REVIEWS

The future of immune checkpoint therapy

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Immune checkpoint therapy, which targets regulatory pathways in T cells to enhance antitumor immune responses, has led to important clinical advances and provided a new weapon against cancer. This therapy has elicited durable clinical responses and, in a fraction of patients, long-term remissions where patients exhibit no clinical signs of cancer for many years. The way forward for this class of novel agents lies in our ability to understand human immune responses in the tumor microenvironment. This will provide valuable information regarding the dynamic nature of the immune response and regulation of additional pathways that will need to be targeted through combination therapies to provide survival benefit for greater numbers of patients.

The field of immune checkpoint therapy has joined the ranks of surgery, radiation, chemotherapy, and targeted therapy as a pillar of cancer therapy. Three new immune checkpoint agents have now been approved by the U.S. Food and Drug Administration (FDA) for the treatment of melanoma, and there is a high expectation that these agents, and others in this class, will also be approved over the next several years for treatment of patients with lung cancer, kidney cancer, bladder cancer, prostate cancer, lymphoma, and many other tumor types. The antibody against CTLA-4 ipilimumab was approved in 2011, and two antibodies against PD-1 (pembrolizumab and nivolumab) were approved in 2014. These drugs represent a radical and disruptive change in cancer therapy in two ways. First, they do not target the tumor cell, but target molecules involved in regulation of T cells, the soldiers of the immune system. And, perhaps in a more radical shift, the goal of the therapy is not to activate the immune system to attack particular targets on tumor cells, but rather to remove inhibitory pathways that block effective antitumor T cell responses. Immune checkpoint therapy, with anti-CTLA-4 having longer follow-up than other agents, leads to durable clinical responses that can last a decade and more, but only in a fraction of patients. There are ongoing studies to identify predictive biomarkers with which to select patients for treatment with a particular agent, but the complexity of the immune response has made this difficult.

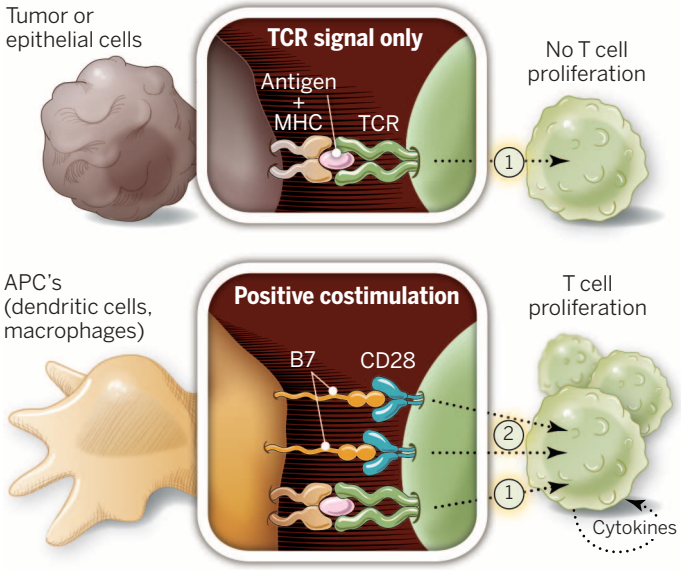

Fig. 1. Activation of T cells requires two signals. T cell activation occurs only after interaction between T cell receptor (TCR) and antigen in the context of MHC (signal 1) plus CD28 costimulation (signal 2).

In the past two decades, remarkable advances in basic science have led to new strategies for the treatment of cancer, which are justifiably generating optimism that it may soon be possible to cure a subset of patients with some types of cancer. We now have detailed knowledge of the molecular basis of cancer to allow a more "personalized" treatment based on genomic sequencing of an individual's cancer cells to identify specific mutations in genes. These mutations can then be targeted with compounds to block the downstream pathways that drive cancer development and progression. Therefore, each specific mutation serves as the predictive biomarker for selecting patients for treatment with a given agent. For example, patients with melanoma whose tumors harbor the BRAFV600E mutation, which enables constitutive activation of the BRAF signaling pathway, would be selected to receive treatment with an agent that inhibits BRAF (*1*, *2*). These targeted therapies have led to promising clinical responses, albeit generally of short duration, in patients whose tumors express the appropriate target biomarker.

The clinical success of genomically targeted agents laid the foundation for other cancer therapies, including the prerequisite to identify predictive biomarkers for selection of patients for treatment. Eventually, as the field of cancer immunotherapy found clinical success with agents based on a greater understanding of how to unleash T cell responses by targeting immune checkpoints, it became clear that the framework used for identification of predictive biomarkers for genomically targeted agents would present a challenge. As opposed to mutated genes in tumors that permanently mark a tumor, the immune response is dynamic and changes rapidly. Therefore, the issue facing the field of cancer immunotherapy may not be the identification of a single biomarker to select a subset of patients for treatment. Instead, we must assess the effectiveness of an evolving immune response, define the immune response that contributes to clinical benefit, and then, hopefully, drive every patient's immune response in that direction through combination therapies.

Tumor microenvironment: Cancer cells and host immune responses

Tumors are composed of many cell types, including the cell of origin with genetic alterations and a myriad of other cells, such as fibroblasts, endothelial cells, and eventually, perhaps, a variety of immune cells. Initially the immune infiltrates may be scarce, but eventually may contain natural killer (NK) cells and macrophages with lytic capacity and, perhaps most importantly, T cells. T cells attack tumor cells that express tumor-specific antigens in the form of complexes of tumor-derived peptides bound to major histocompatibility complex (MHC) molecules on the cell. The tumor antigens can be derived from oncogenic viruses, differentiation antigens, epigenetically regulated molecules such as cancer testes antigens, or neoantigens derived from mutations associated with the process of carcinogenesis (*3*). T cells survey the microenvironment and become activated when tumor antigens are recognized. They then proliferate and differentiate, ultimately leading to the T cell's ability to attack and destroy cells that express relevant antigens. However, regulation of T cell responses is an extremely complex process consisting of both stimulatory and inhibitory cell intrinsic signaling pathways, which limit T cell responses against cancer and prevent eradication of tumors.

Recognition of antigen-MHC complexes by the T cell antigen receptor is not sufficient for

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activation of naïve T cells—additional costimulatory signals (*4*, *5*) are required that are provided by the engagement of CD28 on the T cell surface with B7 molecules (CD80 and CD86) on the antigen-presenting cell (APC) (Fig. 1). Expression of B7 molecules is limited to subsets of hematopoietic cells, especially dendritic cells, which have specialized processes for efficient antigen presentation. With the exception of certain lymphomas, cancer cells do not express B7 molecules, and hence are largely invisible to the immune system. This can be overcome by an inflammatory response, such as the killing of tumor cells, which permits APCs, such as dendritic cells, to take up antigen and present antigen bound to MHC along with B7 molecules for effective activation of T cells.

After encountering tumor antigen in the context of B7 costimulation, initially in tumor-draining lymph nodes, tumor-specific T cells may acquire effector function and traffic to the tumor site to mount an attack on the tumor. Infiltration of T cells into the tumor microenvironment is a critical hurdle that must be overcome for an effective antitumor immune response to occur. However, once T cells are in the tumor microenvironment, the success of the assault is determined by their ability to overcome additional barriers and counter-defenses they encounter from the tumor cells, stroma, regulatory T cells, myeloid-derived suppressor cells, inhibitory cytokines, and other cells in the complex tumor microenvironment that act to mitigate antitumor immune responses.

In the 1980s, tumor antigens from human melanomas were found to elicit T cell responses (*6*), which drove efforts to use vaccination strategies to mobilize the immune system to attack cancer. The vaccines generally consisted of some form of the antigen (for example, peptide or DNA vaccines), as well as additional components to enhance responses (for example, cytokines). While there were anecdotal successes, in hundreds of trials there was scant evidence of reproducible clinical responses (*7*). This failure to induce effective immune responses by attempting to turn T cell response "on" with antigenic vaccines led many to become skeptical of the potential of immunotherapy as a strategy for cancer treatment.

Regulation of T cell responses

Further insights into the fundamental mechanisms that regulate early aspects of T cell activation may provide one of many possible explanations for the limited effectiveness of these early vaccine trials. By the mid-1990s, it was becoming clear that T cell activation was even more complex, and in addition to initiating proliferation and functional differentiation, T cell activation also induced an inhibitory pathway that could eventually attenuate and terminate T cell responses. Expression of *ctla-4*, a gene with very high homology to CD28, is initiated by T cell activation, and, like CD28, CTLA-4 binds B7 molecules, albeit with much higher affinity. Although CTLA-4 was first thought to be another costimulatory molecule (*8*), two laboratories independently showed that it opposed CD28 costimulation and down-regulated T cell responses (*9*, *10*). Thus, activation of T cells results in induction of expression of CTLA-4, which accumulates in the T cell at the T cell–APC interface, reaching a level where it eventually blocks costimulation and abrogates an activated T cell response (Fig. 2).

Based on knowledge of the function of CTLA-4, we proposed that blocking its interaction with the B7 molecules might allow T cell responses to persist sufficiently to achieve tumor eradication. We hypothesized that this could be achieved by releasing the endogenous immune responses, perhaps even without specific knowledge of the antigenic targets of those responses or even the type of cancer. We also proposed that combination treatment with an antibody against CTLA-4 and agents that directly killed tumor cells to release antigens for presentation by APCs to T cells would improve antitumor responses. Our hypotheses were tested in many different experiments in mice (*11*–*15*), with data generated to support the concept, leading to the development of ipilimumab, an antibody against human CTLA-4 for clinical testing. Ipilimumab led to considerable improvement in overall survival for patients with metastatic melanoma (*16*, *17*), which led to FDA approval in 2011.

The preclinical successes of anti-CTLA-4 in achieving tumor rejection in animal models and the ultimate clinical success opened a new field of immune checkpoint therapy (*18*, *19*). It is now known that there are many additional immune checkpoints. Programmed cell death–1 (PD-1) was shown in 2000 to be another immune checkpoint that limits the responses of activated T cells (*20*). PD-1, like CTLA-4, has two ligands, PD-L1 and PD-L2, which are expressed on many cell types. The function of PD-1 is completely distinct from CTLA-4 in that PD-1 does not interfere with costimulation, but interferes with signaling mediated by the T cell antigen receptor (*4*). Also, one of its ligands, PD-L1 (B7-H1), can be expressed on many cell types (Fig. 2), including T cells, epithelial cells, endothelial cells, and tumor cells after exposure to the cytokine interferon-γ (IFN-γ), produced by activated T cells (*21*). This has led to the notion that rather than functioning early in T cell activation, the PD-1/PD-L1 pathway acts to protect cells from T cell attack.

Immune checkpoint therapy in the clinic

Ipilimumab, a fully human antibody to human CTLA-4, entered clinical trials in the late 1990s and early 2000s. As predicted, tumor regression was observed in patients with a variety of tumor types. Phase I/II trials showed clinical responses in

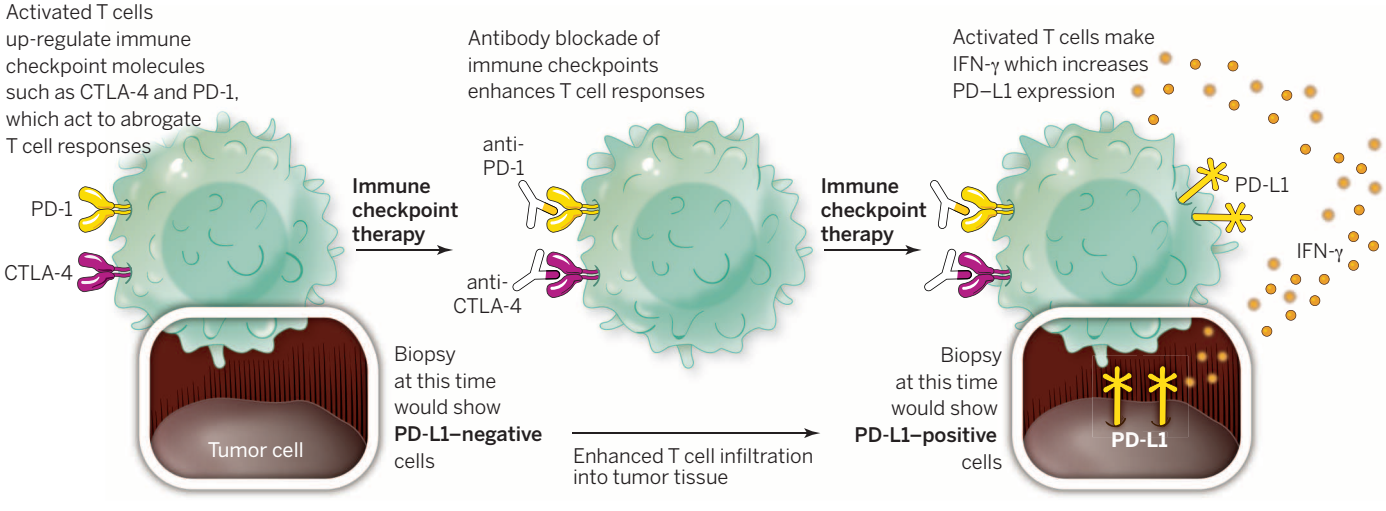

Fig. 2. Blockade of immune checkpoints to enhance T cell responses. After T cell activation, T cells express immune checkpoints such as CTLA-4 and PD-1. A biopsy of tumors taken from patients before treatment with immune checkpoint therapy (so prior to infiltration of activated T cells into tumor tissues) may indicate lack of PD-L1 expression. However, upon T cell activation, T cells can traffic to tumors, up-regulate expression of immune checkpoints such as CTLA-4 and PD-1, and produce cytokines such as IFN-γ, which leads to expression of PD-L1 on tumor cells and other cells, including T cells, within the tumor tissues.

patients with melanoma (*22*), renal cell carcinoma (*23*), prostate cancer (*24*), urothelial carcinoma (*25*), and ovarian cancer (*26*). Two phase III clinical trials with anti-CTLA-4 (ipilimumab) were conducted in patients with advanced melanoma and demonstrated improved overall survival for patients treated with ipilimumab (*16*, *17*). Importantly, durable responses were observed in about 20% of patients living for more than 4 years, including a recent analysis indicating survival of 10 years or more for a subset of patients (*27*).

Antibodies targeting the PD-1/PD-L1 axis have also shown clinical responses in multiple tumor types. Anti-PD-L1 antibodies led to tumor regression in patients with melanoma, renal cell carcinoma, non–small cell lung cancer (*28*), and bladder cancer (*29*). Phase I clinical trials with anti-PD-1 (nivolumab) demonstrated similar clinical responses (*30*). Recently, a large phase I clinical trial with the anti-PD-1 antibody MK-3475 was shown to lead to response rates of ~37 to 38% in patients with advanced melanoma (*31*), with a subsequent study reporting an overall response rate of 26% in patients who had progressive disease after prior ipilimumab treatment (*32*), which led to FDA approval of MK-3475 (pembroluzimab) in September 2014. A phase III trial of a different anti-PD-1 antibody (nivolumab) also showed clinical benefit in patients with metastatic melanoma. In this trial, the objective response rate was 40% and overall survival rate was 72.9% for patients treated with nivolumab as compared to an objective response rate of 13.9% and overall survival rate of 42.1% for patients treated with dacrabazine chemotherapy (*33*). Nivolumab received FDA approval in December 2014 as a treatment for patients with metastatic melanoma. In addition, nivolumab was FDA-approved in March 2015 for patients with previously treated advanced or metastatic non–small cell lung cancer based on a phase III clinical trial, which reported an improvement in overall survival for patients treated with nivolumab as compared to patients treated with docetaxel chemotherapy.

That CTLA-4 and PD-1 regulate distinct inhibitory pathways and have nonoverlapping mechanisms of action suggested that concurrent combination therapy with both might be more efficacious than either alone. This was indeed shown to be the case in preclinical studies in murine models (*34*). In 2013, a phase I clinical trial with anti-CTLA-4 (ipilimumab) in combination with anti-PD-1 (nivolumab) demonstrated tumor regression in ~50% of treated patients with advanced melanoma, most with tumor regression of 80% or more (*35*). There are ongoing clinical trials with anti-CTLA-4 plus anti-PD-1, or anti-PD-L1, in other tumor types, with preliminary data indicating promising results, which highlight this novel combination as an effective immunotherapy strategy for cancer patients.

Tissue-based immune monitoring: Anti-CTLA-4 therapy

Properly designed presurgical or tissue-based trials, where treatment is administered before surgical resection of tumors, can provide valuable insight into the cellular and molecular mechanisms of immune checkpoint therapy by providing sufficient tissues to conduct a battery of analyses. Data gathered from analysis of tumor tissue can then guide rational searches for relevant markers in the blood. We designed the first presurgical clinical trial with anti-CTLA-4 (ipilimumab), which was administered to 12 patients with localized bladder cancer prior to radical cystectomy (*36*). The endpoints of this study were safety and access to samples for immune monitoring. We did not view this trial as a neoadjuvant study, which administers therapy prior to surgery for clinical benefit, but as a presurgical study to provide mechanistic insights regarding the impact of anti-CTLA-4 therapy on the tumor microenvironment. Unexpectedly, the trial enabled us to detect a clinical signal for anti-CTLA-4 as a therapeutic agent for patients with bladder cancer since three patients had no residual tumors identified within the cystectomy samples. This trial was also successful in establishing the safety of anti-CTLA-4 in the presurgical setting, which would be important for future trials, and obtaining patients' matched tumor and blood samples for immune monitoring. This work laid the foundation for using presurgical trials as an important tool to evaluate human immune responses in the tumor microenvironment, which should be included in the current paradigm of phase I, II, and III clinical trials.

"Because of the very nature of immune checkpoint therapy, the development of pharmacodynamic, predictive, or prognostic biomarkers faces unique challenges."

The collection of fresh tumor samples at the time of surgery can provide sufficient tissue for genetic, phenotypic, and functional studies, as well as material for immunohistochemical (IHC) analyses, which can provide extensive insight into the biologic impact of the immunotherapy agent on the tumor microenvironment. For example, high-quality mRNA can be obtained for gene expression studies comparing posttreatment tumor tissues to pretreatment tumor tissues or untreated samples obtained from a stage-matched control group of patients. These types of studies allow unbiased analyses of the samples to identify novel genes and pathways that are affected by therapy. In our ipilimumab trial, gene array data revealed that most of the differences between treated and untreated samples could be attributed to pathways involved in T cell signaling, which is not surprising given the large increases in T cell infiltrates in tumor tissues after CTLA-4 blockade (*25*, *26*). The most pronounced difference was an increase in T cells that express inducible costimulator (ICOS), a T cell surface molecule that is a closely related member of the extended CD28/CTLA-4 family. We confirmed our gene expression studies by flow cytometry. $ICOS^+$ T cells were increased in tumor tissues from patients treated with ipilimumab (*36*). The increase in the frequency of $ICOS^+$ T cells in tumor infiltrates was accompanied by similar increases in the blood. These data, coupled with other studies, showed that an increase in the frequency of $ICOS^+$ CD4 T cells served as a pharmacodynamic biomarker of anti-CTLA-4 treatment (*37*).

To test our hypothesis that $ICOS^+$ CD4 T cells might play a role in the therapeutic effect of CTLA-4 blockade, we conducted studies in mice. In wild-type C57BL/6 mice, anti-CTLA-4 treatment resulted in tumor rejection in 80 to 90% of mice, but in gene-targeted mice that were deficient for either ICOS or its ligand, the efficacy was less than 50% (*38*). The loss of efficacy of CTLA-4 blockade in the absence of an intact ICOS pathway indicates the critical importance of ICOS to the therapeutic effects of treatment with anti-CTLA-4 antibodies. The important role played by ICOS in the effectiveness of CLTA-4 blockade suggested that providing an agonistic stimulus for the ICOS pathway during anti-CTLA-4 therapy might increase its effectiveness. To test this notion, we conducted studies in mice to provide an agonistic signal through ICOS in combination with CTLA-4 blockade. We found that combination therapy resulted in an increase in efficacy that was about four to five times as large as that of control treatments (*39*). Thus, ICOS is a stimulatory checkpoint that provides a novel target for combination immunotherapy strategies. Antibodies for ICOS are being developed for clinical testing, which are expected to start within the next year.

Whereas some presurgical and tissue-based trials are focused on evaluating human immune responses in the tumor microenvironment, other studies have focused on evaluating components of the cancer cells that may contribute to clinical benefit with anti-CTLA-4. Genetic analyses of melanoma tumors revealed that higher numbers of mutations, termed "mutational load," and creation of new antigens that can be recognized by T cells as a result of these mutations, termed "neoantigens," correlated with clinical responses to anti-CTLA-4 therapy (*3*, *40*). These studies provide a strong rationale to integrate genetic analyses of the tumor with immune profiling of the tumor microenvironment for a more comprehensive evaluation of mechanisms that contribute to clinical responses with anti-CTLA-4 therapy.

Tissue-based immune monitoring: Anti-PD-1/PD-L1 therapy

Given that immune checkpoint therapy only benefits a fraction of patients, there are ongoing efforts to identify predictive biomarkers that could be used to select patients for treatment.

Because the PD-1 ligand PD-L1 (and sometimes PD-L2) can be expressed on tumor cells and immune cells in the tumor microenvironment, there have been efforts to use expression of PD-L1 as a criterion for selecting patients for treatments with antibodies targeting the PD-1/PD-L1 pathway.

The initial phase I trial with anti-PD-1 therapy (nivolumab) reported that PD-L1 expression on tumor cells, measured on pretreatment archival samples by immunohistochemical (IHC) methods, may potentially serve as a predictive marker to indicate which patients would benefit from treatment (*30*). Patients with PD-L1–positive tumors (≥5% staining for PD-L1 on tumor cells) had an objective response rate of 36% (9 of 25 patients) whereas patients with PD-L1–negative tumors did not show any objective clinical responses (0 of 17 patients). However, in subsequent trials, some patients whose tumors were deemed to be PD-L1–negative had clinical responses to anti-PD-1 and anti-PD-L1 treatments with either tumor regression or stabilization of disease. For example, on a phase I trial with anti-PD-1 (nivolumab), patients with PD-L1–positive tumors had an objective response rate of 44% (7 of 16) and patients with PD-L1–negative tumors had an objective response rate of 17% (3 of 18) (*41*). Although PD-L1 expression in tumor tissues does correlate with higher response rates, it is not predictive for clinical benefit. Furthermore, current data indicate that the differences in response rates do not translate to differences in survival benefit. For patients with metastatic melanoma who received treatment with nivolumab on a phase III trial, the median overall survival had not been reached for either PD-L1 subgroup, and both subgroups had improved overall survival as compared to patients who received dacarbazine chemotherapy (*33*).

In a phase I study of anti-PD-L1 (MPDL3280A), patients with bladder cancer were considered to have PD-L1–positive tumors if their pretreatment archival tumor samples contained ≥5% PD-L1–positive tumor-infiltrating immune cells (*29*). Twenty-one patients with PD-L1–positive tumors were enrolled onto the trial prior to enrollment of patients with PD-L1–negative tumor samples. Data were reported after a minimum of 6 weeks of follow-up. An objective response rate of 43.3% (13 out of 30 patients) and stable disease rate of 26.7% (8 of 30) was reported for patients with PD-L1-positive tumors, which was compared to an objective response rate of 11.4% (4 of 35 patients) and stable disease rate of 37.1% (13 of 35) for patients with PD-L1–negative tumors. Because the patients with PD-L1–positive tumors received treatment for a longer period of time as compared to patients with PD-L1–negative tumors, it is unclear if the difference in response rates in this study was due to PD-L1 expression or time on treatment. However, for patients with metastatic bladder cancer whose disease had progressed after first-line chemotherapy and in a setting where there are no approved second-line treatments, an objective response rate of 11% and stable disease rate of 37.1% are clinically relevant.

Similarly, in another phase I study of anti-PD-L1 (MPDL3280A) in multiple tumor types, objective response rates were reported as 46% in the cohort of patients whose tumors had the highest PD-L1-expression, 17% in the cohort of patients whose tumors had moderate expression of PD-L1, 21% in the cohort of patients whose tumors had minimal PD-L1 expression, and 13% in the cohort of patients whose tumors had no detectable level of PD-L1 expression (*42*). Thus, this trial also showed that patients whose tumors were deemed as PD-L1–negative can have objective responses. Interestingly, the cohort of patients whose tumors were categorized as moderate expression of PD-L1, which correlates with PD-L1-positive status, had objective responses (17%) and median progression-free survival (18 weeks) that were similar to the objective responses (21%) and median progression-free survival (17 weeks) of the cohort of patients whose tumors had minimal expression of PD-L1, which correlates with PD-L1–negative status. Additional studies will be needed to determine whether PD-L1 expression in the tumor microenvironment affects survival outcomes for patients treated with anti-PD-L1.

On the basis of data reported thus far, it seems fair to conclude that expression of PD-L1 in tumor tissues should not be used as a predictive biomarker for selection or exclusion of patients for treatment with either anti-PD-1 or anti-PD-L1 antibodies. In a study of primary and

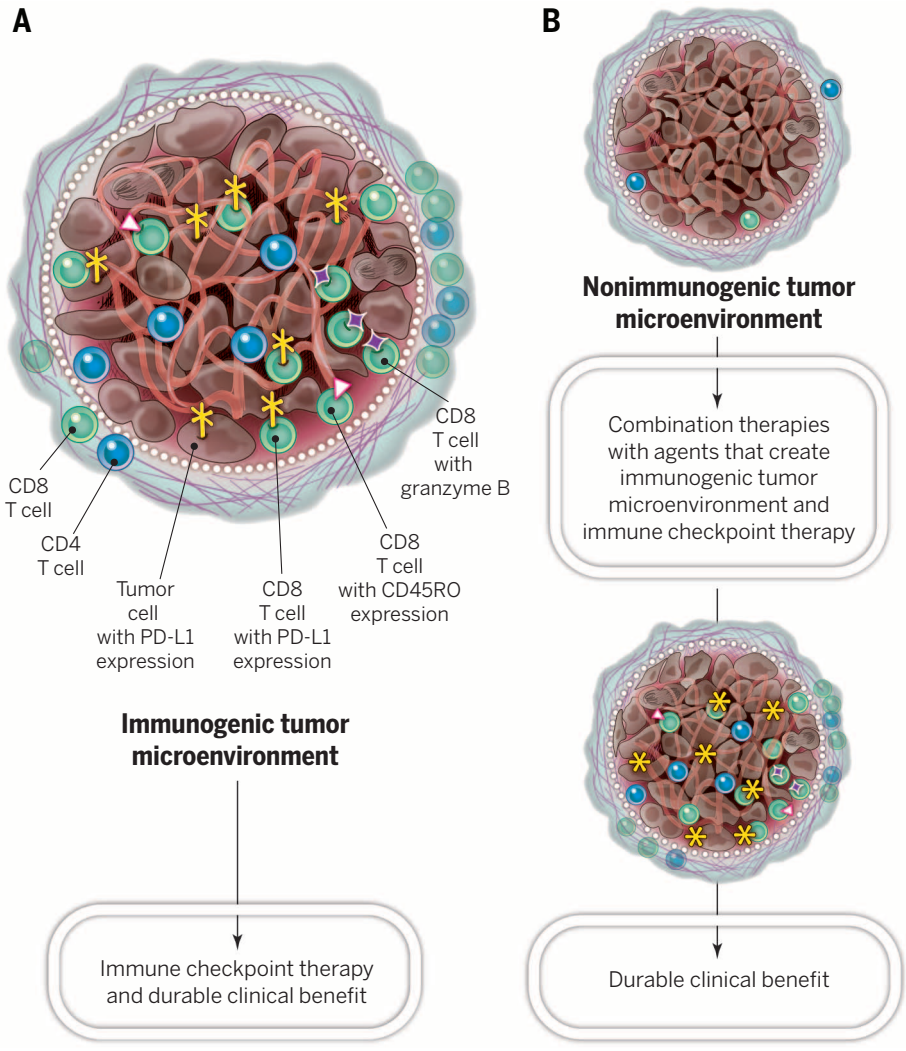

Fig. 3. Potential characteristics of immunogenic and nonimmunogenic tumors. (**A**) Tumor tissue depiction indicating tumor cells and an invasive margin (dotted line), which may delineate separation of tumor cells from stromal components. Evaluation of tumor tissues may reveal an immunogenic tumor microenvironment consisting of many immunologic markers, including CD8 T cells, CD4 T cells, PD-L1, granzyme B, and CD45RO, which may be effectively treated with immune checkpoint therapy to elicit clinical benefit. (**B**) Tumor tissues that lack expression of many immunologic markers may indicate a nonimmunogenic tumor microenvironment, which may require combination therapies consisting of an agent to create an immunogenic tumor microenvironment plus an immune checkpoint agent to further enhance the immune response for clinical benefit.

metastatic melanoma samples, many taken from the same patient, it was shown that PD-L1 expression was discordant between primary tumors and metastases and between intrapatient metastases. In addition, patients whose tumor tissues were positive for both PD-L1 expression and infiltration of T cells were found to have improved overall disease-specific survival as compared to patients who had only one of the two features or lacked both features (*43*). Similarly, in a study with anti-PD-1 (pembrolizumab), it was reported that while expression of PD-L1 in pretreatment tumor tissues correlated with clinical outcomes, the preexisting density of CD8 T cells in the invasive margin of the tumor was more predictive of clinical response to anti-PD-1 (*44*). These data suggest that PD-L1 expression in the tumor is most compelling when it is observed in the context of an active T cell response, and that the ongoing T cell response itself, not PD-L1 expression, is the key factor.

Taken together, these data indicate the complexity of determining the PD-L1 status of a patient's tumor by examination of a single pretreatment tumor sample (Fig. 2). It also raises questions as to whether clinical decisions regarding treatment of patients who have failed conventional therapies and for whom no other treatments are available should be based on static assessment of PD-L1 expression in pretreatment tumor samples.

However, in some settings, expression of PD-L1 in tumors is constitutive and is neither associated with T cell infiltration nor induced by IFN-γ. In these settings, assessment of PD-L1 expression in tumor tissues may be very useful in guiding treatment. In Hodgkin's lymphoma, Reed-Sternberg cells are known to harbor amplification of chromosome 9p24.1, which encodes PD-L1 and PD-L2 and leads to their constitutive expression. Anti-PD-1 (nivolumab) was shown to elicit an objective response rate of 87% in a cohort of 20 patients with Hodgkin's lymphoma (*45*). Therefore, in the setting of Hodgkin's lymphoma, and possibly other malignancies that harbor amplification of chromosome 9p24 or up-regulate PD-L1 or PD-L2 in response to an oncogenic signal, the expression of these ligands may indeed serve as a predictive biomarker.

In addition to evaluation of PD-L1 expression, tumor tissues can also be studied to identify patterns of expression of multiple immunologic components, including other checkpoints and their ligands. T cells that coexpress PD-1 together with other inhibitory molecules such as LAG-3 or Tim-3 may be even more profoundly hyporesponsive than those expressing PD-1 alone and indicate the need for the blockade of multiple checkpoints (*46*, *47*). Given the complexity of regulation of T cell responses by multiple signaling pathways, both negative and positive, it will be necessary to determine the patterns of expression of the receptors, as well as the ligands on T cells, tumor cells, myeloid cells, and other components of the tumor microenvironment, for development of combination strategies with greater clinical benefit.

Additional biomarkers that play a role in antitumor responses elicited by anti-PD-1 therapy and anti-PD-L1 therapies may also be identified through genetic analyses of tumor cells. Similar to previous reports with anti-CTLA-4 therapy, higher numbers of mutations, including mutations in DNA repair pathways, with subsequent increase in numbers of neoantigens, was found to correlate with clinical responses in patients with non–small cell lung cancer who received treatment with anti-PD-1 (pembrolizumab) (*48*). These data highlight the complex interplay between cancer cells and the immune system, which will need further elucidation, to guide rational development of combination therapies.

Combination therapy to increase clinical benefit

Given the dynamic nature of immune responses to tumors and the complexity of regulation of expression of multiple immune checkpoints and their ligands, it may be difficult to rely on any single immunologic biomarker to select patients for treatment. It may be necessary to evaluate multiple components within the tumor microenvironment, which may enable us to distinguish between an immunogenic (hot) tumor microenvironment (Fig. 3A) that is comprised of infiltrating T cells, cytokines such as granzyme B, memory T cell markers such as CD45RO and PD-L1 expression versus a non-immunogenic (cold) tumor microenvironment that lacks these components (Fig. 3B). Patients whose tumors are immunogenic would be treated with immune checkpoint therapy to elicit durable clinical benefit but, patients whose tumors are non-immunogenic would receive combination therapies designed to create an immunogenic tumor microenvironment that would respond to treatment with subsequent durable clinical benefit (Fig. 3).

Substantial data already exist to indicate that certain combination therapies may overcome the limitations of anti-CTLA-4 and anti-PD-1/PD-L1 monotherapies. For example, anti-CTLA-4 seems to drive T cells into tumors, resulting in an increase in the number of T cells and a concomitant increase in IFN-γ. This, in turn, can induce expression of PD-L1 in the tumor microenvironment, with subsequent inhibition of antitumor T cell responses, but also increase the chance of benefit from anti-PD-1 and anti-PD-L1 therapies. Therefore, combination treatment with anti-CTLA-4 plus anti-PD-1 or anti-PD-L1 should enable the creation of an immunogenic tumor microenvironment with subsequent clinical benefit for patients regardless of whether their pretreatment tumor tissues have infiltrating T cells or express PD-L1. Data from a recent phase I clinical trial with anti-CTLA-4 (ipilimumab) plus anti-PD-1 (nivolumab) demonstrated that patients with metastatic melanoma had similar response rates in the setting of concurrent therapy regardless of PD-L1 expression in pretreatment tumor tissues (*35*). For patients with PD-L1–positive tumors, the objective response rate was 46% (6 of 13 patients), which was similar to the objective response rate of 41% (9 of 22 patients) for those patients with PD-L1–negative tumors. Similar data were reported for a combination study with anti-PD-1 (nivolumab) plus anti-CTLA-4 (ipilimumab) in patients with metastatic renal cell carcinoma (mRCC) (*49*).

Conventional cancer therapies (Table 1) may also lead to tumor cell death and release of antigens to initiate activation of T cells, which may then migrate into tumor tissues. Therefore, combination studies with these conventional agents and immune checkpoint therapies should create an "immunogenic" tumor microenvironment with subsequent clinical benefit for patients. There are multiple ongoing trials with radiation therapy in combination with anti-CTLA-4 or anti-PD-1/PD-L1 antibodies, which will provide valuable information regarding schedule, safety, and efficacy of these combinations for future studies (*50*, *51*). In addition, combination treatment with anti-PD-1 (nivolumab) plus pazopanib or sunitib in patients with mRCC resulted in promising clinical responses, with response rates that were similar across all patients regardless of PD-L1 expression in pretreatment tumor tissues (*52*).

Other combination strategies, such as vaccines plus anti-CTLA-4 (ipilimumab), are also being developed and have shown promising results in patients with pancreatic cancer, which has

Table 1. Potential agents for combination therapy. List of some conventional cancer therapies, inhibitory immune signals and stimulatory immune signals that can be considered for combination strategies to improve antitumor responses and durable clinical benefit.

CONVENTIONAL THERAPIES	INHIBITORY IMMUNE SIGNALS	STIMULATORY IMMUNE SIGNALS
Chemotherapy	CTLA-4	ICOS
Radiation	PD-1/PD-L1	OX40
Surgery	LAG-3	41BB
Genomically targeted	TIM-3	Vaccines
Anti-angiogenic	VISTA	Cytokines
Hormonal	BTLA	Oncolytic virus

been consistently viewed as a nonimmunogenic tumor type (*53*). Combination treatments are also being developed to enable blockade of multiple inhibitory pathways, such as LAG-3 (*54*, *55*), TIM-3 (*56*, *57*), VISTA (*58*, *59*), and BTLA (*60*, *61*), or blockade of an inhibitory pathway while providing an agonistic signal through a stimulatory pathway, such as ICOS (*39*), OX40 (*62*), 41BB (*63*), vaccines (*24*, *53*), cytokines (*64*), and oncolytic virus (*65*). The development of these combinations and others are critical for driving antitumor immune responses in many cancer patients, even those who are deemed to have nonimmunogenic or PD-L1–negative tumors.

Discussion

Because of the very nature of immune checkpoint therapy, the development of pharmacodynamic, predictive, or prognostic biomarkers faces unique challenges. Agents that block immune checkpoints unleash dynamic and complex immune responses. Anti-CTLA-4 antibody overcomes a block in essential costimulatory signals that are required for activation of both naïve T cells and resting clones, whereas PD-1/PD-L1 blockade seems to remove a barrier to the function of T cells later in the response and in the tumor tissue. Therefore, there is a fundamental difference in the predictive value of preexisting tumor inflammation for PD-1/PD-L1 and CTLA-4 blockade. The existence of a T cell infiltrate and select biomarkers, such as expression of PD-L1, which indicate a "hot" tumor microenvironment, does correlate with clinical benefit for patients treated with anti-PD-1 or anti-PD-L1. However, in the setting of a "cold" tumor microenvironment, it seems that anti-CTLA-4 therapy can drive T cells into the tumor and induce expression of PD-L1, thus creating a tumor microenvironment that may be responsive to anti-PD-1 or anti-PD-L1 therapy, which provides a strong rationale for combination therapy.

There are many ongoing efforts to identify predictive biomarkers of immune checkpoint therapy. It may be that germline differences in immune genes and pathways or host microbiome may affect host immune responses and clinical outcomes in the setting of immune checkpoint therapy. Also, the nature of the tumor itself can also affect the outcome of immune checkpoint therapy. Tumor types differ considerably in their mutational load, which may affect the number of neoantigens that can serve as targets of antitumor T cell responses (*66*). Patients with tumors at the high end of the mutational spectrum may be more likely to respond to immune checkpoint therapy. For example, anti-PD-1 therapy was thought to be ineffective against colon cancer, but it appears that colon cancer with microsatellite instability, and consequently a higher overall mutational load, may be responsive to treatment with anti-PD-1 (*67*). However, this concept may not hold true for all tumor types, because patients with kidney cancer, which has relatively low numbers of mutations, have had notable clinical responses to immune checkpoint therapy (*28*, *30*).

There are multiple immunologic pathways, both positive and negative, with new checkpoints and ligands that emerge as an immune response develops. Because of the constant evolution of an immune response, it is unlikely that a single immunologic biomarker can be identified at baseline that can predict responses to any agent. It will probably be necessary to develop panels of markers based on patterns of expression of relevant markers, and use these to guide development of combination therapies that will increase the response rate. These combinations will not be limited to agents that target immune checkpoints, because it is apparent that small molecules that target signaling pathways involved in cancer can affect antitumor immune responses (*68*). This can occur at the level of the T cells by enhancing activation signals, but also at the level of the tumor by inducing tumor antigen expression and presentation, thus making the tumors more susceptible to T cell killing. The goal then should be to use panels of markers to guide development of combination therapies, and then examine tumor tissues for changes in markers elicited by the combinations to guide decisions about additional treatment to further increase efficacy, and, hopefully, durable clinical responses.

Immune checkpoint therapies and combination strategies with immunotherapy have provided cancer patients with novel treatments that have the potential to elicit durable control of disease and even cures. The specificity, adaptability, and memory response that are inherent to the immune system give us the opportunity to measure multiple components, not just a single biomarker, that can be targeted over time to provide curative treatments for many patients. The ability of an activated immune response to generate a diverse T cell repertoire that adapts to heterogeneous and genetically unstable tumors and the persistence of memory T cells with specificity for tumor antigens, which provide efficient recall responses against recurrent disease, make it absolutely essential to expand our efforts to find rational combinations to unleash antitumor immune responses for the benefit of cancer patients. Properly done, it seems likely that cures for many types of cancer will soon become reality.

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ACKNOWLEDGMENTS

P.S. and J.P.A. are founders and advisors for Jounce Therapeutics. P.S. also serves as a consultant for Bristol-Myers Squibb, Amgen, and GlaxoSmithKline. J.P.A. is an inventor of intellectual property owned by the University of California, Berkeley, and licensed to Bristol-Myers Squibb and has received royalties from Bristol-Myers Squibb. Our research is supported by a PCF Challenge Grant in Immunology (J.P.A. and P.S.), NCI/NIH 1-R01 CA1633793-01 (P.S.), Cancer Prevention Research in Texas grants (J.P.A. and P.S.), and a Stand Up To Cancer–Cancer Research Institute Cancer Immunology Dream Team Translational Cancer Research Grant (P.S. and J.P.A.). Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

10.1126/science.aaa8172

The future of immune checkpoint therapy

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Science **348** (6230), 56-61.
DOI: 10.1126/science.aaa8172