

Clinical Development of Immunostimulatory Monoclonal Antibodies and Opportunities for Combination

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Abstract

Immune system responses are under the control of extracellular biomolecules, which express functions in receptors present on the surface of cells of the immune system, and thus are amenable to be functionally modulated by monoclonal antibodies. Some of these mechanisms are activating and dictate whether the response ensues, while others play the role of powerful repressors. Antagonist antibodies acting on such repressors result in enhanced immune responses, a goal that is also achieved with agonist antibodies acting on the activating receptors. With these simple logics, a series of therapeutic agents are under clinical development and one of them directed at the CTL-associated antigen 4 (CTLA-4) inhibitory receptor (ipilimumab) has been approved for the treatment of metastatic melanoma. The list of antagonist agents acting on repressors under development includes anti-CTLA-4, anti-PD-1, anti-PD-L1 (B7-H1), anti-KIR, and anti-TGF- β . Agonist antibodies currently being investigated in clinical trials target CD40, CD137 (4-1BB), CD134 (OX40), and glucocorticoid-induced TNF receptor (GITR). A blossoming preclinical pipeline suggests that other active targets will also be tested in patients in the near future. All of these antibodies are being developed as conventional monoclonal immunoglobulins, but other engineered antibody formats or RNA aptamers are under preclinical scrutiny. The "dark side" of these immune interventions is that they elicit autoimmune/inflammatory reactions that can be severe in some patients. A critical and, largely, pending subject is to identify reliable predictive biomarkers both for efficacy and immune toxicity. Preclinical and early clinical studies indicate a tremendous potential to further improve efficacy, using combinations from among these new agents that frequently act in a synergistic fashion. Combinations with other more conventional means of treatment such as radiotherapy, chemotherapy, or cancer vaccines also hold much promise. *Clin Cancer Res*; 19(5); 997-1008. ©2013 AACR.

Introduction

Immunostimulatory monoclonal antibodies

Cancer immunotherapy attempts to stimulate the immune system to reject and destroy tumors. Monoclonal antibodies (mAb) are familiar to oncologists because of the routine use of some of them, such as rituximab and trastuzumab (1, 2). These antibodies direct cytolytic mechanisms in tumors or interfere with growth factor receptors. mAbs can also act on cancer through indirect mechanisms such as targeting vascularization (3) and stimulating the immune system.

Recently, the term immunostimulatory mAbs (Fig. 1) was coined for mAbs that enhance immune responses (4). These agents are either antagonists of immune-repressor molecules or agonists of immune-activating receptors (Fig. 1). The long half-life and broad extracellular fluid biodistribution offer many advantages for antibody therapies. Antibodies block molecular interactions or mediate protein-to-protein interactions that mimic agonist ligands. Because of ligand bivalency, antibodies can cross-link/aggregate the targeted receptors on the plasma membrane.

Manipulation of the immune response with mAbs has found successful application in the field of autoimmunity, and this approach is perceived as a major opportunity to treat cancer and chronic viral diseases. Most of the conceptual framework comes from the concepts of costimulation and coinhibition (5). A T lymphocyte that has engaged in antigen recognition needs to receive further signal from cell-to-cell contacts, termed immune synapses. Some of these accessory receptors reinforce the immune response (costimulatory receptors), whereas others downmodulate the intensity of the response (coinhibitor receptors; ref. 4; Fig. 2). The prefix "co-" refers to the fact that these receptors act in concert with the antigen receptor [T-cell receptor (TCR)] machinery (Fig. 2). The

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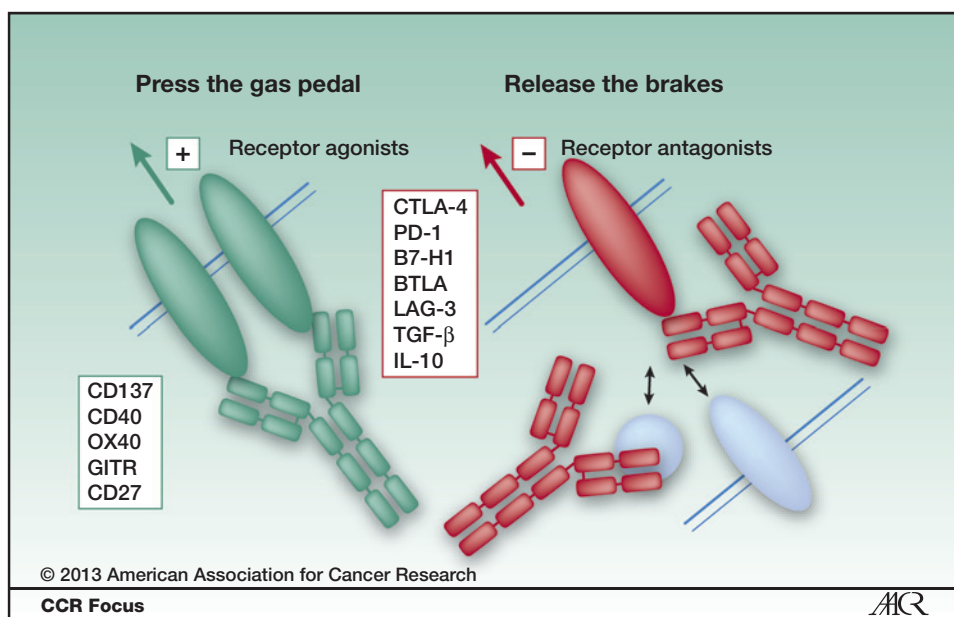


Figure 1. Schematic representation of the concept of immunostimulatory mAbs. Concept and examples of agonist and antagonist mAbs directed toward activatory or inhibitory receptors of immune system cells. Green means activatory receptor and agonist antibody, whereas red means inhibitory receptor and antagonist antibody. Examples of immunostimulatory mAbs in advanced development of each kind of antibodies are framed in the corresponding color.

relative abundance or absence of such ligand–receptor pairs, either as soluble molecules or membrane-attached glycoproteins, is critical to determine the antigen-primed T-cell fate, ranging from programmed cell death to the acquisition of proinflammatory and cytolytic or memory differentiation.

In the textbooks of pharmacology, cytokines have pioneered in the field of immunostimulatory agents with only limited success in oncology (6). Immunostimulatory mAbs are the next family of agents on the list currently holding much hope for efficacy. A major advantage of cancer immunotherapy with these immunotherapeutic agents is the prospect of a long-lasting clinical benefit, but the difficulty is that so far only a prospectively unidentified proportion of patients (approximately <25%) experiences clinical benefit. Table 1 summarizes the agents of this kind of class undergoing clinical development, including those reviewed in this *CCR Focus* section, highlighting the most clinically relevant pros and cons.

Building on a Story of Success

Ipilimumab is an anti-CTLA-4 antibody molecule at the T-lymphocyte surface. CTLA-4 is a glycoprotein that appears in secretory granules and on the surface of the T cells upon activation to play a critical role in downregulating adaptive immune responses. For this purpose, the critical costimulatory receptor CD28 and the coinhibitory receptor CTLA-4 share the same ligands (CD80 and CD86) in professional antigen-presenting cells (APC). Ligation of CTLA-4 suppresses T-lymphocyte responses by a variety of mechanisms (7), which chiefly include negative signaling at the immune synapse, out-competing CD28 for ligand binding, and kidnapping these CD80 and CD86 shared ligands from the surface of APCs, internalizing them into the T cell. Blocking the inhibitory activities of CTLA-4 with mAbs stimulates

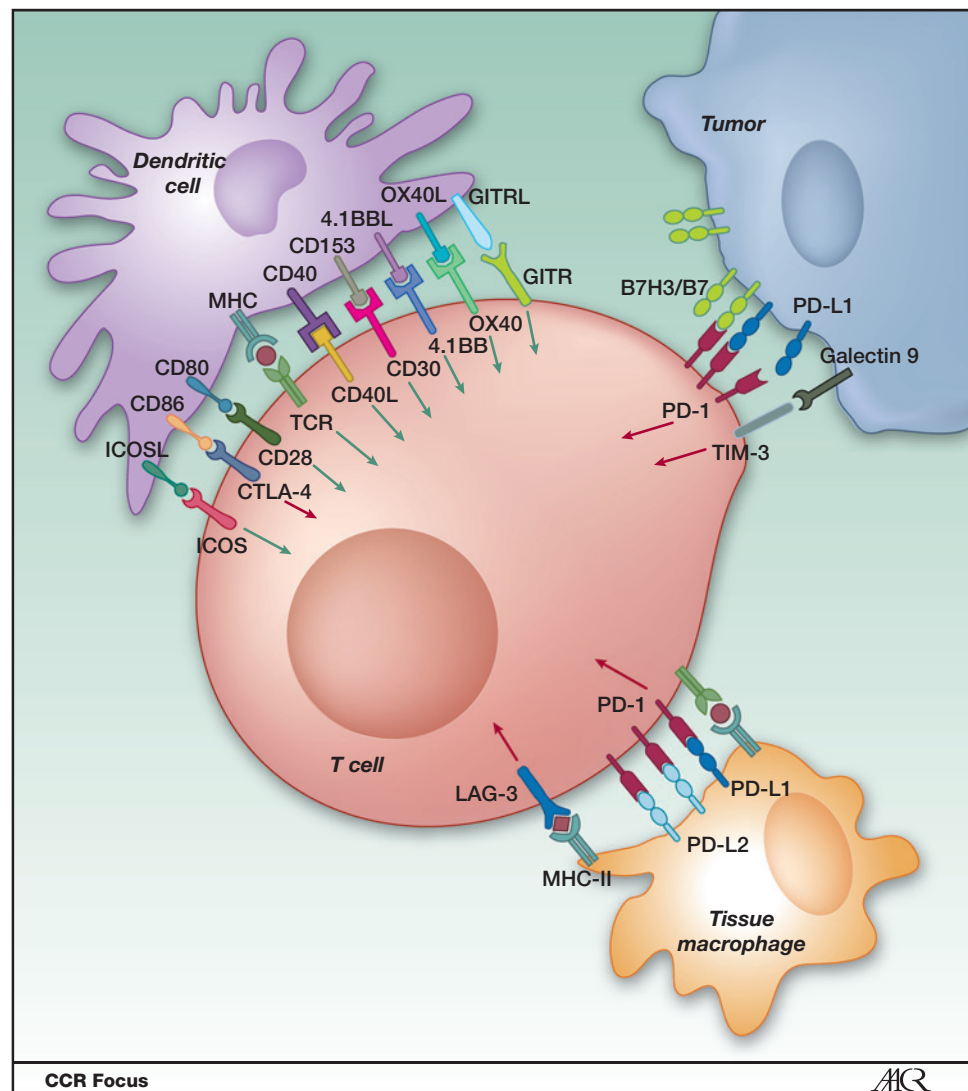
the immune system to fight against cancer, as reported by Chambers and colleagues for the first time in mouse models (8). Three early phase II clinical trials reported 1-year survival rates of 47% to 51% in patients with stage III or IV melanoma treated with ipilimumab, almost doubling the average historical survival (9–11).

Ipilimumab has been tested in phase III clinical trials as monotherapy and in combination with vaccines (12) and chemotherapy (such as dacarbazine; ref. 13). Combinations with other immunotherapies for melanoma, such as interleukin (IL)-2 were previously tested, showing a 17% complete response rate, all of which were sustained in the long term (14).

Overall response rates range from 13% with ipilimumab plus vaccine in patients with stage IV disease to 17% and 22% with ipilimumab plus dacarbazine or IL-2, respectively, in patients with metastatic melanoma. These studies also indicate that more than one third of patients with advanced melanoma treated with ipilimumab benefit in terms of survival (13, 14), and this is considered as a landmark success in the treatment of this deadly disease. In fact, ipilimumab has been the first treatment in history that has shown a survival benefit in advanced melanoma.

Importantly, the hyperstimulation of the immune system explains the peculiar safety profile seen with ipilimumab, characterized by eliciting autoimmunity and inflammation. Immune-related adverse events (irAE) is the new term coined for this particular kind of side effects, which frequently involve the skin, gastrointestinal apparatus, liver, and endocrine system (Fig. 3). It is still controversial whether such adverse events correlate with clinical benefit (15–17). Some authors hold that the development of an irAE may not be predictive of ipilimumab activity (15). The Italian group reported a large experience with ipilimumab from the European Italian Expanded Access Program (EAP; ref. 16). Among 887 patients with advanced

Figure 2. Costimulatory and coinhibitory ligand receptor pairs that are amenable to manipulation with immunostimulatory mAbs. Schematic representation of immune synapses formed by a tumor-reactive T lymphocyte with a dendritic cell, a tumor cell, and a macrophage in the tumor microenvironment. Costimulatory and coinhibitory receptors modulate the function of both the antigen-presenting and the antigen-sensing lymphocytes. Tampering with these interactions underlies the mechanism of action of immunostimulatory mAb conceivably acting mainly in the malignant tissue and in its draining lymph nodes. Adapted from Sharma (110).



melanoma, who received the treatment, no correlation between toxicity and efficacy was found. The immune-related response rate in patients with drug-related toxicity was 35.5% (84 of 255) and 33% (176 of 532) in patients without drug toxicity (16).

Tremelimumab, another anti-CTLA-4 antibody, failed to show a survival benefit in a phase III trial of advanced melanoma (18). The overall survival (OS) of patients treated with tremelimumab was 12.6 months, quite similar to the ipilimumab phase III studies (12, 13), as compared with 10.71 months in the control arm (dacarbazine). Several explanations have been posited for these results, including the long interval between administrations (90 days) and that the control arm fared better on this trial than expected. Some authors (18, 19) argue that the phase II data of ipilimumab at 10 mg/kg every 3 weeks and tremelimumab at 15 mg/kg every 90 days were similar in terms of objective response rates when assessed by independent radiology review committees

(20, 21). However, it should be considered that the main benefit of anti-CTLA-4 is not on response rate but on OS. In addition, other explanations could be: (i) the concurrent availability of ipilimumab in several studies and 2 expanded access protocols, which allowed a significant cross-over to ipilimumab in the control arm (18); (ii) a premature assessment of the results by the external review committee, before the survival curves had time to separate; and (iii) the restriction of the lactate dehydrogenase (LDH) level to twice the upper limit of normality (ULN) in the tremelimumab phase III trial, which favored the control arm and was not included in the ipilimumab phase III trials. Of note, very recently another phase III trial, which compared Abraxane (a new chemotherapeutic agent) with dacarbazine and in which there were the same exclusion criteria for patient enrollment about the LDH > 2 × ULN level, the control arm showed a median OS of 10.7 months (22), as in the tremelimumab data.

Table 1. Toxicity, positive comments, and caveats pertaining to immunomodulating mAbs

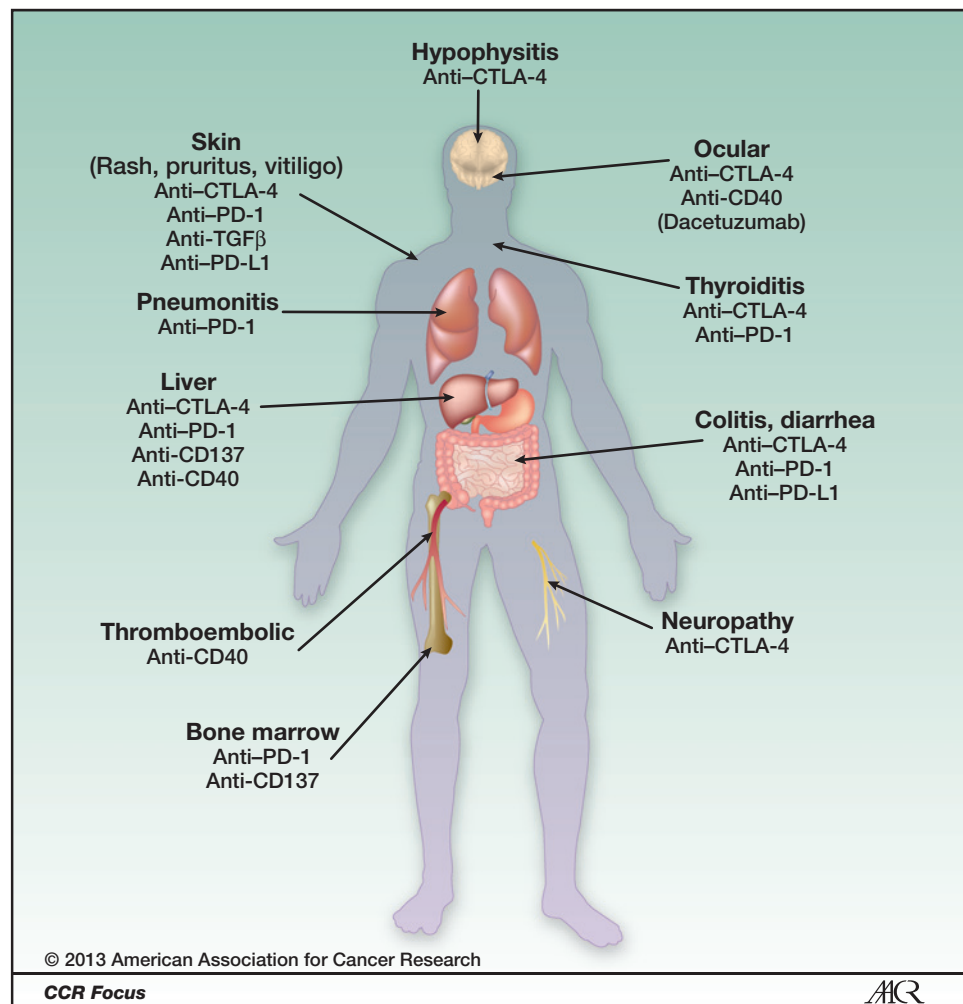
Agent	Toxicity	Positive comments	Caveats
Ipilimumab (anti-CTLA-4; refs. 12, 13)	Rash, colitis, diarrhea, hepatotoxicity, endocrinopathies, neuropathies	Two randomized phase III trials showed improvement in OS in patients with melanoma as first- and second-line therapy. First therapy ever to show a survival improvement in metastatic melanoma.	More efficacious schedule and dosage to be defined. Increase in tumor lesion size due to lymphocyte infiltrates might be confused with disease progression and makes decision making difficult. Lack of predictive biomarkers.
Tremelimumab (anti-CTLA-4; ref. 18)	Colitis, diarrhea, rash, pruritus, endocrinopathies	Treatment schedule with administrations every 90 days (too long interval) with the same safety profile as ipilimumab. Confirmed clinical activity in some patients.	Negative results in the melanoma phase III study, compared with standard chemotherapy.
Nivolumab (anti-PD-1; refs. 42–44)	Relevant toxicity is uncommon. Some patients develop fatigue, rash, diarrhea, pruritus, pneumonitis; rare decreases in appetite and hemoglobin, pyrexia.	Dramatic and sustained clinical responses in 20%–30% of patients with melanoma, renal cell cancer, and non-small cell lung cancer. Effective at low doses (1 mg/kg). Better safety profile as compared with ipilimumab and tremelimumab.	Not yet studied in phase III trials. Lack of confirmed biomarkers (PD-L1 expression under study, pending confirmation).
MK-3475 (anti-PD-1; ref. 46)	Fatigue, pruritus, dyspnea, nausea, anorexia	Promising efficacy and safety profile (no grade 3–4 adverse events in the phase I trial)	Early phase of development.
BMS936559 (anti-PD-L1; ref. 108)	Fatigue, infusion-related reaction, diarrhea, arthralgia, rash, nausea, pruritus	Theoretically a better inhibition PD-1/PD-L1 because directly inside the malignant tissue (where there is the higher PD-L1 expression from the tumor and PD-1 expression on TILs). Good safety profile.	Early phase of development.
Anti-CD40 (58) Dacetuzumab (67)	Cytokine release syndrome, thromboembolic syndromes, transient cytopenias, depletion of T cells in multidose trial	CP-870,893 has shown clinical efficacy in a number of settings of patients with advanced cancer. Dacetuzumab has shown single-agent activity in DLBCL. No tumor regression was observed in multiple myeloma. Possible combination with a checkpoint blockade mAbs (i.e., anti-CTLA-4, anti-PD-1), as well as rituximab, and chemotherapeutic agents.	It is necessary to improve our understanding of the mechanism of action of different CD40 mAb and understand which of the many mechanisms is the most appropriate for the clinical use.
Urelumab (anti-CD137; ref. 31)	Fatigue, rash, fever, rare cytopenias, and hepatotoxicity.	Possible combination with anti-immune checkpoint blockade mAbs (ipilimumab, nivolumab).	Severe hepatic toxicity (seems dose related, not observed at lower doses). Early phase of development.
Anti-OX40	Fatigue, transient lymphopenia	Excellent for combination with other molecules.	Early phase of development.
Anti-TGF- β (GC1008) Fresolimumab (109)	Rash, gingival bleeding, SCC, keratoacanthomas	Promising result in phase I trial.	Early phase of development.

Antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced cancer

Antigen-specific T-cell responses are regulated by coinhibitory molecules categorized as "checkpoint molecules" (23). Following the path of ipilimumab, a blocking anti-

body directed at PD-1 (CD279), termed nivolumab, is predicted to reach regulatory approval based on overwhelming phase I results and ongoing pivotal phase III trials. PD-1 (24) is a member of the B7-CD28 family whose main role is to control immune responses in healthy tissues

Figure 3. Tissue distribution of the most frequent irAEs observed with immunostimulatory mAbs. This new group of agents may cause or exacerbate immune reactions that can functionally or structurally damage different tissues. In some cases, immunity against self-antigens reaches a clinically meaningful threshold, whereas in others an inappropriate response to saprophyte flora may be involved in the observed syndromes. Fortunately, the large majority of these conditions are self-limited and respond to steroids and conventional immunosuppressive regimens.



to prevent autoimmunity and collateral tissue damage (25, 26). PD-1 becomes expressed upon lymphocyte activation in CD4⁺ and CD8⁺ T cells, natural killer (NK) T cells, B cells, as well as in monocytes and dendritic cells (DC). The PD-1 receptors are B7-H1 (PD-L1; refs. 27, 28) and B7-DC (also called PD-L2; refs. 29, 30), which have a different cellular distribution. In healthy individuals, PD-1 signaling in T cells regulates immune responses in healthy bystander tissue and prevents autoimmunity by promoting tolerance to self-antigens (31). PD-1 is also actively involved in the exhaustion process of activated T cells (32). PD-1 ligation by B7-H1 inhibits production of several cytokines and promotes apoptosis via inhibition of the cell survival factor Bcl-xL (33) in T cells. At a molecular level, ligated PD-1 brings tyrosine phosphatases to the immune synapse, thus interfering with a variety of activating signals (34). Although PD-1 primarily controls the function of effector cells, this pathway may also have a role in modulating T-cell priming and on regulatory T cells.

B7-H1 is frequently detected in malignant cells of poor-prognosis cancers; bright PD-1 expression is often present in tumor-infiltrating lymphocytes (TIL; ref. 35). However,

the data for a poor prognosis correlation of B7-H1 expression in tumor cells is not universal, as several reports also indicate that B7-H1 expression lacks prognostic association (36–38) or can be even associated with improved survival (39). Chen and colleagues observed that PD-1/B7-H1 can be targeted with mAbs in mouse models of cancer to achieve therapeutic tumor-eradicating immune responses (40). Agents of this kind are so far the most promising agents of the family and have extended the realm of immunotherapy beyond melanoma and renal cell carcinoma to some of the deadliest tumors, including lung and colorectal cancer (41).

Nivolumab (BMS-936558) is a fully human monoclonal immunoglobulin G (IgG) 4 antibody that binds PD-1 with high affinity, blocking its interaction with both B7-H1 and B7-DC. This antibody was initially evaluated in a standard phase I dose-escalation trial with intravenous escalating doses from 0.3 to 10 mg/kg (39). Nivolumab showed promising activity, achieving durable clinical responses in several tumor types including colorectal cancer, melanoma, renal cancer, lung cancer, and ovarian cancer. The response rate in non-small-cell lung cancer was 42%, despite the widespread perception that this

malignancy cannot be addressed with immunotherapy. In patients with metastatic melanoma, response rates were comparable among the different dose groups (1, 3, and 10 mg/kg), suggesting that even the lowest tested doses reach full-receptor occupancy.

In a subsequent large phase I trial in 296 patients with different tumor types, nivolumab was administered in doses ranging from 0.1 to 10 mg/kg i.v. every 2 weeks (42–44). The response rate was also encouraging, reaching 28% in pretreated patients with metastatic melanoma. Nivolumab was again well tolerated at all dose levels. In a subset of 42 patients in whom pretreatment paraffin-embedded tumor tissue was assessed for B7-H1 expression using the 5H1 antibody (39), no patients with B7-H1 tumors responded, whereas 9 of 25 (36%) patients with B7-H1–positive tumors (defined as >5% of cells with membrane expression) responded ($P = 0.006$). These data suggest a role for tumor cell B7-H1 expression as a potential predictive biomarker of response to PD-1 pathway blockade, but further prospective confirmation will be required in larger patient populations.

Other anti-PD-1 mAb are also under early clinical development (41, 45). MK-3475, a humanized IgG4 with high-affinity binding for PD-1 has completed a phase I clinical trial. It was well tolerated in doses ranging from 1 to 10 mg/kg for 13 patients with melanoma treated in the dose-escalation phase, and in the 10 mg/kg expansion cohort, 7 responses were observed (46). CT-011 is a humanized IgG1 anti-PD-1 mAb that promotes human NK and T-cell function *in vitro*. Development of CT-011 has focused primarily on hematologic malignancies and has been tested in combination with selected chemotherapies. Nonetheless, a single-agent phase II trial is being conducted in patients with metastatic melanoma (41). A phase I trial exploring the PD-1–binding B7-DC-Fc fusion protein (AMP-224) is also ongoing. Therefore, this molecular pathway is attracting a great deal of attention from the pharmaceutical industry, targeting both PD-1 and its ligands (41).

Antibodies blocking the PD-1 ligand B7-H1 (PD-L1) are also under clinical development. In the next few years, we eagerly expect results with several compounds (BMS-936559, MPDL3280A/RG7446, MEDI4736, etc.).

Recent murine studies (47) suggest that concurrent blockade of other exhaustion/co-inhibitory molecules (CTLA-4, TIM-3, LAG3, etc.) and PD-1 can result in powerful synergistic antitumor activity, and preclinical study suggested incremental activity by blocking both PD-1 and B7-H1 (42). Solid preclinical data in mice (47) have been the rationale that supports an ongoing landmark clinical trial combining ipilimumab and nivolumab (NCT01024231). CTLA-4 expression increases in response to PD-1 blockade both in TILs and peripheral blood lymphocytes (PBL) as observed in animal tumors and in patients (41). Assuming that CTLA-4 ligands are present in the tumor microenvironment, these data provide a rationale for the ongoing phase I trial that combines nivolumab and ipilimumab. Preclinical studies have also supported combinations of anti-PD-1 with a variety of agents including chemotherapy, cytokines,

and other immunostimulatory agents, such as anti-CD137 mAb (48). Although combinations with chemotherapy will be preferred in diseases where chemotherapy is standard of care, it will also be important to assess the activity of PD-1 blockade alone and in combination with other immune therapies. If additional data confirm that tumor B7-H1 expression is a predictive biomarker for the activity of PD-1 blockade alone, the lack of expression should not preclude testing combinations of PD-1 with other agents that could drive T cells into the tumor microenvironment. Combinations with anti-CTLA-4, anti-CD137, IFNs (which also induce surface B7-H1 expression), and signaling antagonists, such as B-Raf inhibitors (e.g., vemurafenib), are expected to synergistically interact.

Agonistic CD40 antibodies and cancer therapy: the power of enabling antigen-presenting cells

A long quest for a master switch to turn on cellular immune responses led to a series of findings pertaining to the CD40 receptor. This molecule interacting with its natural ligand (CD40L) is critical for the communication of T lymphocytes and dendritic cells as well as in the communication between T-helper cells and B lymphocytes in the humoral immune response (49). Indeed, CD40L is highly restricted to activated T cells and is a critical molecule to enhance antigen presentation, macrophage bacterial killing, and antibody responses. Immunostimulatory mAb offers an attractive way to boost anticancer responses acting on this molecule and might be used to potentiate existing responses as adjuvants for cancer vaccines (50) or in combination with anticheckpoint blockade molecules. Glennie and colleagues were pioneers in the field treating experimental mouse B-cell malignancies expressing CD40. However, the most prominent antitumor effect CD40 is the activation of the antigen-presenting dendritic cell network. A comprehensive review is available in this same issue (49).

CD40 is a TNF receptor (TNFR) superfamily member expressed in APC, such as dendritic cells, B cells, and monocytes as well as many nonimmune cells and a wide range of tumors (51–53). Interaction with its trimeric ligand on activated T-helper cells results in APC activation as required to induce adaptive immunity (51–53). In preclinical models, rat anti-mouse CD40 mAb show important therapeutic activity in the treatment of CD40⁺ B-cell lymphomas (54, 55) and are also effective in various CD40⁺ tumors (55–57).

Four CD40 mAb have been investigated in clinical trials: CP-870,893 (Pfizer and VLST; ref. 58), dacetuzumab (Seattle Genetics; ref. 59), Chi Lob 7/4 (University of Southampton, Southampton, United Kingdom; ref. 60), and lucatumumab (Novartis; ref. 61). These reagents show different activities on the CD40 targets ranging from strong agonism (CP-870,893) to antagonism (lucatumumab; ref. 62). Even if the rationale to use agonistic CD40 mAb is to activate host dendritic cell in the induction of antitumor T-cell responses in patients, other immune

mechanisms, not necessarily mutually exclusive, have been proposed. These include T-cell-independent but macrophage-dependent regressions of pancreatic cancer (63). Indeed, CD40-activated macrophages are active against cancer cells and deplete tumor stroma-inducing tumor regression *in vivo*, at least in pancreatic cancer (64).

Extensive efforts have been made to develop CD40 agonists as a new class of drug for cancer treatment (62). These approaches primarily include agonistic CD40 mAb but also include recombinant CD40L and CD40L gene therapy. In the first clinical trial with CD40 agonists, recombinant human CD40L showed clinical activity and led to long-term complete remission in a patient with advanced squamous cell carcinoma (SCC) of the head and neck (65). The agonistic mAb CP-870,893 is a fully human IgG2 molecule, against CD40, not mediating cell-mediated cytotoxicity (CMC) or antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 66). In contrast, other human CD40 mAbs are of the IgG1 isotype, and therefore able to mediate complement-dependent cytotoxicity (CDC) and ADCC against CD40⁺ tumors. CP-870,893 has shown clinical efficacy in patients with advanced cancer, but no objective clinical responses have been reported perhaps CP-870,893 has been frequently tested in combination with carboplatin and paclitaxel. A trial of gemcitabine with CP-870,893 for patients with resectable pancreatic cancer recently opened at the University of Pennsylvania (Philadelphia, PA). CP-870,893 is also being tested in combination with an anti-CTLA-4-blocking mAb (tremelimumab) for patients with metastatic melanoma, thereby pioneering an interesting combined immunotherapy approach.

Dacetuzumab, a weaker CD40 agonist than CP-870,893, has shown single-agent activity when administered intravenously every week, especially in patients with diffuse large B-cell lymphoma (DLBCL; ref. 49). No tumor regression was observed with dacetuzumab in multiple myeloma (67) and chronic lymphocytic leukemia (CLL; ref. 68). In a phase Ib study of dacetuzumab in combination with rituximab and gemcitabine in patients with relapsed or refractory DLBCL, complete response rate was 20% and partial response rate was 27% (69). Moreover, in a randomized, double-blind phase IIb clinical trial of dacetuzumab versus placebo in combination with rituximab plus ifosfamide, carboplatin, and etoposide chemotherapy for patients with relapsed or refractory DLBCL (stopped early based on a futility analysis), a late analysis showed a trend for an increased of OS with the use of dacetuzumab (49). Currently, there are no registered trials with dacetuzumab. The third agonistic CD40 mAb, Chi Lob 7/4, again less agonistic than CP-870,893, is undergoing initial clinical testing.

In some cases (low-grade B-cell malignancies as for normal B cells), CD40 may be a strong activator for the tumor cell and perhaps growth signal. For this reason, patients with low-grade B-cell malignancy were excluded from clinical trials of agonistic CD40 mAb. The blocking of the potential CD40-CD40L tumor growth signal was the rationale for developing the CD40 antagonistic mAb luca-tumumab in diseases such as CLL (49).

The development of agonistic CD40 mAb as a novel cancer therapy has not been universally endorsed. However, this class of drug is encumbered by a worrying safety profile including cytokine release syndromes (70), autoimmune reactions, thromboembolic syndromes (because CD40 is expressed by platelets and endothelial cells), hyperimmune stimulation leading to activation-induced cell death or tolerance (71, 72), and proangiogenesis (73). Clinical trials of agonistic CD40 mAb based on robust preclinical investigations have shown clinical activity in the absence of disabling toxicity. In fact, some clinical responses have been dramatic and very durable, but response rates remain around 20% or less. It is critical to improve our understanding of the mechanism of action of different CD40 mAb and understand which of the many mechanisms is the most suitable for a given disease case. Such understanding will allow the design of more appropriate and more potent CD40 agonists for different diseases as well as for combination with other anticancer therapies.

Agonist antibodies to TNFR molecules that costimulate T and NK cells

A number of molecules that belong to the TNFR family are endowed with stimulating properties without eliciting apoptosis. When expressed on the surface of NK and T lymphocytes, the ligation of these molecules dictates the survival, effector function, and memory persistence of the lymphocytes (74). Agonist antibodies against members of TNFR family show very interesting results in preclinical trials as reviewed by Melero and colleagues in this issue (75), but are not expected to obtain frequent complete regressions in patients with cancer as monotherapy. However, this class of antibodies can be efficiently combined with radiotherapy, chemotherapy, and/or immunotherapy. Clinical trials are needed to find the right combinatorial strategy with anti-TNFR family antibodies.

CD137-based cancer immunotherapy. CD137 (4-1BB, TNFRSF9), a surface protein discovered in activated T cells, has only 1 confirmed ligand (CD137-Ligand, TNFSF9) expressed in macrophages, activated B cells, and dendritic cells (76, 77). Agonist antibodies to CD137 and CD137-ligand costimulate T cells after TCR stimulation, enhanced cytolytic effector functions, and protecting lymphocytes from programmed cell death (78, 79). Furthermore, anti-CD137 mAb immunotherapy can be combined with other treatments: in preclinical models very encouraging results were obtained combining this class of drug with radiotherapy, chemotherapy, and immunotherapy (80–82).

Fully human and chimeric mAbs against CD137 have been produced (urelumab or BMS-663513, PF-05082566, GTC Biotherapeutics), but only urelumab has been tested in phase I and multiple dose phase II clinical trials, showing interesting clinical activity although severe liver toxicity has been reported (31, 83). More recently, PF-05082566 has initiated clinical testing and its potential

synergy with rituximab is being tested in patients with lymphoma.

OX40-based cancer immunotherapy. OX40 (CD134 or TNFRSF4) is a costimulatory molecule expressed on the surface of activated T lymphocytes (84). Agonists of OX40 have been used successfully in a variety of preclinical tumor models with promising results (85–88), and a variety of combinatorial strategies to increase anti-OX40 antibody therapy with vaccines, chemotherapy, radiotherapy, and immunotherapy have been explored (79–91). A mouse anti-human OX40 mAb has shown activity in nonhuman primates and has been tested in phase I clinical trials in 30 patients at different dosages with minimal toxicity although patients showed elevated levels of neutralizing human anti-mouse antibodies (92). Therefore, humanized anti-OX40 antibodies will be required to further develop this agent.

GITR-based cancer immunotherapy. The glucocorticoid induced TNFR (GITR) is upregulated in activated T cells, and as a costimulatory molecule, increases proliferation, activation, and cytokine production of CD4⁺ and CD8⁺ T cells after TCR activation (93, 94). In preclinical models, an agonist monoclonal rat anti-mouse GITR antibody (DTA-1) obtained very interesting results (95, 96). A humanized agonist anti-human GITR mAb (TRX518) has been developed, and similarly to DTA-1, provides potent costimulation to human lymphocytes *in vitro*, and for this reason, a dose-escalation phase I clinical trial has been recently initiated (97).

Use of oligonucleotide aptamer ligands to modulate the function of immune receptors

Aptamers are high-affinity single-stranded nucleic acid ligands, specific for a given target molecule with remarkable affinity and specificity comparable, or exceeding, those of antibodies, which can inhibit proteins or activate receptors which they bind to (98–100).

A number of aptamers have been developed to target immune regulator molecules as CTLA-4 (monomeric), CD137, OV-40 (both dimeric) to potentiate immunity, and in preclinical models, these showed to be as effective as the corresponding immune-regulating antibody in terms both of enhancing immune function and therapeutic impact. Further development of the protocols will generate higher affinity aptamers with enhanced bioactivity and better therapeutic potential. Several features commented on by Gilboa and colleagues in this issue (101) indicate that these biomolecules are an alternative platform to mAbs with some unique features.

High-affinity aptamers can even be used instead of immune modulating antibodies to reduce their toxicity. CD137-targeting with antibodies is associated with grade 3 or higher neutropenia and elevated liver enzymes (102) and severe hepatic toxicity at higher doses that led to the suspension of a clinical trial. To stimulate tumor-specific T cells while at the same time limit the activation of auto-reactive T cells, costimulation should be restricted to the tumor site. In preclinical models, aptamers are able to

potentiate tumor immunity but apparently with a superior therapeutic index (103).

Emerging Targets for Immunostimulatory mAb

The list of immunostimulatory mAb is not complete, as more will be discovered in the future. Many more target molecules that are extracellularly accessible are candidates for manipulation with mAbs in patients with cancer. Unfortunately, antibodies cannot reach intracellular targets and some lymphocyte-repressing systems such as signaling molecules or transcriptional factors never reach the cell surface.

If the target is expressed only in the T cells dealing with tumor antigens (in an inducible fashion) or in the tumor itself the situation is far more convenient, for it will be less likely to render on-target side effects.

Among the new emerging targets 2 important aspects must be considered: (i) are they functionally expressed in the tumor microenvironment or in the lymphoid tissues of the patient with cancer? (ii) Does the mouse lacking these genes suffer from propensity to autoimmunity? A positive answer to these 2 questions makes the target attractive. In this regard, LAG-3, TIM-3, killer inhibitory receptors, NKG2D and its ligands, CD69, TGF- β , and IL-10, offer much hope and are under early clinical or translational development. Some of these agents exert only modest effects as monotherapy in mouse models, but the "trick of the trade" is that they can act in synergy when combined with other treatments. It is worth mentioning that the immune system of the mouse and the human are different, and therefore some of the potentially valuable targets cannot be tested preclinically in mice.

Where Do We Go from Here?

If immunotherapy with mAbs were a day, in our opinion, we are still early at dawn. Much is expected to happen in the next 3 to 4 years. The road to success should focus on:

1. Identification and validation of new targets or new ways to optimally act on already known targets.
2. Predictive biomarker identification and increased ability to evaluate the correlates with survival benefit.
3. Greater knowledge of the effect of combinations of these agents among themselves and with conventional therapies.
4. T-lymphocyte penetration of malignant tissue seems to be a limiting factor.

The old metaphor of the car is still valid (104): we should simultaneously or sequentially press the gas pedal (costimulators), release the brakes (coinhibitors), and guide the wheel to handle autoimmunity and to steer toward the tumor antigens. An important element in an engine is the ignition system, and by that we mean starting the immune response by immunization against tumor antigens (i.e., vaccines) or by rendering tumor cells more immunogenic so the patient mounts immune responses to cancer-driver

mutations. Such usually weak immune responses are those that must be amplified by the armamentarium of immunostimulatory mAbs.

At this time point, there is fierce industrial competition to develop this kind of agent currently focused on the PD-1/B7-H1 interaction. The 2 advantages of such competition are that it might help to bring reasonable prices and that it will hasten progress. We can foresee that cost will be a clear limiting factor for drug access, as already perceived for ipilimumab. Although industrial stakeholders should be able to profit from risky investments in the field, if the indications for immunostimulatory mAbs keep expanding to extremely prevalent diseases the cost of treatment will be impossible for health care systems to absorb.

As clearly detailed in the accompanying *CCR Focus* reviews, the pace of preclinical and clinical research is fast. New targets and combinations are being evaluated preclinically and combination regimens are being tested in the clinic (105–107). In our opinion, "combination" is the key word.

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Disclosure of Potential Conflicts of Interest

I. Melero has commercial research grant from Bristol Myers Squibb, has honoraria from speakers bureau from Bristol Myers Squibb, and is a consultant/advisory board member of Bristol Myers Squibb and Medimmune. P.A. Ascierto has honoraria from speakers bureau from Bristol Myers Squibb, Merck Sharp & Dohme, and Roche-Genentech, and is a consultant/advisory board member for Bristol Myers Squibb, Merck Sharp & Dohme, Roche-Genentech, GlaxoSmithKline, Amgen, Celgene, Medimmune, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: P.A. Ascierto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Perez-Gracia, P.A. Ascierto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Perez-Gracia, P.A. Ascierto
Writing, review, and/or revision of the manuscript: I. Melero, A.M. Grimaldi, J.L. Perez-Gracia, P.A. Ascierto
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Clinical Cancer Research

Clinical Development of Immunostimulatory Monoclonal Antibodies and Opportunities for Combination

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