Combinatorial approach to cancer immunotherapy: strength in numbers

Anna E. Vilgelm,*†,‡ Douglas B. Johnson, and Ann Richmond*†

Abbreviations: 5-AZA-CdR = 5-aza-2'-deoxycytidine, AML = acute myelogenous leukemia, ASCO = American Society of Clinical Oncology, BTLA = B and T lymphocyte attenuator, CD40L = cluster of differentiation 40 ligand, DAMP = damage-associated molecular pattern, DNMT = DNA methyltransferase, FDA = U.S. Food and Drug Administration, Gal-9 = Galectin-9, GITR = glucocorticoid-induced TNFR-related protein, HDAC = histone deacetylase, ICT = immunotherapy, Ipilimumab, mAb = monoclonal antibody, LAG-3 = lymphocyte activation gene-3, MHC = major histocompatibility complex, NCI = National Cancer Institute, NY-ESO-1 = New York esophageal squamous cell carcinoma antigen, OX40 = CD134, PD-1 = programmed death 1, TIM-3 = T cell immunoglobulin and mucin-domain containing 3, TIGIT = T cell immunoreceptor with Ig and ITIM domains, TLR = Toll-like receptor, Tregs = regulatory T cells, TIGIT = T cell immunoreceptor with Ig and ITIM domains, TIGIT = T cell immunoreceptor with Ig and ITIM domains, VEGF = vascular endothelial growth factor, VISTA = V-domain containing免疫-inhibitory receptor with a cysteine-rich domain and Ig superfamily motifs 1

ABSTRACT

Immune-checkpoint blockade therapy with antibodies targeting CTLA-4 and PD-1 has revolutionized melanoma treatment by eliciting responses that can be remarkably durable and is now advancing to other malignancies. However, not all patients respond to immune-checkpoint inhibitors. Extensive preclinical evidence suggests that combining immune-checkpoint inhibitors with other anti-cancer treatments can greatly improve the therapeutic benefit. The first clinical success of the combinatorial approach to cancer immunotherapy was demonstrated using a dual-checkpoint blockade with CTLA-4 and PD-1 inhibitors, which resulted in accelerated FDA approval of this therapeutic regimen. In this review, we discuss the combinations of current and emerging immunotherapeutic agents in clinical and preclinical development and summarize the insights into potential mechanisms of synergistic anti-tumor activity gained from animal studies. These promising combinatorial partners for the immune-checkpoint blockade include therapeutics targeting additional inhibitory receptors of T cells, such as TIM-3, LAG-3, TIGIT, and BTLA, and agonists of T cell costimulatory receptors 4-1BB, OX40, and GITR, as well as agents that promote cancer cell recognition by the immune system, such as tumor vaccines,IDO inhibitors, and agonists of the CD40 receptor of APCs. We also review the therapeutic potential of regimens combining the immune-checkpoint blockade with therapeutic interventions that have been shown to enhance immunogenicity of cancer cells, including oncolytic viruses, RT, epigenetic therapy, and senescence-inducing therapy. J. Leukoc. Biol. 100: 275–290; 2016.

Introduction

The ultimate goal of immunotherapeutic cancer treatment is to make the immune system more efficient in killing tumor cells.

Early approaches to cancer immunotherapy included peptide vaccination and high-dose cytokines and had modest clinical activity overall. The breakthrough in the field of cancer immunotherapy came from the basic immunology research investigating fundamental mechanisms of T cell function. These studies have led to the clinical development and licensing of therapeutics targeting 2 distinct inhibitory receptors of T cells, CTLA-4 and PD-1, which induce durable responses in a small but significant proportion of patients. These agents have transformed the therapy of metastatic melanoma, a disease notorious for promptly developing resistance to traditional systemic treatments. Inspired by this success, a major focus of current translational cancer research is exploring the clinical use of manipulating other molecules involved in T cell regulation, as well as finding other ways of rendering tumors responsive to immunotherapies.

IMMUNE-CHECKPOINT INHIBITORS

Approved for Treatment of Cancer

Ipilimumab, a mAb targeting the coinhibitory receptor CTLA-4 (CD152), was the first immune-checkpoint inhibitor to be approved for treatment of human cancer. CTLA-4 plays a critical role in negative regulation of T cell function. It attenuates T cell responses by interfering with their costimulation via costimulatory receptor CD28 [1]. CD28 has 2 ligands, B7-1 (CD80) and B7-2 (CD86), which are expressed on APCs. When a T cell recognizes an MHC-bound peptide antigen via the TCR, CD28 provides the second signal critical for activation of naïve T cells, which results in their proliferation, production of cytokines, and survival [1]. CTLA-4 shares structural similarity with CD28 and can bind to the ligands of CD28, B7-1 and B7-2, albeit with much higher affinity [2, 3]. Furthermore, CTLA-4 is known to be critical for inhibitory function of Tregs [4]. The blocking of interactions between CTLA-4 and the B7 molecules leads to persistent T cell responses and tumor eradication [5, 6]. The anti-tumor effect of anti-CTLA-4 therapy is attributed to the enhancement of T cell priming, which is associated with
generation of new tumor-responsive T cells [7], as well as the Fc-dependent depletion of Treg in the TME [8].

Anti-CTLA-4 therapy with ipilimumab significantly improved OS for patients with metastatic melanoma in clinical trials [9, 10] and was approved by the FDA for treatment of this disease in 2011. One large Phase 3 study of 676 patients receiving ipilimumab and/or a peptide vaccine reported a significant benefit in OS for ipilimumab-treated cohorts, with a median survival of 10.0 mo for ipilimumab plus vaccine versus 10.1 mo for ipilimumab alone versus 6.4 mo for peptide vaccine alone [9]. Furthermore, in a Phase 3 trial of tremelimumab—another anti-CTLA-4 antibody in development—the response duration was longer for patients who received tremelimumab compared with the chemotherapy-treated cohort (35.8 vs. 13.7 mo), although the difference in OS between treatment groups did not meet statistical significance [11]. Despite the demonstrated OS advantage, the ORRs in multiple clinical trials of anti-CTLA-4 antibodies are typically only ~10% [12]. Some patients may experience prolonged stable disease or atypical immune-related responses (including tumor growth, followed by shrinkage).

On the downside, the treatment with anti-CTLA-4 antibodies is often associated with significant side-effects related to aberrant immune activation in multiple organ systems, including skin (dermatitis, pruritus), gastrointestinal tract (diarrhea, colitis), liver (hepatitis), and endocrine organs (hypophysitis, thyroiditis, hypothyroidism, adrenal insufficiency) [9]. Common treatment-related toxicities together with modest response rates prompted an extensive search for biomarkers to guide the selection of patients who are likely to respond to therapy without developing serious complications. Snyder et al. [13] sequenced exomes in 64 melanoma tumors and showed that a high level of mutations in tumors correlates with a sustained clinical benefit from the CTLA-4 blockade. However, high mutational load alone is not sufficient to predict clinical benefit, as not all of the tumors with a high mutational burden responded to therapy. The authors then identified candidate tumor neoantigens that arose from the somatic tumor mutations using genome-wide somatic neoepitope analysis and found that these predicted neoantigens activated T cells from the patients treated with ipilimumab. Likewise, Van Allen et al. [14] showed that the number of nonsynonymous mutations per tumor correlated with clinical responses in ipilimumab-treated melanoma patients in their whole exome analysis of 110 pretreatment melanoma tumor biopsies and matching germline tissue samples. However, no recurrent neoantigen peptide sequences that can predict patients’ benefit to the CTLA-4 blockade were found. Among other potential biomarkers, absolute lymphocyte counts and increased frequency of ICOS+ CD4 T cells after the first 2 doses of ipilimumab therapy have been associated with therapeutic benefit, whereas baseline serum concentrations of soluble CD25 predicted resistance to therapy [15–17].

Superior clinical outcomes in a wider range of cancer types have been demonstrated by the antibodies that target PD-1, another immune-checkpoint receptor that inhibits responses of activated T cells [18]. The ligands of PD-1, PD-L1 and PD-L2, are expressed on many cell types, including T cells, epithelial cells, endothelial cells, and tumor cells. These ligands are induced in response to IFN-γ, which is produced by activated T cells [19]. Therefore, unlike CTLA-4 that functions early in T cell activation to block costimulation, the PD-1/PD-L1 pathway inhibits T cells that have already been activated, thus protecting healthy host cells from T cell attack. Tumors often use the PD-1/PD-L1 immune checkpoint to escape immune surveillance by overexpressing PD-L1 (B7-H1) in the TME [20]. Consequently, the blocking of the PD-1/PD-L1 axis in such tumors is expected to “release the breaks” on the anti-tumor immune response.

Several PD-1 antagonists, such as nivolumab and pembrolizumab, demonstrate excellent activity against melanoma, RCC, and NSCLC, leading to significant improvement of patients’ OS. PD-1- and PD-L1-blocking antibodies are currently being tested against a spectrum of other solid and hematologic malignancies, including bladder, prostate, head and neck, breast cancer, and Hodgkin lymphoma [21]. In a Phase 3 study involving 418 previously untreated patients with metastatic melanoma without a BRAF mutation, nivolumab outperformed chemotherapy with a 1 yr OS rate of 72.9% and an ORR of 40% (compared with 42.1% 1 yr OS and 13.9 ORR in dacarbazine group) [22]. Likewise, pembrolizumab showed a significant improvement of response rates compared with ipilimumab (33.7 vs. 11.9%), as well as improved estimated 12 mo survival rates (74.1 vs. 58.2%) [23]. Furthermore, anti-PD-1 therapy with pembrolizumab or nivolumab is effective after ipilimumab failure [24, 25]. Notably, compared with CTLA-4, substantially fewer serious immune-related adverse events were reported with anti-PD-1 therapy. Anti-PD-1 immunotherapy has been cleared for clinical use in the United States, as well as in several other developed countries, including Europe, Australia, and Japan. Despite the efficacy and relative lack of toxicities of anti-PD-1 therapy, the rise of its global use is exciting. However, the high cost of this therapy will be a barrier for many patients and will need to decrease substantially before it is accessible to low-income countries. Identification of biomarkers that accurately pinpoint potential responders would certainly help to facilitate the deployment of anti-PD-1/PD-L1 therapy in low-income settings.

There are several potential biomarkers being evaluated for their ability to predict clinical response to PD-1/PD-L1 therapy. Among those, the level of PD-L1 expression in the tumor is, perhaps, the potential biomarker of most debate. The analysis of tumors from patients on an anti-PD-1 clinical trial identified absence of tumor PD-L1 expression as a potential predictor of poor therapeutic response. In this study, 9 out of 25 patients with PD-L1-positive tumors responded to the PD-1 blockade, whereas none of the 17 patients with undetectable tumor PD-L1 expression responded [26]. Likewise, in a clinical study of anti-PD-L1, therapeutic response was associated with high levels of PD-L1 in tumors, especially when PD-L1 was expressed by tumor-infiltrating immune cells [27]. However, whereas many other
trials confirmed that PD-L1-positive tumors tend to respond better to the PD-1/PD-L1 blockade, collective evidence demonstrates that PD-L1 status alone is not always an accurate predictor of response. A recent meta-analysis of 20 distinct trials of anti-PD-1 and anti-PD-L1 agents, involving 1475 patients collectively, reported a statistically significant association between the tumor PD-L1 expression and the response to PD-1 therapy; however, there were responders in both PD-L1-positive and negative groups. Specifically, an ORR of 34.1% was found in patients with PD-L1-positive and 19.9% in patients with PD-L1-negative tumors [28]. Moreover, recently reported results from randomized Phase 3 clinical trials of PD-1 antagonists in melanoma, RCC, and NSCLC demonstrated efficacy in patients with either PD-L1-positive or -negative tumors [29–31]. Based on these facts, PD-L1 should not be used as a prerequisite for prescribing anti-PD-1/PD-L1 therapy. Clearly, additional predictive biomarkers are needed to guide anti-PD-1 treatment in adherence with the principals of precision medicine. A study of 46 metastatic melanoma tumors biopsied before and during anti-PD-1 therapy demonstrated that responsive patients have higher numbers of CD8+, PD-1+, and PD-L1-expressing cells at the invasive margin and inside the tumor and increased clonality in antigen specificity of T cells within the tumor [32]. Furthermore, we have recently demonstrated that expression of MHC II on melanoma cells can predict response to anti-PD-1/PD-L1 therapy in patients. Collectively, these findings point out that the therapeutic benefit from the PD-1/PD-L1 blockade requires hindered pre-existing immunity that gets reinvigorated by the treatment.

Emerging combinations of immune-checkpoint inhibitors

Besides CTLA-4 and PD-1, TILs express a diverse array of additional inhibitory coreceptors that function as immune-checkpoint regulators and can be targeted to boost tumor immunity. Many promising emerging immunotherapeutic modalities are based on targeting these molecules in the TME (Fig. 1).

**TIM-3.** TIM-3 is a co-inhibitory receptor known to negatively regulate T cell responses. It was initially identified as a specific marker of fully differentiated IFN-γ-producing CD4 Th1 [38] but later has been shown to play a significant biologic role in other cells, including CD8 T cells, T<sub>reg</sub>, dendritic cells, macrophages, and MDSCs [39–40]. TIM-3 is activated by binding its ligand Gal-9 that is ubiquitously expressed in a variety of tissues. This binding induces aggregation and death of TIM-3+ Th1 cells [41]. The blocking of the TIM-3/Gal-9 interaction induces hyperproliferation of effector T cells and increases Th1 cytokine production and CD8 T cell cytotoxicity [41–42]. Interestingly, Gal-9-independent activation of TIM-3 was also reported. Chiba et al. [43] showed that TIM-3 expressed on tumor-infiltrating dendritic cells binds HMGB1, thus preventing HMGB1 from binding the DNA released from dying tumor cells and delivering it to the innate cells. The loss of HMGB1 binding to DNA ultimately dampens the innate immune response in tumor tissue. Not surprisingly, the targeting of TIM-3 is currently being developed as a promising, emerging modality of cancer immunotherapy [44]. There is experimental evidence that TIM-3 antagonists will be an excellent therapeutic partner for PD-1/PD-L1-blocking antibodies. Coexpression of TIM-3 and PD-1 marks exhausted T cells [45–46], and this coexpression links to loss of function of TA-specific CD8<sup>+</sup> T cells in melanoma patients [47]. In addition, intratumoral forkhead box P3<sup>+</sup> T<sub>reg</sub> that coexpress TIM-3 and PD-1 are highly suppressive [48]. The cotargeting of TIM-3 and PD-1 pathways can reverse T cell exhaustion and restore anti-tumor immunity in experimental and carcinogen-induced mouse models of melanoma, sarcoma, and colon cancer [49–50]. Furthermore, the combination of the blockade of the TIM-3/PD-1 pathways extended survival in mice with AML [46]. Early-phase clinical trials of this combination are ongoing for patients with advanced malignancies (Table 1).

**LAG-3.** Another targetable inhibitory receptor that is expressed on activated CD4 and CD8 T cells is the LAG-3 (CD223) [41]. This surface molecule is highly homologous to

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**COMBINATIONS OF IMMUNOTHERAPEUTIC AGENTS**

**Dual CTLA-4 and PD-1 blockade**

In view of the success of anti-PD-1 and anti-CTLA-4 as single agents, combining these agents was considered as a potential strategy. Indeed, early preclinical studies supported this concept [33] and led to several clinical trials. In the first clinical trial of combined immunotherapy with CTLA-4 and PD-1 antagonists involving 53 patients with advanced melanoma, an ORR of 53% was achieved with acceptable level of adverse events [34]. Likewise, another Phase 2 study of 142 patients with previously untreated advanced melanoma reported improved ORR and the PFS with combined nivolumab/ipilimumab compared with ipilimumab monotherapy. A subsequent large Phase 3 study involving 945 previously untreated patients with unresectable Stage 3 or 4 melanoma revealed a median PFS of 11.5 mo with nivolumab plus ipilimumab compared with 2.9 mo with ipilimumab and 6.9 mo with nivolumab [35]. As a result, the FDA has granted accelerated approval to the combination of the PD-1 inhibitor nivolumab and the CTLA-4 inhibitor ipilimumab to treat advanced melanoma [36]. Based on these exciting findings in melanoma, nivolumab/ipilimumab is now undergoing clinical trials in many other human malignancies (see Table 1 for examples). The synergy of ipilimumab and nivolumab is likely a result of the collaboration of 2 nonredundant mechanisms that boost anti-tumor immunity via the amplification of anti-tumor T cells and broadening of their repertoire in response to CTLA-4 targeting and through overcoming immunosuppression induced in the TME by blocking PD-1/PD-L1 interaction. Notably, combination therapy demonstrated increased immune-related adverse events compared with either agent alone [37]. Overall, however, toxicities were similar in nature and were well-managed with standard treatment algorithms. In addition to the obvious benefit of the combined CTLA-4/PD-1 blockade, there are several potential drawbacks, including added toxicities and the high cost of therapy. Therefore, the detection of ways to identify patients who will gain a long-term benefit from single-agent treatment alone remains a priority of current translational melanoma research. Furthermore, a number of additional immunostimulatory approaches are being tested in combination with the CTLA-4 or PD-1 immune-checkpoint blockade in an effort to achieve high rates of durable responses while increasing the “benefit/risk” ratio.
<table>
<thead>
<tr>
<th>Method</th>
<th>Combination therapy and drug used</th>
<th>Immunotherapy target and drug used</th>
<th>Trial phase</th>
<th>Tumor types</th>
<th>Trial reference</th>
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CD4 in structure and like CD4, can bind to MHC II molecules, but the binding affinity for LAG-3 is much higher than for CD4 [51, 52]. It negatively regulates T cell proliferation, homeostasis, and function via the inhibition of TCR signaling following antigen activation [53, 54]. LAG-3 is also necessary for the optimal function of immunosuppressive Tregs, and its expression on conventional T cells makes them more susceptible to Treg-based suppression [55, 56]. Interestingly, LAG-3 and PD-1
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<th>Method</th>
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<th>Tumor types</th>
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GM.CD40L, GM-CSF-producing and CD40L-expressing bystander cell line; BCG, bacillus Calmette-Guérin; IDO, BRAFi, MEKi, EGFRi, DNMTi, and HDACi, IDO, BRAF, MEK, epidermal growth factor, DNMT, and HDAC inhibitors.
were found to be coexpressed on TILs of ovarian cancer patients [57], as well as on TILs derived from mouse melanoma, fibrosarcoma, and colon adenocarcinoma tumors [58]. Furthermore, whereas single PD-1 and LAG-3 knockout had minimal immunopathological consequences in C57BL/6 mice, dual knockout mice abrogated self-tolerance, resulting in autoimmunity infiltrates in multiple organs and lethality [58]. These findings suggest that LAG-3 and PD-1 act synergistically to control immune homeostasis and mediate tumor-induced tolerance. Notably, combined anti-LAG-3/anti-PD-1 antibody treatment cured most mice of established fibrosarcoma and colon adenocarcinoma tumors that were largely resistant to single-antibody therapy [58]. Likewise, in a mouse model of recurrent melanoma, the simultaneous immune-checkpoint blockade with combination therapy with PD-L1 and LAG-3 antagonists caused regression of aggressive B16F10 tumors [59].

TIGIT. TIGIT is an emerging tumor immunotherapy target in preclinical development. It is an inhibitory receptor of the Ig superfamily expressed on subsets of activated T cells and NK cells [60]. One study showed that TIGIT is up-regulated on TA-specific CD8+ T cells and CD8+ TILs from patients with melanoma and cooperates with PD-1 to impair anti-tumor activity of these cells [61]. In contrast to these findings, another recent study reported that TIGIT primarily suppresses anti-tumor immunity via its ability to stimulate Tregs rather than by inhibiting CD8+ T cells [62]. A recent report demonstrated that cotargeting TIGIT and PD-L1 results in complete regression of subcutaneous tumors formed by mouse colorectal carcinoma cells [63], highlighting the potential of this treatment strategy.

BTLA. BTLA is another inhibitory receptor found on B and T cells. BTLA may also be targeted for cancer therapy. Up-regulation of BTLA and PD-1 in the TME was shown to inhibit the function of TA-specific CD8+ T cells, and dual antagonism of these receptors ex vivo enhanced the expansion, proliferation, and cytokine production in these cells [64]. However, a more recent study reported that the role of BTLA in anti-tumor response may be complex, as it can act as a dual signaling molecule, inhibiting the activation of T cells and at the same time, enhancing the survival of CD8+ T cells [65].

Combinations of immune-checkpoint inhibitors with agonists of costimulatory receptors
An alternative to the checkpoint blockade approach for reactivation of anti-tumor immune responses is to activate costimulatory receptors of T cells that in conjunction with TCR signaling, promote their proliferation, as well as proinflammatory and cytotoxic activity [66]. Costimulatory receptors are often constitutively expressed on the surface of T cells, including resting antigen-naïve T cells, or become transiently available in response to antigen recognition. A classic example of costimulatory receptors is CD28, which plays a crucial role in T cell activation [67]. Antibody agonists of CD28 are used routinely in laboratory practice for stimulation and expansion of T cells in vitro. Unfortunately, these agents may be too potent to be used as therapeutics, as they can induce a very severe systemic inflammatory syndrome driven by a cytokine storm with multiple organ failure, even when administered at extremely low doses [68].

CD27. More clinical potential has been demonstrated for another constitutive costimulatory receptor expressed on T and plasma cells, CD27. Activation of this receptor via the ligation with its ligand CD70 promotes cell survival, expansion, and effector functions of T and B cells [69]. Costimulation of T cells with the CD27 agonist antibody in vitro can activate memory CD4+ and CD8+ T cells but not purified Tregs [70]. Furthermore,
costimulation with CD27 and OX40 was shown to synergize with the anti-PD-L1 blockade by forcing exhausted CD8+ T cells to exit quiescence [71]. A fully humanized antibody agonist of CD27, varilumab, has completed a Phase 1 dose-escalation study. The manufacturer of the drug (Cellnex Therapeutics, Hampton, NJ, USA) reported potent immunologic and anti-tumor activity in patients with advanced, refractory disease. Furthermore, minimal toxicity was observed, and the maximal tolerated dose was not reached (http://www.celldex.com/science/publications.php#cdx1127). Furthermore, the company reports improved survival after combining varilumab with immune-checkpoint inhibitors in animal models, and clinical trials of varilumab in combination with the CTLA-4, PD-1, or PD-L1-targeting antibody are ongoing (Table 1). However, another study has shown that in some tumors, the CD27–CD70 interaction may promote tumor growth and inhibit, rather than activate, anti-tumor immune response [72]. Currently, there are several antagonists of CD70 undergoing clinical development for cancer treatment [73]. Therefore, clinical development of therapeutics acting on the CD27–CD70 pathway may require particular precision to achieve potent anti-tumor activity without exaggerating tumor-immune escape.

Several agonists are being developed for therapeutic targeting of costimulatory receptors specific for activated T cells, such as 4-1BB, OX40, GITR, and others (Fig. 1). These receptors, in contrast to ubiquitous CD28 and CD27, are exclusively expressed or substantially up-regulated on a surface of T cells within a few days following TCR engagement.

4-1BB. Lymphocyte costimulatory receptor 4-1BB (CD137/TNF superfamily 9) possesses an unequalled capacity to promote survival, expansion, and enhanced effector function of activated T cells [74]. It is expressed on many subtypes of immune cells and plays a critical role in sustaining effective T cell immune responses and generating immunologic memory [75]. The anti-tumor potential of 4-1BB activation was first shown in 1997 by Melero and colleagues [76], who reported that antibodies against 4-1BB eradicate established tumors in a murine model of mastocytoma and sarcoma. Therapeutic stimulation of 4-1BB was also effective in mouse model of squamous cell cancer [77], lymphoma [78], hepatocellular carcinoma [79], melanoma [80], and colon cancer [81]. A subsequent Phase 1 study of urelumab (BMS-663513), a fully human anti-CD137 agonist mAb, in patients with advanced cancer (NCT0030902), reported evidence of clinical activity, as well as generally manageable adverse effects, including fatigue and neutropenia [82]. However, a follow-up Phase 2 (NCT00612664) had to be terminated as a result of unusually high incidence of Grade 4 hepatitis. Consequently, a number of other trials of CD137 agonists were terminated or withdrawn (reviewed in ref. [83]). Studies in mice confirmed hepatotoxicity, resulting from 4-1BB activation that was associated with the influx of inflammatory cells into the liver of the treated animals [84, 85]. It is plausible that using a low dose of urelumab in combination with other immunotherapies could potentially augment anti-tumor immune response while limiting the severity of therapy-associated adverse events. Recently, a number of trials were initiated using a putatively safer low dose of urelumab in combination with other therapeutics (Table 1). There is another CD137 agonist PF-05082566 currently undergoing clinical development, which in contrast to urelumab, has been reported to be well tolerated [86].

Several animal studies have demonstrated a promising therapeutic benefit of combining 4-1BB agonists with the PD-1 and CTLA-4 blockade. For instance, the combination of 4-1BB agonist and PD-1 antagonist promoted anti-tumor effector/memory CD8+ T cells in a poorly immunogenic, aggressive B16F10 murine melanoma model, resulting in tumor inhibition that was dependent on IFN-γ and CD8+ T cells [87]. Likewise, tumor rejection and enhanced anti-tumor immunity after combined anti-PD-1 and anti-4-1BB therapy were seen in mouse models of colon and ovarian cancer [88, 89]. Anti-4-1BB antibody treatment also augmented the therapeutic response to CTLA-4 antagonist in MC38 colon cancer tumors but not in B16 melanoma tumors [90]. Another study found that the combination of CTLA-4 and 4-1BB agonists had a synergistic effect on tumor rejection in the context of a Fcγ3 ligand-based B16 melanoma vaccine [91].

OX40. OX40 (CD134) is another receptor that serves costimulatory functions. It is expressed on activated CD4+ (including both Th1 and Th2 cells) and CD8+ T cells within 24–96 h following TCR engagement [92, 93]. OX40 is activated by its ligand OX40L (CD252) that is expressed on APCs; this binding augments proliferation and activity of activated T cells [93]. This receptor has been detected on tumor-infiltrating leukocytes and in tumor-draining lymph nodes in patients with melanoma, head and neck, breast, and colorectal cancer [94–97]. Moreover, in colorectal and melanoma tumors, high expression of OX40 on tumor-infiltrating leukocytes was associated with decreased metastasis and longer survival [96, 97]. Interestingly, OX40 has also been implicated in inhibiting activity of Treg cells [98, 99]. Agonists of OX40 have demonstrated promising anti-tumor activity in preclinical development [100]. A Phase 1 clinical trial of the OX40 agonist antibody demonstrated increased anti-tumor activity of T and B cells in melanoma patients. This study also reported tumor regression in 12 of 30 patients and an acceptable level of toxicity [101]. Preclinical evidence also suggests that addition of OX40 agonists may enhance the clinical benefit of an immune-checkpoint blockade. For instance, antibodies specific for OX40 combined with the CTLA-4 blockade demonstrated synergistic anti-tumor activity in mouse lymphoma, leukemia, breast, and sarcoma tumors [98, 102, 103]. Furthermore, OX40 activation combined with the PD-1 blockade was effective against murine ovarian cancer [104]. Phase 1/2 clinical studies, combining the OX40 agonist humanized antibody MEDI6583 with anti-PD-L1 and anti-CTLA-4 antagonists, are ongoing (Table 1).

GITR. Another targetable costimulatory receptor of T cells is GITR. It is expressed at a low level on naive cells and is induced after TCR engagement in CD4+ and CD8+ subsets. Activation of GITR by its ligand (GITRL), which is highly expressed on activated APCs and endothelial cells, promotes survival, proliferation, and effector function of T cells [93, 105, 106]. GITR is constitutively expressed on Treg cells and blocks their inhibitory function [107, 108]. In mice, GITR agonists showed activity against CT26 colon tumors and small B16 melanoma tumors [109, 110]. An agonist of GITR synergized with the CTLA-4 blockade to inhibit the growth of sarcoma and colon tumors.
Likewise, the combination of the PD-1 blockade with the GITR agonist induced potent anti-tumor immunity in mice with peritoneal ovarian cancer tumors [112]. Humanized antibody agonists of GITR are undergoing clinical evaluation.

Combination of immune-checkpoint inhibitors with therapies that promote tumor cell recognition by T cells

Stimulation of APCs. APCs play a critical role in the establishment of effective anti-tumor immunity and are attractive therapeutic targets. For instance, CD40 is a costimulatory molecule essential for activation of APCs, such as dendritic cells, B cells, and macrophages [113, 114]. CD40 is activated when ligated to CD40L (expressed on CD4+ T cells), which substantially increases antigen presentation and costimulatory capacity of APCs. Notably, a synergistic response was observed after combined treatment with the CD40 agonist and PD-1 antagonist, inducing rejection of colon and breast tumor implants in 50% of the tumor-bearing mice [115]. This synergistic response suggests that CD40 may be used potentially as a therapeutic partner for immune-checkpoint inhibitors.

Tumor vaccines. In light of the exceptional success of preventative vaccination against infectious diseases, including polio, tetanus, measles, and many others, the vaccination of patients against antigens expressed by tumors to boost antitumor immunity seems like an attractive therapeutic approach. Somatic mutations in tumors can give rise to neoantigens. Interestingly, Snyder et al. [13] reported significant similarities of tumor neoantigens to certain viral and bacterial antigens with tetrapeptide sequences of tumor neoantigens matching those in known antigenic peptides of pathogens [116]. Response to neoantigens is especially relevant in malignancies with high-mutation burden, such as melanoma. A large number of therapeutic vaccination studies in patients with nonviral cancers have been conducted. As summarized in recent reviews, many Phase 1/2 cancer vaccine studies showed modest clinical benefit, and objective durable tumor regressions were rarely seen [117, 118]. Use of appropriate cotreatments, for instance, immune-checkpoint inhibitors, can potentially alleviate immunosuppressive mechanisms in the TME and boost vaccine performance, and there are numerous clinical studies currently testing checkpoint blockade-vaccine combinations in a variety of tumor types. However, despite the promising reports from mouse model studies, no strong evidence of therapeutic potential for these approaches has been demonstrated in the clinic so far (reviewed recently in refs. [119, 120]). For instance, no evidence of clinical synergy from vaccine plus ipilimumab treatment was shown in a clinical trial, where melanoma patients received CTLA-4 antagonist ipilimumab plus gp100 peptide vaccine, ipilimumab alone, or gp100 alone. In fact, survival was similar for the ipilimumab, with or without the GP100 vaccine, and exceeded survival for the GP100 alone [9]. Furthermore, peptide vaccination did not add to the clinical activity of the anti-PD-1 therapy in a clinical study of the PD-1 antagonist nivolumab in combination with a multipeptide vaccine in melanoma patients [121].

IDO inhibitors. IDO1 is a critical enzyme in the metabolic pathway that converts l-tryptophan to l-kynurenine [122]. It plays an important role in the establishment of local immunosuppression and is responsible for protection of allogeneic fetuses from the attack of maternal T cells [123]. IDO1 is expressed by distinct subsets of macrophages, monocytes, and dendritic cells with potent immunosuppressive activity [124–126] and is often found in tumors, where it is implicated in enabling immunologic tolerance and immune escape; however, the exact mechanism of these effects is still a subject of debate (reviewed in ref. [127]). Inhibition of IDO1 demonstrated anti-tumor activity in a mouse model, which was associated with the induction of an anti-tumor immune response [128], and enhanced the efficacy of chemotherapy [129]. In clinical studies, IDO inhibitors epacadostat (INCB024360) and indoximod (NLG-8189) were well tolerated by the majority of enrolled patients. Disease stabilization was often reported; however, tumor eradication from stand-alone IDO targeting was not achieved [130, 131]. More exciting results were obtained in studies combining IDO inhibitors with the immune-checkpoint blockade. For instance, epacadostat showed promising clinical activity in combination with ipilimumab with ORR of 31% (10 out of 32 immunotherapy-naive patients) and was well tolerated [132]. Furthermore, a number of clinical trials of epacadostat, in combination with PD-1/PD-L1 targeting agents, are ongoing (Table 1). The level of optimism for these approaches is high, based on preliminary results from a Phase 1/2 study of an epacadostat and pembrolizumab (anti-PD-1) combination, reported at the 2015 Society for Immunotherapy of Cancer Annual Meeting, which demonstrated an ORR of 53% (10 out of 19 patients) and a disease control rate of 74% (15 out of 19 patients) across multiple malignancies, with an even more profound effect in patients with melanoma [133].

Combination of immune-checkpoint inhibitors with therapies that enhance tumor immunogenicity

Inducers of ICD. It is well established that a certain type of cell death, ICD, induced by some anti-tumor agents, carries immunostimulatory potential. ICD results from ordered activation of stress-response pathways associated with the emission of danger signals by dying cancer cells, called DAMPs, which promote recognition of dying tumor cells by the innate and adaptive immune system, ultimately eliciting tumor-targeting immune responses [134–136]. Among the most studied of these are the following: membrane exposure of the endoplasmic reticulum chaperone calreticulin and various heat-shock proteins, production of type I IFNs, secretion of ATP, and the release of the nuclear protein HMGB1 into the extracellular space (reviewed in refs. [136–138]). Notably, outside of its cancer-related effect, DAMP release plays a key role on the induction of a systemic inflammatory response syndrome in response to injury [139]. Among the most studied inducers of ICD are several chemotherapeutic drugs and RT. Furthermore, ICD has been reported in response to treatment with oncolytic viruses and drugs that target HDAC [137]. Notably, a recent review of the clinical data on cancer patients, with a variety of different malignancies, showed the prognostic and predictive value of ICD markers, DAMPs and DAMP-associated stress responses [140].
It is plausible that the immunogenic potential of therapies that promote DAMPs can be exploited in combination with immune-checkpoint blockade drugs to stimulate further immune-mediated tumor destruction (Fig. 1). The synergistic relationship between RT and the CTLA-4 blockade was shown in a preclinical model of breast cancer [141, 142], with the PD-1 blockade in melanoma, breast, and glioma models [143, 144], as well as with anti-PD-L1 in melanoma, breast, and colon cancer [145, 146]. In 2012, several anecdotal cases have reported tumor regression of multiple distant, unirradiated metastases (abscopal effect) in patients treated with radiation and anti-CTLA-4 [147, 148]. Later, a small study of 21 melanoma patients, who received RT after progressing on ipilimumab, suggested that RT after ipilimumab leads to abscopal responses in some patients, which correlates with prolonged OS [149]. Another study of 47 patients with metastatic melanoma, treated with ipilimumab and RT, showed an abscopal effect in 68% of cases [150]. Recently, Twyman-Saint Victor and colleagues [151] reported major tumor regression in a subset of patients with metastatic melanoma treated with an anti-CTLA-4 antibody and radiation. However, the majority of patients was resistant to this treatment. The authors recapitulated these results in a mouse model and showed that resistance was a result of up-regulation of PD-L1 on melanoma cells and was associated with T cell exhaustion. Notably, improved responses in the mouse model were seen when RT and anti-CTLA-4 cotreatment was combined with the anti-PD-L1/PD-1 blockade [151]. Multiple clinical trials of immunotherapy/RT regimens for treating patients with melanoma and other cancers are ongoing (examples in Table 1).

In contrast to immune-stimulating effects of modern RT that target tumors with precision, systemic chemotherapy is generally associated with leukopenia and immunosuppression, as it negatively impacts normal proliferation of cells. Nevertheless, a number of clinical studies that test combinations of immunotherapy with chemotherapeutic drugs are ongoing, with a yet-unknown outcome (Table 1). In particular, low-dose chemotherapy (e.g., cyclophosphamide) has demonstrated a proinflammatory effect by depleting Treg and other immune-suppressive cellular subsets [152].

Oncolytic viruses. Oncolytic viruses can be engineered so that they preferentially replicate in, and kill, tumor cells. Compelling evidence has demonstrated that oncolytic viruses can stimulate anti-tumor responses. In 1999, the induction of systemic anti-tumor immunity by replication-competent, attenuated HSV was shown in a syngeneic murine model of neuroblastoma. In this study, experimental mice were protected from tumor rechallenge and had elevated anti-tumor activity of the specific CTL [153]. Furthermore, oncolytic viral therapy is capable of producing regression, not only in injected but also in distant, un.injected tumor lesions. In a murine model of glioma, tumor cells infected with oncolytic rodent parvovirus activated APCs, such as dendritic cells and microglia, resulting in anti-tumor immunity, demonstrated in rechallenge experiments using uninfected tumor cells, and this effect of oncoviral therapy was abrogated in immunodeficient mice [154]. Another study using viral therapy with Newcastle disease virus showed long-term survival and the establishment of the anti-tumor immune memory in a murine glioma model [155]. Furthermore, Zamarin et al. [156] found that injection of oncolytic Newcastle disease virus into subcutaneous melanoma, colon, and prostate tumors in immunocompetent mouse models augments the response to the CTLA-4 blockade via the induction of tumor inflammation. Notably, virotherapy promoted lymphocyte recruitment and tumor regression not only in the tumor that was injected with the virus but also in the un injected tumor that was grown on the opposite flank of the mouse.

A modified herpes virus T-VEC was the first oncolytic virus approved in the United States for therapy of cancer. T-VEC is designed to replicate exclusively in the tumor cells and produces GM-CSF to enhance anti-tumor immune responses. This virus is injected directly into the tumor; therefore, only superficial cutaneous or nodal lesions, but not the distant metastatic lesions, could be treated with this therapy. In a Phase 3 clinical trial, T-VEC demonstrated durable responses lasting >6 mo in 16% of advanced melanoma patients with an ORR of 26% [157]. Combinations of CTLA-4 and PD-1 antagonist antibodies, T-VEC, and other injectable oncolytic viruses are currently in clinical development (Table 1). Preliminary results of the Phase 1b trial of T-VEC plus ipilimumab, reported at the 2015 ASCO Annual Meeting, were very promising, with an ORR of 56%, including 33% complete responses in 18 patients, receiving this combination [158]. The early report from the T-VEC-pembrolizumab study highlights the relatively low toxicity of this therapeutic combination, and although the efficacy has not been officially published as of May 2016, a Phase 3 arm of this trial is already recruiting participants [159].

Epigenetic therapy. Epigenetic silencing is a common strategy used by cancer cells to escape immune surveillance. It leads to down-regulation of tumor-associated antigens or molecules that are required for processing and presentation of these antigens and thereby, interferes with recognition of neoplastic cells by the immune system. Maintenance of gene silencing requires continued activity of DNMT and HDAC; therefore, “epigenetic drugs” that target these enzymes may restore/improve immunologic recognition of cancer cells [160, 161]. Indeed, DNA hypomethylating agent 5-aza-deoxycytidine up-regulates expression of tumor-associated antigens [162], as well as HLA class I molecules [163, 164], in various models. HDAC inhibitors can also restore expression of HLA molecules in cancer cells [165, 166]. Furthermore, HDAC inhibitors have been shown to promote recognition and lysis of cancer cells by NK cells by inducing neoplastic cell expression of MICA, MICB, and/or ULBP1–3, the ligands for the NK cell receptor NKG2D [167, 168]. West et al. [169] demonstrated that an intact immune system was required for the robust anticancer effects of the HDAC inhibitors, vorinostat and panobinostat, against a colon adenocarcinoma and leukemia/lymphoma. In this study, the authors reported signs of ICD, including surface calreticulin exposure, ATP, and HMGB1 release in vorinostat-treated cells. Likewise, calreticulin on the cellular membrane was expressed in cells from childhood brain tumors after exposure to HDAC inhibitors [170].

Several studies have reported synergistic anti-tumor activity of combined epigenetic and immune-checkpoint blockade therapies. For instance, in mouse tumor models, treatment with 5-azacytidine (DNMT inhibitor) and entinostat (HDAC
inhibitor), combined with anti-PD-1- and anti-CTLA-4-targeted antibodies, was able to produce a significantly improved outcome [171]. Intriguingly, the primary targets of the epigenetic modulators were MDSCs and not the tumor cells. Furthermore, mice bearing B16F10 tumors that received combination therapy with HDAC inhibitors and the PD-1 blockade exhibited slower progression of tumors and increased survival compared with control and single-agent treatments [172]. Recently, Covre et al. [173] demonstrated significant immune-related anti-tumor activity of 5-aza-deoxycytidine combined with the anti-CTLA-4 therapy in a mouse model of mammary carcinoma and mesothelioma. Several ongoing human studies of immunotherapy/epigenetic therapy are shown in Table 1.

**Senescence-inducing therapy.** Senescent cells are characterized by metabolic activity in "permanent" cell-cycle arrest. Senescence is induced by aberrant oncogene activation and other cellular stressors. Senescent cells are characterized by enhanced secretion of many proinflammatory cytokines and chemokines, described as the SASP [174, 175], which has been shown to attract cells of the innate and adaptive immune system that kill and clear senescent cells [176, 177]. Based on these findings, it was proposed that senescence and SASP are essential steps in the process of immunosurveillance and elimination of pre-malignant cells in the body [178]. Notably, senescence can be induced in established tumors by overexpression of p53 or in response to certain therapies. It has been shown that senescence subjects tumors to immune targeting [176, 179, 180]. We have reported that senescence induced in melanoma tumors by treatment with the inhibitors of cell-cycle kinases aurora kinase A and cyclin-dependent kinase 4/6 promotes secretion of chemokines that recruit T cells and APCs into the tumor [181, 182]. This activity is potentiated further in cells treated with a combination of senescence-inducing drugs and p53-activating therapy. Furthermore, senescent melanoma cells overexpress TNF family death receptors that make them vulnerable to cell death induced by immune cells secreting death receptor ligands [183]. Importantly, we have recently demonstrated that senescence-inducing therapy can synergize with the agonist antibody to T cell costimulatory receptor 4-1BB (CD137), leading to complete tumor regression in a significant proportion of melanoma-bearing mice [182]. Whereas senescence-inducing and immune therapy combinations have not yet entered clinical development, this approach has proved promising in preclinical studies.

**Targeted therapy**

Targeted therapy is designed to disrupt specific pathways critical for survival and proliferation of cancer cells. The inhibition of oncogenic kinase BRAF (BRAFV600E), activated in ~½ of melanoma cases, has been among the most successful targeted approaches to date. Specific small-molecule inhibitors of BRAF, combined with inhibitors of its downstream signaling mediator MEK, are widely used for patients with BRAFV600E mutations (see ref. [184] for review). Whereas response rates from these drugs are high, prompt acquisition of resistance is a major drawback [35, 185]. It is suggested that combining targeted therapy with immunotherapy may induce durable responses in a large subset of patients [186]. Recent reviews on the subject highlight the immunostimulatory effects of several targeted therapeutics and compile impressive evidence for the potent anti-tumor activity of targeted/immunotherapy combinations [187, 188]. However, higher than expected incidences of toxicity, observed with targeted/immunotherapy combinations, have been hindering the clinical development of these strategies. For instance, severe liver toxicity was responsible for terminating a clinical trial combining the BRAF inhibitor vemurafenib with ipilimumab [189]. In addition, several episodes of severe colitis with bowel perforation in response to the BRAF inhibitor dabrafenib, the MEK inhibitor trametinib, and ipilimumab led to another study closure [190]. Notably, a 2015 ASCO report from an ongoing Phase 1 study that tested combinations of BRAF and MEK inhibitors with anti-PD-L1 therapy in melanoma patients showed strong evidence of clinical activity with a manageable safety profile [191]. These exciting results suggest that PD-L1 blockers may be better combinatorial partners for BRAF/MEK-targeted therapy compared with ipilimumab. Sequential administration is another solution proposed to address toxicity issues with targeted/immunotherapy combinations (see ref. [187] for review).

**CONCLUDING REMARKS**

Just a few decades ago, aggressive malignancies, such as metastatic melanoma and NSCLC, were terminal diseases with very low life expectancy. The development of immune-checkpoint agents, such as CTLA-4- and PD-1-targeting antibodies, has allowed patients to achieve a durable therapeutic response and in some cases, even complete regression of advanced disease. Furthermore, the knowledge gained in cancer immunotherapy research has led to significant advances in the treatment of nonmalignant diseases, including getting closer to a cure of historically devastating, but currently manageable, HIV-1 infections [192]. One current goal of translational cancer research is to capitalize on the success of novel immunotherapeutics to improve further the incidence and durability of patients’ responses. As we have described here, a large number of promising immunotherapeutic combinations are transitioning into clinical practice, including 1 approved combination (dual-checkpoint inhibition with anti-CTLA-4 and anti-PD-1). Furthermore, numerous emerging drug combinations are being investigated in clinical trials for treatment of aggressive malignancies, such as melanoma, lung, breast cancer, liver, renal, brain, and pancreatic cancer. The immune-checkpoint blockade is commonly combined with other antagonists of inhibitory receptors of T cells or with agonists of their costimulatory receptors. Other promising therapeutic partners of the checkpoint blockade are treatments that promote presentation and recognition of TAs by effector T cells and therapies that increase antigenicity of tumor cells. Early clinical and preclinical studies have highlighted the major challenges of combining immunotherapies with other immune-stimulatory agents or targeted therapies, including unexpected toxicities and the importance of treatment dose and schedule. In summary, whereas current immunotherapeutic agents have already demonstrated their impressive potential in
immunogenic tumors, such as melanoma, rational combina-
tions with these agents hold promise to improve further clinical
response rates and overall patient survival. We envision an
extended use of immunotherapy for the treatment of a wide
spectrum of malignancies in the immediate future.

AUTHORSHIP
A.E.V., D.B.J., and A.R. wrote the paper.

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