background

One of the most important decisions made by the immune system involves modulating both the breadth and magnitude of an evolving response. As a whole, the immune system is capable of sterilizing immunity against a wide variety of pathogens and maintains memory responses for future encounters. Therefore, an immune response is tightly regulated, and multiple mechanisms are in place to prevent autoimmune reactions to self-proteins. The devastating and life-long effects of many autoimmune diseases evidence the importance of these mechanisms. Over the past 20 years, a broad class of extracellular "checkpoint molecules" has been found to modulate T-cell responses to self-proteins (1). However, many of these molecules also have a role in regulating T-cell responses to chronic infections and tumor antigens. Checkpoint molecules include cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), and T-cell immunoglobulin and mucin protein-3 (TIM-3) as well as several others (1, 2). Recent clinical data on single-agent CTLA-4 (3) and PD-1 (4, 5) blockades in patients with cancer show that these pathways play a critical role in the maintenance of tumor tolerance in humans because single-agent

Authors' Affiliation: Departments of Oncology, Immunology, and Urology, Johns Hopkins Sidney Kimmel Comprehensives Cancer Center, Baltimore, Maryland

Corresponding Author: Charles G. Drake, Johns Hopkins University, 1650 Orleans Street, CRB I Rm. 416, Baltimore, MD 21231. Phone: 410-502-7523; Fax: 443-287-4653; E-mail: cdrake@jhmi.edu

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checkpoint blockade is associated with objective tumor responses and improved overall survival. Furthermore, very recent data combining PD-1 and CTLA-4 blockade in patients with melanoma showed an increased rate of objective tumor responses as compared with blocking either checkpoint alone, supporting the notion that combinatorial checkpoint blockade may result in increased clinical benefit (6).

Signaling through Immune Checkpoint Molecules

Although the precise molecular pathways by which these checkpoint proteins signal are poorly understood, preclinical data from studies in which multiple checkpoints were blocked simultaneously suggest that the pathways used by different checkpoint proteins may be relatively unique and potentially nonredundant. This may provide a clinical rationale for blocking multiple checkpoints to enhance antitumor immunity. Among checkpoint molecules, CTLA-4 blockade was first shown to augment antitumor immunity (7) and is the checkpoint molecule for which signaling is best understood. CTLA-4 is a homolog of CD28 and plays a significant role in the development of peripheral tolerance to self-proteins, as shown by studies of CTLA-4 knockout mice (8, 9). These animals are moribund by 3 to 4 weeks of age, have significant upregulation of T-cell activation markers, and exhibit severe pancreatitis, myocarditis, and T-cell infiltration of the liver, heart, lung, and pancreas. In terms of signaling, the major ligands for CTLA-4 are B7–1 (CD80) and B7–2 (CD86), which transmit an inhibitory signal to CTLA-4–expressing T cells. Initial data suggesting that the signaling pathway for CTLA-4 directly involves events downstream of T-cell activation also came from studies using knockout mice and showed that in the absence of CTLA-4 signaling, there was constitutive activation of the protein tyrosine kinases FYN, LCK, and ZAP-70 (10). To regulate the function of these kinases (and downmodulate T-cell function), CTLA-4 recruits two phosphatases, SHP2 (10) and PP2A (11). As shown in Fig. 1, the association of CTLA-4 with SHP2 results in dephosphorylation of the CD3ζ chain, reducing the signaling potential of the T-cell receptor (TCR). Furthermore, CTLA-4 recruitment of PP2A results in decreased downstream AKT phosphorylation (12), further dampening the signaling cascade initiated by TCR engagement. Taken together, these data show that CTLA-4 signaling dampens T-cell activation through both proximal and distal mechanisms.

PD-1 is a 55 kDa transmembrane protein that, like CTLA-4, downregulates T-cell function (13, 14). Consistent with that role, PD-1 knockout mice show some evidence of autoimmunity; they have elevated serum levels of immunoglobulin IgG2b as well as IgA and develop mild lupus-like autoimmunity (15) as well as dilated cardiomyopathy (16), although this phenotype has not been universally observed. In addition, these disease phenotypes are strain specific, occur later in life, and are markedly less prominent than those observed in CTLA-4 knockout animals (2). PD-1 signaling involves binding to several discrete ligands, including PD-L1 and PD-L2, as well as to the costimulatory molecule B7-1 (17). Under certain (inflamed) conditions, PD-L1 can be expressed on most cell types, including cancer cells, epithelial cells, lymphoid cells, myeloid cells, and professional antigen-presenting cells. PD-L2, in contrast, is expressed primarily on professional antigen-presenting cells, although recent data from several labs, including ours, suggest that PD-L2 may be expressed on several cancer cell lines (C.J. Nirschl, unpublished data). Structurally, PD-1 has a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), as well as an immunoreceptor tyrosine-based switch motif that has been found to be capable of recruiting the phosphatases SHP-1 and SHP-2 (18), although only SHP-2 recruitment has been confirmed *in vivo* (Fig. 1). Furthermore, PD-1 signaling may result in dephosphorylation of the CD3ζ chain, mediating decreased TCR signaling (19). Taken together, these data support a model in which PD-1 and CTLA-4 both inhibit T-cell function in part by inhibiting Akt activation, although PD-1 may operate primarily at a more membrane-proximal level (20). Despite these similarities in the known signaling pathways of PD-1 and CTLA-4, early experiments by Blazar and colleagues showed that these two inhibitory pathways do not serve fully redundant roles. In a murine model of graft-versus-host disease in which heavily irradiated hosts were given MHC mismatched bone marrow, blockade of either PD-1 or CTLA-4 exacerbated the disease by an IFN-γ–dependent mechanism (21). However, combinatorial blockade had the greatest effect, showing that these two pathways have distinct effects in maintaining self-tolerance.

A third immune checkpoint molecule that may be important in the immune response to cancer (22) is LAG-3, a CD4 homolog with four extracellular Ig-like domains (23). Like CD4, LAG-3 has been found to bind MHC class II molecules (24). However, unlike CTLA-4 or PD-1 knockout animals, LAG-3 knockout mice do not develop overt autoimmunity (25), suggesting that LAG-3 plays a more subtle role in modulating T-cell function than either CTLA-4 or PD-1. Nevertheless, LAG-3 clearly restrains T-cell function under several conditions (26). This is particularly notable in the nonobese diabetic (NOD) model of diabetes, where knocking out LAG-3 results in significantly accelerated disease, marked by increased $CD4^+$ and $CD8^+$ T-cell infiltration of the pancreas (27). Furthermore, LAG-3 knockout CD4 and CD8 T cells show increased expansion in response to staphylococcal enterotoxin B (SEB) activation, *in vivo* peptide stimulation, and to *Sendai virus* (28), suggesting that LAG-3 may function by regulating T-cell expansion in immune reactions that have already been initiated. Other important data suggest a more prominent role for LAG-3 in regulatory T-cell (Treg) function (29), in that enforced expression of LAG-3, but not a LAG-3 mutant, enhanced Treg suppressive capacity *in vitro*. The molecular pathways that mediate LAG-3 signaling are still largely unknown, although it is clear that the unique intracellular

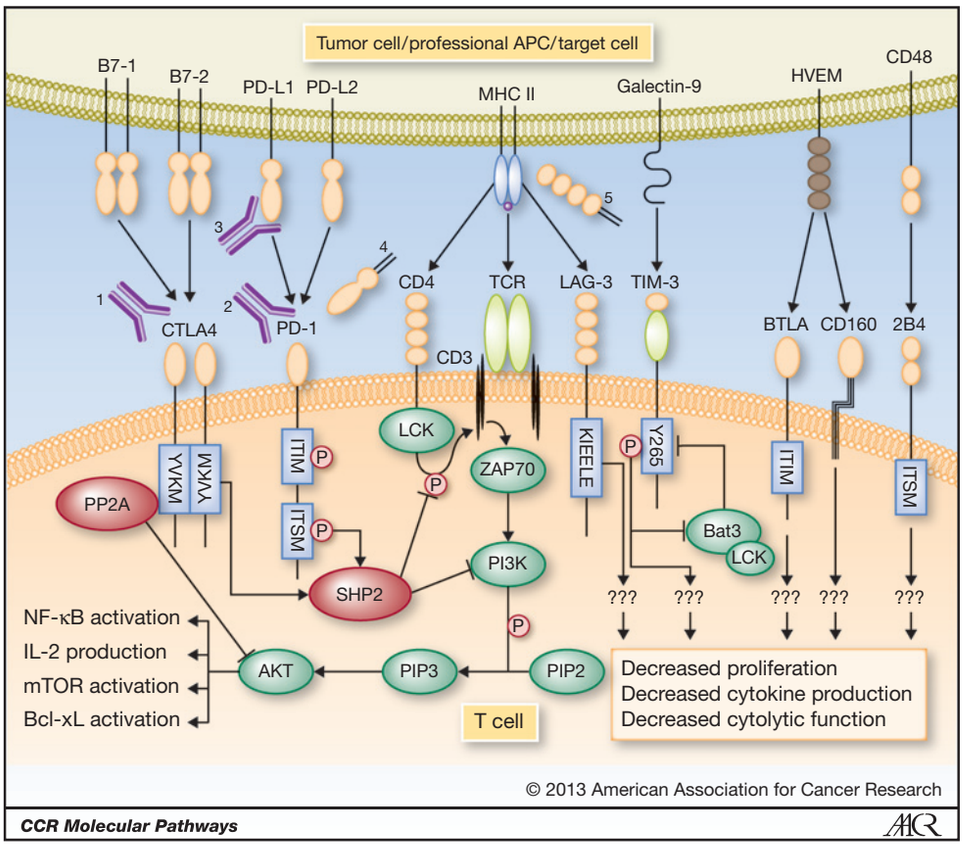

Figure 1. Known signaling pathways of selected checkpoint molecules and current therapeutics. Upon binding B7-1 or B7-2, CTLA-4 recruits the phosphatases SHP2 and PP2A via the YVKM motif in its cytoplasmic domain. SHP2 recruitment results in attenuation of TCR signaling by dephosphorylating the CD3ζ chain. PP2A recruitment results in downstream dephosphorylation of AKT, further dampening the T-cell activation pathway. PD-1 ligation by PD-L1 or PD-L2 also recruits SHP2 to the ITSM domain, resulting in membrane proximal decreases in TCR signaling. LAG-3 signaling is dependent on interaction with its ligand, MHC II, as well as its intracellular KIEELE domain. TIM-3 binds to Galectin-9, as well as other ligands. In the absence of ligand binding, TIM-3 is associated with Bat3, protecting the cell from TIM-3–mediated inhibition and allowing for greater activation. However, once TIM-3 binds to Galectin-9, Y265 is phosphorylated and the interaction with Bat3 is disrupted, allowing TIM-3 to deliver inhibitory signals to the T cell. BTLA and CD160 bind to herpes virus entry mediator. BTLA contains an intracellular ITIM domain that may be important in signaling. 2B4 has four intracellular ITSM domains and binds to CD48, but further signaling mechanisms are poorly understood. Ig domains are depicted in orange, mucin domains in green, cysteine-rich domains in brown, and GPI anchors as bolded black lines. Current therapeutics to block checkpoint-signaling molecules include both monoclonal antibodies and Ig fusion proteins: (i) anti-CTLA-4: ipilimumab (BMS-734016), tremelimumab (CP-675206), (ii) anti-PD-1: nivolumab (BMS-936558, MDX1106), lambrolizumab (MK-3475), CT-011 (iii) anti PD-L1: BMS-936559 (MDX1105), MEDI4736, (iv) PD-L2 Ig: AMP224, and (v) LAG-3 Ig: IMP321.

KIEELE domain is required for its function (ref. 28; Fig. 1). On the basis of the currently available data, it is not possible to determine whether PD-1 and LAG-3 signaling pathways overlap significantly, although recent data in several models (to be discussed below) would suggest that this is not the case.

A fourth immune checkpoint molecule with potential relevance to cancer immunology is TIM-3, a glycoprotein that has both immunoglobulin and mucin domains on its extracellular portion. Like LAG-3 knockout mice, TIM-3 knockout animals do not develop overt autoimmunity (30), suggesting that TIM-3 and LAG-3 have similarly subtle effects in controlling T-cell function. In concordance with this hypothesis, TIM-3 blockade also accelerates the disease phenotype in models prone to the development of autoimmunity, including NOD mice (30) as well as experimental autoimmune encephalitis (31). 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 T_H1 cells in a TIM-3–dependent manner (32). Furthermore, Galectin-9 treatment *in vivo* suppresses T_H1-mediated experimental autoimmune encephalitis by inducing the death of IFN-γ–producing CD4 T cells. TIM-3 signaling is dependent on Y265 phosphorylation by inducible T-cell kinase (33), and recent data in autoimmune models suggest that the cytoplasmic protein Bat3, important in modulating cellular proliferation, serves as an important adaptor protein (34). In this model, Bat3 is bound to TIM-3 at rest and protects the T cell from TIM-3 signaling. However, when TIM-3 binds to Galectin-9, Bat3 dissociates from TIM-3 and TIM-3 can now downmodulate production of IFN-γ and T-cell proliferation.

Immune Checkpoint Molecules in Infectious Disease

Immunologically, chronic infections are in some ways quite similar to tumors, in that lymphocytes are persistently exposed to their cognate antigens, resulting in nonfunctionality or tolerance. In models of chronic viral infection, checkpoint molecules have been individually found to play a role in downmodulating a pathogen-specific immune response. However, recent studies have begun to home in on the expression of a checkpoint signature, wherein multiple checkpoint molecules are coexpressed on the same T cell. Many of these studies have focused on a murine model of chronic infection (lymphochoriomeningitis virus or LCMV), in which CD8 T cells specific for viral epitopes persist but lose their lytic function as well as the capacity to secrete cytokines over time (35). Using the LCMV model, a seminal study by Blackburn and colleagues showed that nonfunctional antigen-specific CD8 T cells coexpress multiple checkpoint molecules, including PD-1, LAG-3, 2B4, and CD160 (36). Expression of multiple checkpoint molecules was correlated with decreased cytokine production, in which virus-specific CD8 T cells first lost lytic ability, then their ability to secrete interleukin (IL)-2, TNF-α, and IFN-γ in this order. In this model, certain combinations of immune checkpoint molecules were more commonly coexpressed; in particular PD-1 was commonly expressed, along with LAG-3, 2B4 and/or CD160. Of potential clinical relevance, it was noted that combination PD-1/LAG-3 blockade was superior in terms of restoring IFN-γ secretion and viral clearance than blocking either checkpoint alone (36). A related study in the LCMV model also showed that coexpression of PD-1 and TIM-3 (37) was correlated with decreased production of IFN-γ, TNF-α, and IL-2. In both studies, there was a clear hierarchy of checkpoint expression: In addition to dual expressing cells (cells expressing PD-1 and either LAG-3 or TIM-3), PD-1 single-positive cells could be found, but LAG-3 or TIM-3 single-positive cells were relatively rare. We found similar results for PD-1 and LAG-3 in a model of self-antigen tolerance *in vivo* (38). While those results focused mostly on CD8 T cells, in a model of chronic parasitic infection (*Plasmodium yoelii*), CD4 T cells were also found to coexpress PD-1 and LAG-3, and as was the case with the LCMV model, blocking both checkpoint molecules was superior in restoring production of IFN-γ and TNF-α, leading to increased clearance of the parasite (39). Taken together, these data support the notion that immune checkpoint molecules are often coexpressed in response to persistent antigens from infectious agents and that blocking multiple checkpoints may significantly improve T-cell immune responses.

Combined Checkpoint Blockade in Cancer: Preclinical Models

As tumors represent a fairly obvious example of persistent antigen expression, one might reason that tumor-specific lymphocytes should express multiple immune checkpoints and that combination checkpoint blockade might mediate increased therapeutic benefit. Indeed, early data showed that combinatorial blockade of PD-1 and CTLA-4 resulted in significantly increased antitumor immunity when compared with blocking either single checkpoint alone (40). Data supporting this hypothesis were generated in a murine melanoma model, in which PD-1 and CTLA-4 blockade was combined with vaccination (41). In these studies, vaccination with irradiated tumor cells expressing Flt3 ligand was important, most likely to initiate an antitumor response to a poorly immunogenic tumor. The combination of vaccination plus dual PD-1/CTLA-4 blockade resulted in increased survival of mice bearing B16 melanoma flank tumors in comparison with vaccination alone or to vaccination combined with single-agent blockade of either CTLA-4 or PD-1. In terms of immunologic mechanism, the combination of vaccination along with dual CTLA-4/PD-1 blockade significantly increased the ratios of both CD4 and CD8 effector T cells to Tregs. Further studies in the MB49 bladder cancer model showed that combined blockade of PD-1 and CTLA-4 increased survival and decreased tumor growth in both small and large established flank tumors without additional vaccination (42). However, more recent studies blocking PD-1 and CTLA-4 in a model of ovarian cancer also required vaccination for optimal preclinical benefit (43). Taken together, these studies are important as they confirm the potential of blocking multiple immune-checkpoint molecules in cancer models; however, they also raise the issue of whether specific vaccination might be required for maximal clinical benefit.

In other recent studies, the role of the immune checkpoint molecule TIM-3 was studied in several murine cancer models (44), including CT26 colon carcinoma, 4T1 mammary carcinoma, and B16 melanoma. Interestingly, TIM-3 was nearly universally coexpressed with PD-1 and TIM-3/PD-1 double-positive cells represented the majority of infiltrating T cells. Coexpression of both checkpoint molecules corresponded to a more exhausted phenotype, defined as a T cell's ability to proliferate and secrete IFN-γ, IL-2, and TNF-α. Combined blockade was more effective in controlling tumor growth than blocking either checkpoint alone, confirming the notion that combined immune checkpoint blockade could be a potential treatment strategy to a wide variety of cancers and that, besides CTLA-4, other checkpoints might synergize with PD-1 to downmodulate T-cell responses to tumors.

In related work, we examined the relationship between the immune checkpoints LAG-3 and PD-1. In previous studies, we found that LAG-3 is relatively overexpressed on nonfunctional CD8 T cells in models of both self-tolerance and tumor tolerance (26). In those studies, blocking LAG-3 alone resulted in a significant, but incomplete, recovery of function, with evidence for a cell-intrinsic effect on CD8 T cells. On the basis of emerging data underscoring the importance of the immune checkpoint PD-1, we crossed LAG-3 knockout mice to PD-1 knockout animals. Unlike either single knockout animal, loss of both LAG-3 and PD-1 resulted in multiorgan lymphocytic infiltration and in death

of the animal between 6 and 8 weeks of age (45). Nearly identical results were obtained earlier by a group studying autoimmunity (46), reinforcing the notion that LAG-3 and PD-1 are potentially synergistic in regulating T-cell function. Mechanistically, adoptive transfer of CD4 and CD8 T cells from double-knockout mice into mice lacking B and T cells ($Rag^{-/-}$) resulted in a similar, fatal autoimmune phenotype, confirming that the primary drivers of this autoimmunity are CD4 and CD8 T cells. Interestingly, in three separate tumor models (Sa1N, MC38, and B16), we found significant expression of PD-1 and/or LAG-3 on both CD4- and CD8-expressing tumor-infiltrating lymphocytes (TIL). Tumors implanted onto PD-1/LAG-3 double-knockout mice were mostly rejected, whereas PD-1 single-knockout mice showed delayed tumor growth. In that regard, LAG-3 knockout mice were not significantly different from wild-type mice in terms of tumor growth, underscoring the more subtle nature of the LAG-3 checkpoint. In preclinical studies, we treated established Sa1N and MC38 tumors by blocking either LAG-3, PD-1, or both. As was the case in studies with TIM-3, anti-PD-1 monotherapy showed some efficacy (including a small percentage of "cured" animals), anti-LAG-3 monotherapy delayed tumor growth, and quite strikingly, combined blockade resulted in the majority of tumors being rejected, without any evidence of autoimmune side effects. These results were mathematically synergistic and seemed to be mediated by increased secretion of effector cytokines such as IFN-γ and TNF-α by TILs.

Clinical–Translational Advances: Coexpression of Immune Checkpoints on Human T Cells

Recent studies of virus-specific T cells in humans corroborate the results discussed above involving murine models of chronic infection. Specifically, in patients with chronic hepatitis C (HCV), CD8 T cells specific for HCV coexpressed combinations of PD-1, 2B4, and CD160 (47). Furthermore, cells coexpressing multiple checkpoint proteins expressed low levels of CD127, indicating that these cells were actively responding to the virus. As was the case in the murine models, coexpression of multiple checkpoint molecules was correlated with decreased proliferative capacity *in vitro*. TIM-3 has also been found on HCV-specific CD8 T cells. Surprisingly, in patients transitioning from acute to chronic HCV infection, a significant increase was observed in the expression of TIM-3 on HCV-specific CD8 T cells in the peripheral blood, as well as significant coexpression of PD-1 and TIM-3 (48). Furthermore, the majority of intrahepatic CD8 T cells expressed PD-1 and TIM-3, followed by a population expressing PD-1 alone, mirroring the data in TILs. Blocking TIM-3 and PD-1 during *in vitro* restimulation also restored proliferative function of T cells to HCV peptides, suggesting that combinatorial blockade could also be of clinical use in chronic infections.

Another chronic infection in which checkpoint proteins have been implicated is HIV. A recent report examining the role of checkpoint proteins on HIV-specific CD8 T cells found increases in PD-1, CD160, and 2B4 expression (49). Curiously, no significant increase was observed in LAG-3 expression on these CD8 T cells, suggesting once again that while checkpoint molecules act in concert, their signaling and expression is likely not redundant. Expression of PD-1 and CD160 decreased following highly active antiretroviral therapy in these patients and as in the preclinical models, distinct patterns of combinatorial expression were evident. Also similar to the murine models, the most prevalent subpopulations expressed PD-1 and a combination of other markers, in this case CD160. Furthermore, the number of checkpoint proteins expressed was correlated with an inability to produce IFN-γ upon restimulation *in vitro*. Together, these data mirror the preclinical murine data and suggest a potential clinical strategy involving combinatorial checkpoint blockade to treat chronic infectious diseases in patients.

In cancer, recent studies have begun to investigate coexpression of immune checkpoint molecules on either tumor-infiltrating or tumor-specific T cells. Some of the earliest studies involved isolation of peripheral blood lymphocytes and TIL from women with ovarian cancer (50). Cells specific for the cancer-testis antigen NY-ESO-1 were found to coexpress LAG-3 and PD-1, with the double-positive cells being most impaired in terms of IFN-γ secretion. Of clinical relevance, blocking both immune checkpoint molecules during *in vitro* T-cell priming augmented both proliferation and cytokine secretion, again suggesting combined checkpoint blockade as a potential therapeutic intervention. Similar results have been reported for the combination of TIM-3 and PD-1 in patients with melanoma (51). Perhaps the most comprehensive analysis of immune checkpoint coexpression was recently reported by Baitsch and colleagues, who examined the expression of CTLA-4, PD-1, LAG-3, and TIM-3, in addition to CD160, 2B4, and BTLA (52). These data are fascinating, suggesting that naïve T cells are controlled primarily by TIM-3 and BTLA, whereas effector T cells that infiltrate tumors coexpressed a wide variety of combinations of checkpoint molecules, depending to some degree on anatomical location. The conclusion of those studies was that further work is necessary to define the relative role of different checkpoint molecules in patients.

Clinically, a variety of checkpoint blocking agents are being developed to block PD-1 and CTLA-4 signaling. These include a wide variety of monoclonal antibodies blocking CTLA-4, PD-1, or PD-L1 as well as PD-L2 and LAG-3 fusion proteins (Fig. 1). Currently, several early-stage, ongoing clinical trials are exploring combined monoclonal antibody-based immune checkpoint blockade in patients with cancer and a phase III trial in melanoma has been announced (Table 1). These studies all involve the combination of anti-CTLA-4 (ipilumumab), which is approved by the U.S. Food and Drug Administration for treatment of patients with melanoma and anti-PD-1 (nivolumumab), which is currently in phase III trials in several tumor types. Recently, a study investigating stage III or IV unresectable melanoma (NCT01024231) was published with quite striking results (6). Across all dose levels, concurrent delivery of anti-PD-1 (nivolumumab) and anti-CTLA-4 (ipilimumab) resulted in objective responses in 40% of patients. When the

Table 1. Combined immune checkpoint blockade: current clinical trials

Trial ID	Target disease	Agents	Description	Phase
NCT01024231	Unresectable stage III or IV malignant melanoma	Nivolumab (BMS-936558) Ipilimumab (BMS-734016)	Phase I dose escalation trial	I
NCT01472081	Metastatic renal cell carcinoma (clear cell)	Nivolumab (BMS-936558) Ipilimumab (BMS-734016)	Experimental arms with ipilimumab and nivolumab at doses of 1 mg/kg + 3 mg/kg and 3 mg/kg + 1 mg/kg, respectively	I
NCT01454102	Non–small cell lung cancer	Nivolumab (BMS-936558) Ipilimumab (BMS-734016)	Various dose combinations of ipilimumab and nivolumab	I
NCT01783938	Advanced or metastatic melanoma	Nivolumab (BMS-936558) Ipilimumab (BMS-734016)	Randomized phase II study: sequencing trial of ipilimumab followed by nivolumab versus nivolumab followed by ipilimumab	II
NCT01844505	Previously untreated unresectable or metastatic melanoma	Nivolumab (BMS-936558) Ipilimumab (BMS-734016)	Randomized phase III trial of ipilimumab, nivolumab, or combination	III

combination was given at the maximum tolerated dose, 53% of patients had objective responses. Furthermore, these responses were rapid: All responding patients had a tumor reduction of 80% or more by their first scheduled assessment. Studies in kidney cancer (NCT01472081) and non–small cell lung cancer (NCT01454102) recently opened combined anti-CTLA-4/anti-PD-1 arms, and it will be interesting to see whether the melanoma results extend to the histology of other diseases. It also remains to be seen whether this combination will prove tolerable, or whether further dose and schedule optimization is necessary.

Conclusions

Preclinical models of chronic infection, self-tolerance, and tumor tolerance have illuminated a role for combination of checkpoint molecules in regulating the immune response. Remarkably, despite the differences in these models, several broad conclusions have emerged. First, in many preclinical models of T-cell tolerance and exhaustion as well as in human disease, multiple immune checkpoint molecules are coexpressed on CD4 and CD8 T cells. Second, certain combinations of checkpoint molecules are expressed more frequently than are other combinations, in many cases involving coexpression of PD-1 with other molecules. A potentially central role for PD-1 in tumor tolerance is supported by data showing expression on TIL in many tumor types (1), in both mice and humans, as well as by data showing that PD-1 is upregulated at the first division in a tolerogenic environment (53). Although patterns of checkpoint coexpression have only begun to be analyzed in patients with cancer, these accumulating data could be quite valuable in designing combination regimens; in fact it could very well turn out that combination checkpoint blockade requires a personalized approach to achieve maximal efficacy. Finally, and perhaps most importantly, combinations of individual checkpoint blockades can result in increased clinical benefit, as highlighted by recent clinical data in patients with melanoma; a great deal of additional clinical work is required to understand the potential for combined checkpoint blockade to induce long-term clinical responses in patients with cancer.

Authors' Contributions

Conception and design: C.J. Nirschl, C.G. Drake
Development of methodology: C.J. Nirschl, C.G. Drake
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Nirschl, C.G. Drake
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.J. Nirschl, C.G. Drake
Writing, review, and/or revision of the manuscript: C.J. Nirschl, C.G. Drake
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.G. Drake

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References

1. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12:252–64.
2. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007;8:239–45.
3. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711–23.
4. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates

of anti-PD-1 antibody in cancer. N Engl J Med 2012;366:2443–54.
5. Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Kefford R, et al. Safety and tumor responses with lambrolizumab (Anti–PD-1) in melanoma. N Engl J Med 2013;369:134–44.
6. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;369:122–33.
7. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996;271:1734–6.
8. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995;3:541–7.
9. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995;270:985–8.
10. Marengere LE, Waterhouse P, Duncan GS, Mittrucker HW, Feng GS, Mak TW. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. Science 1996;272:1170–3.
11. Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, et al. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. Immunity 2000;13:313–22.
12. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol 2005;25:9543–53.
13. Chen L. Coinhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004;4:336–47.
14. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677–704.
15. Nishimura H, Minato N, Nakano T, Honjo T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. Int Immunol 1998;10:1563–72.
16. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matrumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001;291:319–22.
17. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity 2007;27:111–22.
18. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J Immunol 2004;173:945–54.
19. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett 2004;574:37–41.
20. Riley JL. PD-1 signaling in primary T cells. Immunol Rev 2009;229:114–25.
21. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, Carter L, Iwai Y, Yagita H, et al. Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. J Immunol 171:1272–7.
22. Goldberg MV, Drake CG. LAG-3 in cancer immunotherapy. Curr Top Microbiol Immunol 2011;344:269–78.
23. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med 1990;171:1393–405.
24. Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, et al. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. J Exp Med 1992;176:327–37.
25. Miyazaki T, Dierich A, Benoist C, Mathis D. LAG-3 is not responsible for selecting T helper cells in CD4-deficient mice. Int Immunol 1996;8:725–9.
26. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. J Clin Invest 2007;117:3383–92.
27. Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X, et al. Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. J Immunol 2011;187:3493–8.
28. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. Eur J Immunol 2003;33:970–9.
29. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. Immunity 2004;21:503–13.
30. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol 2003;4:1093–101.
31. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 2002;415:536–41.
32. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 2005;6:1245–52.
33. van de Weyer PS, Muehlfeit M, Klose C, Bonventre JV, Walz G, Kuehn EW. A highly conserved tyrosine of Tim-3 is phosphorylated upon stimulation by its ligand galectin-9. Biochem Biophys Res Commun 2006;351:571–6.
34. Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, et al. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3–mediated cell death and exhaustion. Nat Med 2012;18:1394–400.
35. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 1998;188:2205–13.
36. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009;10:29–37.
37. Jin H-T, Anderson AC, Tan WG, West EE, Ha S-J, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci U S A 2010;107:14733–8.
38. Grosso JF, Goldberg MV, Getnet D, Bruno TC, Yen HR, Pyle KJ, et al. Functionally distinct LAG-3 and PD-1 subsets on activated and chronically stimulated CD8 T cells. J Immunol 2009;182:6659–69.
39. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage plasmodium infection. Nat Immunol 2012;13:188–95.
40. Korman A, Chen B, Wang C, Wu L, Cardarelli P, Selby M. Activity of anti-PD-1 in murine tumor models: role of "host" PD-L1 and synergistic effect of anti-PD-1 and anti-CTLA-4. J Immunol 2007;178:48.37.
41. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci U S A 2010;107:4275–80.
42. Mangsbo SM, Sandin LC, Anger K, Korman AJ, Loskog A, Tötterman TH. Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. J Immunother 2010;33:225–35.
43. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T cell rejection function in tumors. Cancer Res 2013;73:3591–603.
44. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. J Exp Med 2010;207:2187–94.
45. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res 2012;72:917–27.
46. Okazaki T, Okazaki IM, Wang J, Sugiura D, Nakaki F, Yoshida T, et al. PD-1 and LAG-3 inhibitory coreceptors act synergistically to prevent autoimmunity in mice. J Exp Med 2011;208:395–407.
47. Bengsch B, Seigel B, Ruhl M, Timm J, Kuntz M, Blum HE, et al. Coexpression of PD-1, 2B4, CD160, and KLRG1 on exhausted

HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. PLoS Pathog 2010;6:e1000947.
48. McMahan RH, Golden-Mason L, Nishimura MI, McMahon BJ, Kemper M, Allen TM, et al. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence and its blockade restores hepatocyte-directed *in vitro* cytotoxicity. J Clin Invest 2010;120: 4546–57.
49. Yamamoto T, Price DA, Casazza JP, Ferrari G, Chattopadhyay PK, Roederer M, et al.Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. Blood 2011;117:4805–15.
50. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, et al. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad Sci U S A 2010;107:7875–80.
51. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med 2010;207:2175–86.
52. Baitsch L, Legat A, Barba L, Fuertes Marraco SA, Rivals JP, Baumgaertner P, et al. Extended coexpression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization. PLoS ONE 2012;7:e30852.
53. Goldberg MV, Maris CH, Hipkiss EL, Flies AS, Zhen L, Tuder RM, et al. Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells. Blood 2007;110:186–92.