Biomarkers for Immunostimulatory Monoclonal Antibodies in Combination Strategies for Melanoma and Other Tumor Types

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Abstract

Modulation of the immune system by targeting coinhibitory and costimulatory receptors has become a promising new approach of immunotherapy for cancer. The recent approval of the CTLA-4–blocking antibody ipilimumab for the treatment of melanoma was a watershed event, opening up a new era in the field of immunotherapy. Ipilimumab was the first treatment to ever show enhanced overall survival (OS) for patients with stage IV melanoma. However, measuring response rates using standard Response Evaluation Criteria in Solid Tumors (RECIST) or modified World Health Organization criteria or progression-free survival does not accurately capture the potential for clinical benefit for ipilimumab-treated patients. As immunotherapy approaches are translated into more tumor types, it is important to study biomarkers, which may be more predictive of OS to identify the patients most likely to have clinical benefit. Ipilimumab is the first-in-class of a series of immunomodulating antibodies that are in clinical development. Anti-PD1 (nivolumab and MK-3475), anti-PD-L1 (BMS-936 559, RG7446, and MEDI4736), anti-CD137 (urelumab), anti-OX40, anti-GITR, and anti-CD40 monoclonal antibodies are just some of the agents that are being actively investigated in clinical trials, each having the potential for combination with the ipilimumab to enhance its effectiveness. Development of rational combinations of immunomodulatory antibodies with small-molecule pathway inhibitor therapies such as vemurafenib makes the discovery of predictive biomarkers even more important. Identifying reliable biomarkers is a necessary step in personalizing the treatment of each patient's cancer through a baseline assessment of tumor gene expression and/or immune profile to optimize therapy for the best chance of therapeutic success. *Clin Cancer Res; 19(5); 1009–20. ©2013 AACR.*

Introduction

It has been known for over 100 years that the immune system can detect and eliminate tumors; however, immunotherapy approaches have only very recently become an option for cancer treatment. This is highlighted by the recent U.S. Food and Drug Administration (FDA) approvals of the CTLA-4–blocking antibody ipilimumab for patients with melanoma (1) and sipuleucel-T (2), a therapeutic cell-based prostate cancer vaccine, which have led to renewed interest in cancer immunotherapy. Although much of the initial focus of recent immunotherapy development has been in patients with melanoma, evidence is rapidly emerging showing the potential of this approach in other malignancies. Currently, ipilumimab is being investigated in prostate, lung, pancreatic, and various hematologic malignancies. Likewise, blockade of another coinhibitory pathway, programmed death-1 receptor (PD-1), has already shown efficacy during an expanded phase I trial in multiple malignancies including renal cancer and non–small cell lung carcinoma (NSCLC; ref. 3).

Even though immunotherapeutic approaches are being studied in many cancer types, correlative studies have thus far been most extensively conducted in melanoma, providing valuable experience and making it a model system for identifying therapeutic biomarkers. Along with monoclonal antibodies (mAb) directed against specific coinhibitory and costimulatory T-cell receptors (TCR), such as anti-CTLA-4 (ipilimumab and tremelimumab), anti-PD1, 4-1BB (anti-CD137), GITR, and OX40 (4), small-molecule inhibitors that specifically target oncogenically activated pathways, such as the BRAF (vemurafenib and dabrafenib; refs. 5, 6), MEK (7, 8) and c-Kit (9), are also rapidly being incorporated into the melanoma treatment paradigm. In fact, the BRAF inhibitor vemurafenib was also approved for patients with late-stage melanoma. A direct comparison of these 2 therapeutic approaches in melanoma highlights the

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doi: 10.1158/1078-0432.CCR-12-2982

importance of incorporating immunotherapy biomarkers in clinical decisions. Vemurafenib treatment results in a dramatic and rapid clinical benefit in patients with tumors harboring BRAF mutations (within 15 days), but most patients relapse within a median of 7 to 8 months. Conversely, ipilimumab therapy is routinely associated with unpredictable and kinetically heterogeneous responses that may not satisfy traditional Response Evaluation Criteria in Solid Tumors (RECIST) or modified World Health Organization response-based endpoint criteria. However, patients who do respond can have prolonged survival and clinical benefit. Stable disease or objective responses are seen in about 30% of patients (1, 10). These findings have resulted in the development of immune-related response criteria (irRC) to reflect the additional response patterns observed with ipilimumab and other immune therapies currently in development for metastatic melanoma and other cancers (11). Given the variability in response to immunotherapy and the desire to extend long-term clinical benefit to more patients, cancer therapy is likely to focus on combinatorial approaches involving targeted inhibitors, immunotherapy, chemotherapy, surgery, radiation, and vaccination, etc. (12). With the increasing use of combination therapy (13), there is an increased need for the development of biomarkers that can help predict treatment outcomes and ensure that these costly new treatments, which sometimes have severe adverse reactions, are targeted at those patients most likely to benefit.

Approaches to Study T-cell Modulation during Immunotherapy

A fundamental objective of immunostimulatory antibodies is to modulate T-cell function and mediate effective antitumor immunity. Accordingly, the development and implementation of appropriate biomarker assays to study T cells is an essential companion objective for clinical trials, which seek to evaluate this promising class of immunotherapeutic agents, particularly in combination. In principle, the efficacy of a compound (in this case immune-target agents) is at least partially dependent on the presence of the target in the tumor [see nivolumab experience (3)]. In an ideal scenario, when complete information on predictive factors and proper selection of patients can be obtained in the early phases of drug development (phase I–II studies), the conduct of subsequent phase III studies could be optimized. Unfortunately, this ideal scenario occurs rarely. When planning a phase III trial comparing an experimental treatment with the standard, we often have evidence supporting a predictive role for response of a biomarker, whereas patients with absence of such expression should not respond. In such a scenario, different strategies are theoretically possible (see Fig. 1; ref. 14): (i) "randomize-all" strategy, that is, randomization between standard and experimental treatment without selection, possibly with stratification based on the status of the biomarker (in this case, "stratified trial design" or "treatment–marker interaction design"); (ii) "targeted" design, that is, randomization between standard and experimental treatment only in patients selected according to the status of the marker (also called "enrichment design"); and (iii) "customized" strategy (also called "marker-based strategy"), that is, randomization between standard arm in which the treatment is the same for all patients, and a personalized arm in which treatment is chosen on the basis of the marker status of each patient (Fig. 1).

A variety of often complementary strategies has been developed to study the presence, identity, phenotype, and function of T cells, and these have been reviewed recently (15). Because the antitumor activity of immunomodulating agents studied to date is thought to principally be a consequence of modulating the quality and quantity of the patient T-cell repertoire, perhaps the most relevant parameters to evaluate in the context of immunomodulating agents are the abundance, antigenic specificity, diversity, activation/suppressive, and functional phenotype of T-cell populations.

T-cell abundance can most readily be assessed by straightforward antibody based (flow cytometric, immunohistochemical) or molecular quantification of CD3ε-positive cells, commonly accompanied by quantification of CD8 and CD4 T-cell subsets. An important consideration for such analyses is the ability to quantify numbers rather than relative percentages of T cells to determine if there is a change in the absolute abundance after therapy. In fact, changes in absolute number of T cells have been found to correlate with clinical benefit after ipilimumab treatment. Measuring induction of antigen-specific responses can be evaluated through the use of multimer HLA-peptide complex reagents specific to individual TCR, which can identify antigen-specific T cells, often at frequencies as low as 0.1% of total $CD3^+$ cells (16, 17).

T-cell diversity can be evaluated in a general manner using flow cytometry or semiquantitatively using PCR to assay TCR Vβ usage (18, 19). More recently, quantitative high-throughput and deep-sequencing–based approaches have afforded the opportunity to evaluate in a systematic and essentially comprehensive manner the entire T-cell repertoire diversity directly *ex vivo* (20, 21). The ability to evaluate in a quantitative and unbiased manner the diversity of T-cell populations holds considerable potential for the mechanistic understanding of immunotherapy with immunostimulating reagents, by providing quantitative information about the breadth and depth of the modulated T-cell repertoire. This also provides important tools that facilitate the ability to study specific T cells modulated by treatment.

Combined with assessing T-cell abundance and diversity, detailed phenotypic and functional evaluation of T cells directly *ex vivo* can enhance our understanding of the changes that occur during immunotherapy. Technical evolutions in polychromatic flow cytometry have facilitated the ability to routinely conduct analyses using 12 markers in research laboratories and 17 markers or more can be conducted by specialized laboratories (22). An important parallel analysis for immunotherapy approaches that use immunostimulatory antibodies is the evaluation of surface

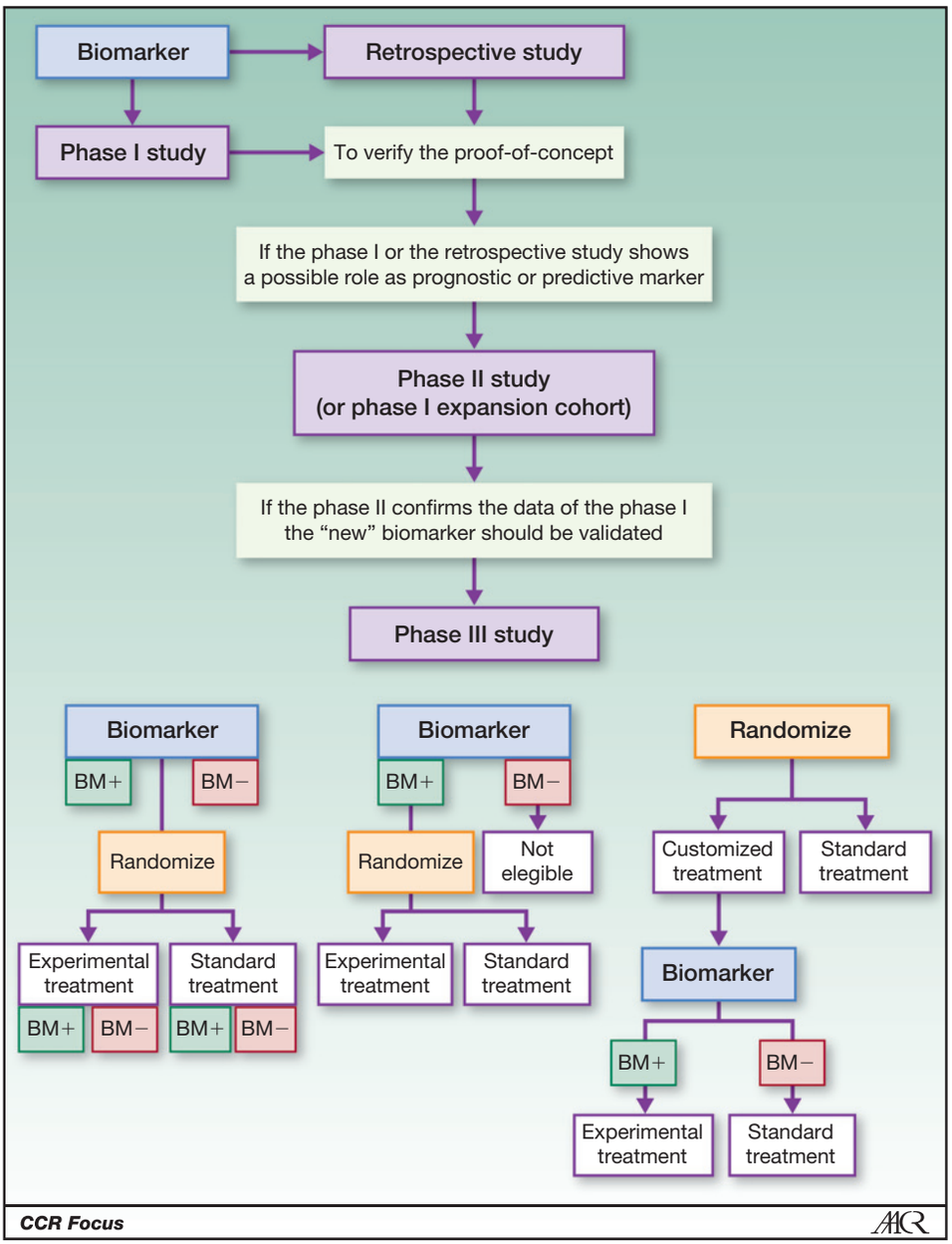

Figure 1. From hypothesis to biomarker. Processes for discovery and validation of biomarkers. Alternative designs of randomized phase III trials in the presence of a potentially predictive marker of efficacy of treatment. BM+, positive biomarker; BM−, biomarker negative. Bottom left, "randomize-all" design with determination and prospective stratification of BM+ and BM− patients. Center, "targeted" design. Right, "customized" design. Modified from Di Maio and colleagues (ref. 14; see in the text for details).

molecules that have been shown to modulate T-cell function. A preliminary list of such surface molecules includes markers associated with T-cell activation, such as CD27 (a marker of activation and memory T cells), CD134 (OX40; a secondary costimulatory molecule, marker of acutely activated T cells and involved in maintaining immune responses postinitial antigenic stimulation), CD137 (4-1BB; principally expressed by CD8 T cells and involved in T-cell proliferative responses postactivation), CD154 (CD40L; principally expressed by acutely activated CD4 T cells), as well as markers such as CD152 (CTLA4), CD272 (BTLA), CD279 (PD-1), and TIM-3, which are expressed following T-cell activation and are associated with the dampening and/or suppression of T-cell responses (23). Evaluation of these surface molecules can be combined with the detection of intracellular effector molecules, such perforin, granzyme-B, and the transcription factor T-β (24), effector cytokines, such as interleukin (IL)-2, IFN-γ, TNF-α, IL-4, MIP-1α/β, and concomitantly the phosphorylation status of intracellular signaling molecules important for T-cell function (25) to more thoroughly characterize the impact on a cell-specific basis of the immunomodulatory treatments (26). Recently published efforts to evaluate the transcriptome of the tumor microenvironment have generated intriguing insights into the transcriptional signature of a productive antitumor immune response (27).

Following appropriate enrichment strategies, the potential exists to apply transcriptional profiling to study the transcriptome of T-cell populations following immunomodulation therapy (28, 29).

Indirect but potentially relevant functional insights about the immunomodulating properties of antibody therapies can be obtained by evaluating the impact of the treatment on circulating systemic levels of soluble factors, such as cytokines, chemokines, and growth factors secreted by immune cells as a consequence of immunomodulation. In this regard, multiplex-based approaches using electrochemiluminescence and cytokine bead arrays, which enable the relatively comprehensive assessment of systemic levels for soluble factors, both directly and indirectly produced as a consequence of T-cell activation (30) have the potential to provide important insights into more general immunomodulation during treatment. A summary of platforms and assays, which often can be applied to provide mechanistic and functional insights about immunotherapy therapy, is presented in Table 1.

An additional and relevant issue to address with regard to the generation of datasets to evaluate T-cell modulation postimmunomodulating therapy relates to the increasing need to be able to objectively and quantitatively interpret parallel biomarker datasets generated across laboratories. This need can be achieved by standardizing assays across laboratories, an often difficult objective to achieve, particularly at the early stages of clinical development. An alternative conceptual approach is assay harmonization, which involves an iterative process that involves participating laboratories to identify critical variables that impact assay variability and for which the ultimate outcome is a set of laboratory-individual protocols, which generate qualitatively and quantitatively comparable biomarker datasets (31).

Finally, the development of uniform data reporting guidelines and repositories is a critical element necessary to facilitate both primary data analyses by collaborating and third party investigators as well as the ability to integrate and meta-analyze larger datasets. The Minimal Information about T Cell Assays initiative in particular (32) is one such effort, which is gaining traction as a mechanism to facilitate these objectives.

Table 1. Summary of platforms and assays to study T-cell modulation

Platform	Assays
High-throughput arrays	• Transcriptional profiling • Proteomic profiling • Cytokine profiling • Immune subset identification
Flow cytometry	• Surface and intracellular marker detection • Functional assays (cytolysis, degranulation, cytokine production)
Deep sequencing	• Detection of specific TCR clonotypes
Biochemical	• Multiplex detection of soluble factors (Luminex, mesoscale, bead array) • ELISA

Ipilimumab and Possible Predictive Markers

Ipilimumab was the first agent approved for the treatment of unresectable or metastatic melanoma that showed an overall survival (OS) benefit in a randomized phase III trial (1). However, to date, no surrogate or predictive marker for clinical response has been found. Thus, the discovery of a consistently reliable biomarker to identify patients who might benefit from ipilimumab treatment remains an important goal. Recent research has shown that that the absolute lymphocyte count (ALC) > 1,000/μL and increases in ALC after 2 ipilimumab treatments (week 7) correlate with clinical benefit and OS (33–35). In addition, maintained expression of the inducible costimulator (ICOS) molecule (36–39) identified those patients most likely to benefit from ipilimumab in a small cohort of patients with melanoma (40). Other potential immunologic markers in patients treated with ipilimumab included lactate dehydrogenase (LDH), c-reactive protein (CRP), circulating regulatory T cells (Treg), combined with ALC and white blood cell (WBC). In a study of 95 patients, responders showed decreased levels of LDH, CRP, and circulating Tregs and increased ALC between baseline and week 12, which was significantly associated with survival (Table 2; ref. 41). Evidence of an active humoral immune response against the cancer testis antigen NY-ESO-1 also correlated with OS in patients treated with ipilimumab (42). Although only 22% of patients showed seropositivity for this single tumor antigen, this subset was more likely to achieve clinical benefit from ipilimumab than those patients who were seronegative at baseline (55% vs. 31%). In addition, integrated NY-ESO-1–specific CD8 T-cell response further identified those patients most likely to see a survival benefit (HR, 0.181; $P = 0.0139$). The most conservative conclusion from these data is that preexisting immunity to NY-ESO-1, as a surrogate antigen, represents a means to identify patients whose immune systems have already been primed against the tumor, yet are not able to mount a therapeutic response. These patients may be the ones who are most likely to benefit from checkpoint blockade or other immunomodulation strategies and could be the same patients who have tumors with an "inflammatory" gene signature. This is an area of active research investigation. Further information will be provided from a clinical study in which ipilimumab is used in patients with advanced melanoma with spontaneous preexisting immune response to NY-ESO-1 (NCT01216696). Another potentially supportive study is a soon-to-be launched trial of NY-ESO-1 vaccination in combination with ipilimumab.

Other cells such as myeloid-derived suppressor cells (MDSC, $CD14^{+}$HLA-DRneg/low) accumulate in the peripheral blood of patients with melanoma from the very

Table 2. Correlation between changes in level of immunologic markers and disease control in patients treated with ipilimumab (41)

Marker		Patients, *n*	Median change between baseline and week 12, % (range)	*P* value
LDH	No response	32	42.3 (−74.7; 411.5)	<0.0001
	Disease control	25	−15.8 (−78.8; 105.9)	
CRP	No response	35	28.6 (−100; 4,940)	<0.0001
	Disease control	27	−60.3 (−100; 400)	
WBC	No response	54	26.7 (−51; 337)	0.13
	Disease control	34	17.6 (−51; 137)	
ALC	No response	55	−9.1 (−79; 505)	0.009
	Disease control	34	10.4 (−38; 244)	
FoxP3	No response	31	−28.6 (−100; 5,900)	0.003
	Disease control	27	−100.0 (−100; 1,400)	

Abbreviation: FoxP3, Forkhead box P3.

start of the disease (stage IIB and IIC) and can be detected as infiltrating components of primary lesions, suggesting a potential involvement of these cells in melanoma progression. As a biomarker for ipilimumab treatment, we have recently shown that the lower pretreatment level of $CD14^+$ HLA-DRneg/low MDSC (less that 20.5% of $CD14^+$ cells) correlated positively with response to therapy in a small study of patients treated with 10 mg/kg of antibody. In the same analysis, increasing MDSC numbers during therapy identified patients that did not show clinical benefit (43).

It is well known that CTLA-4 blockade results not only in clinical benefit but also to a mechanism-based panel of side effects, which have been termed immune-related adverse events (irAE). This represents tissue-specific inflammatory events, which most commonly affect skin, gastrointestinal tracts, and endocrine organs. There is a diversity of opinions about the relationship between irAEs and ipilimumab activity. Some authors show that there is no relationship between irAEs and ipilimumab activity (44, 45), whereas others have described that patients having an irAE are more likely to achieve clinical benefit (46). It was recently found that ipilimumab is able to upregulate Ki67 and ICOS on $CD4^+$ and $CD8^+$ cells (47). Interestingly, the baseline low level of circulating $Ki67^+EOMES^+CD8^+$ T cells was associated with relapse ($P \leq 0.001$), whereas a low level of circulating $Ki67^+EOMES^+CD4^+$ T cells was associated with irAEs. This finding could contribute not only to predicting the outcome of patients treated with ipilimumab but also the occurrence of irAE. Additional preliminary studies have noted a correlation between serum IL-17 levels, Th17 cells, and symptomatic colitis (48). The latter finding suggests a possible specific means to address the toxicity using neutralizing IL-117 antibodies.

Although none of these markers alone can completely identify all patients who are either most likely to benefit or are benefiting from ipilimumab therapy, combining these measurements together into a matrix of correlative markers may provide more power to accurately classify patients and predict outcomes. Table 3 lists the possible biomarkers that could have a role in the monitoring of patients treated with anti-CTLA-4.

The next step will be to focus attention toward those biomarkers, which can predict the response to ipilimumab treatment. In fact, in the majority of the cases, such markers are prognostic and not predictive of response. The next prospective phase III trials and new possible approaches such as single cell network profiling (SCNP; ref. 49), the gene signature, and the immunoscore/immunoprofiling will help aid in the definition of optimal and relevant biomarkers, and consequently in the selection of patients for ipilimumab treatment.

PD-1 Biomarker Development

The promising efficacy observed in response to anti-CTLA-4 therapy has set the stage for the development of additional T-cell immunomodulatory approaches for cancer treatment. These include antibodies against costimulatory molecules CD137 (4-1BB), anti-OX-40 (CD134), anti-GITR, and anti-CD40, along with antagonists of coinhibitory pathways, such as the PD-1, its ligand programmed death ligand-1 (PD-L1, B7-H1; ref. 4).

Table 3. Potential biomarkers useful for the treatment with anti-CTLA-4

Increase in ALC (33–35)
Increased in circulating $CD4^+ICOS^{high}$ T cells (35–40)
Baseline level of $Ki67^+EOMES^+CD8^+$ T Cells (47)
High expression of FOXP3 and indoleamine 2,3-dioxygenase at baseline and an increase from baseline in TILs in tumor biopsies (35)
Decrease in circulating Tregs (41)
Decrease of LDH level (41)
Lower pretreatment level of circulating MDSC (43)

Of the additional immunomodulatory therapies in clinical development, PD-1/PD-L1 blockade has currently advanced the furthest in both clinical development and biomarker research. PD-1 is a surface molecule that delivers inhibitory signals to activated T cells after binding to its ligands: B7-H1/PD-L1 and B7-DC/PD-L2. PD-L1 is expressed in hematopoietic cells and can be found in tissues, such as pancreatic islets, heart, endothelium, small intestine, and placenta. It is believed that tumor cell expression of PD-L1 is used as a mechanism to evade recognition/destruction by the immune system. In fact, expression of PD-L1 in tumors is associated with poor outcome for patients with certain epithelial cancers (50). PD-L2 expression is restricted only to macrophages and dendritic cells and is also upregulated upon activation. Antibodies blocking PD-1 and PD-L1 are in clinical development. Phase I trials with anti-PD-1 antibodies have been very encouraging, particularly those involving repeated doses in which durable objective responses have been observed with a reasonable safety profile in multiple disease types (3, 51).

In a phase I study of the anti-PD-1 antibody nivolumab (formerly MDX-1106, Bristol-Myers Squibb), Topalian and colleagues found an association between pretreatment tumor expression of PD-L1 and objective responses (3). Tumor PD-L1 expression, as detected by immunohistochemistry (IHC), was found in tumor biopsy samples from 25 of 42 patients. The objective response rate for patients with PD-L1–positive tumors was 36% (9 of 25), whereas the response rate in the PD-L1–negative group was 0%. However, unlike biomarkers that reflect mutations in tumor DNA, and thus are relatively fixed, such as the BRAF V600E mutation, tumor expression of PD-L1 may be dynamic, depending upon changes in the tumor microenvironment. In fact, in an evaluation of 150 melanoma tumor samples, tumor PD-L1 expression was highly concordant with immune infiltrate and the presence of IFN-γ in the tumor microenvironment (52). Moreover, this group did not provide an analysis of the PD-L1 expression on tumor-infiltrating antigen-presenting cells, a population likely to have high PD-L1/L2 expression in the tumor microenvironment. Thus, although tumor PD-L1 was suggested to have predictive value in this small group of nivolumab-treated patients, larger prospective studies will be necessary to explore this further.

Other Promising Immunotherapies

Beyond immunomodulatory antibody therapies, there are other promising immunotherapies being investigated for the treatment of melanoma such as vaccines (12), oncolytic immunotherapy (e.g., OncoVEXGM-CSF; ref. 53), darleukine (a fusion protein, consisting of the human vascular targeting antibody L19 and human IL-2; ref. 54), and Treg-depleting agents (as in the experience based on the use of denileukin diftitox; ref. 55). However, the challenge will be to optimize the treatment of individual patients using these active agents sequentially or in combination with each other or with traditional anticancer modalities, such as chemotherapy, radiation, or surgery (12). Therefore, biomarker development and the understanding of how these therapies function *in vivo* again becomes of paramount importance. The clinical development and evaluation of IL-2 provides some instructive lessons. IL-2 treatment of patients with melanoma results in objective tumor response in 16% of patients, with 6% achieving a complete response (56). The response rate in kidney cancer is also low. This was one of the first examples of immunotherapy in which biomarkers were assessed for relationship with therapeutic response. In fact, due to the difficulty in selecting patients for the treatment as well as the toxicity and low response rate, this treatment is not commonly used. Sabatino and colleagues (57) identified pretreatment serum concentrations of fibronectin and VEGF as being important predictive biomarkers for response to IL-2 in patients with melanoma and renal cell carcinoma. Joseph and colleagues (58) have reported data from a retrospective study to identify clinical and molecular characteristics of patients with melanoma to predict response to high-dose IL-2 in a subset of 103 patients (of whom 15% had an NRAS mutation). In this study, the response rate was 47% in patients whose tumors had an NRAS mutation, with a nonstatistically longer progression-free survival and OS versus wild-type patients. These results suggest that the NRAS mutational status could be a possible biomarker for selecting patients with melanoma for IL-2 treatment.

In the area of vaccines, MAGE-A3, a cancer-testis antigen, is expressed by up to 76% of metastatic melanomas (59). After 2 positive phase II trials, phase III trials have now been initiated using a MAGE-A3 protein vaccine in the adjuvant treatment of melanoma (DERMA trial: resected MAGE-A3 plus pIIIB/pIIIC melanoma randomized to rec-MAGE-A3 plus AS15 or placebo—NCT00796445) and NSCLC (MAGRIT trial: resected MAGE-A3+ NSCLC pIB/II/IIIA randomized to rec-MAGE-A3 plus AS15 or placebo with or without prior chemotherapy—NCT00480025). During the clinical development of MAGE-A3 vaccine therapy, a gene signature that may predict the clinical outcomes of MAGE-A3 treatment was investigated. Such gene signatures suggest that the presence of a specific tumor microenvironment, permissive to immune infiltration before MAGE-A3 treatment, influences its efficacy. In fact, both the phase II studies showed that patients with the immunogenic gene signature are more likely to benefit from MAGE-A3 vaccination (Table 4). The gene signature is currently the subject of prospective validation (60).

Combinations of Immunomodulatory Antibodies

A phase I study of ipilimumab in combination with nivolumab is currently ongoing (NCT01024231). Several preclinical studies have shown promising antitumor activity with this combination and identified putative biomarkers for further exploration. Curran and colleagues (61) evaluated combinations of CTLA-4, PD-1, and PD-L1 blockade along with Flt3-ligand transfected tumor cell vaccine (FVAX) in the treatment of the poorly immunogenic B16

Table 4. Gene signature associated to clinical benefit of MAGE-A3 ASCI in phase II studies in melanoma and NSCLC patients [from Ascierto and colleagues (73)]

	Phase II studies evaluating the MAGE-A3 ASCI	
	Phase II NSCLC (NCT 00290355)	Phase II Melanoma (NCT 00086866)
GS−	25% Relative improvement (DFI)	OS of 16.2 months
GS+	53% Relative improvement (DFI)	OS of 28.0 months

Abbreviations: DFI, disease-free interval; GS−, population in which the gene signature was not found; GS+, population for which a specific gene signature has been defined.

mouse melanoma. Combined checkpoint blockade was clearly superior to CTLA-4, PD-1, or PD-L1 blockade alone. Successful combination regimens were associated with higher intratumoral levels of $CD4^+$ and $CD8^+$ effector T cells and higher levels of effector cytokines such as IFN-γ. Mangbo and colleagues (62) explored single or combined antibody blockade of CTLA-4 and PD-1 alone or combined with the Toll-like receptor agonists CpG or bacillus Calmette–Guérin for treatment of murine experimental bladder cancer. Although the combination had no additive effect compared with anti-CTLA-4 alone, there were elevated levels of circulating CD107a-expressing CD8 T cells found in the anti-CTLA-4 plus anti-PD-1 group (62). In addition, levels of antinuclear antibodies correlated inversely with tumor size. Similar effects were seen when CpG were combined with anti-CTLA-4 or anti-PD-1, increasing the numbers of circulating tumor-specific CD107a-expressing CD8 T cells as well as activated ($CD25^+FoxP3^-$) CD4 splenocytes (50). Furthermore, the number of Tregs in the tumor area of treated animals was decreased after anti-CTLA-4 or anti-PD-1 plus CpG therapy. The combination of CpG with CTLA-4 or PD-1 blockade improved long-term survival and led to increased levels of tumor-reactive T cells and reduced numbers of Tregs at the tumor site (62).

The difference in the mechanism of action between the 2 pathways (CTLA-4/B7.1 and PD-1/PD-L1) justifies the possible potentiating of the combination. In fact, whereas the CTLA-4/B7.1 pathway is important in the priming phase, the PD1-PD-L1 interaction is important in the effector phase and in the exhaustation of T cells. It will be important to characterize these tumor-infiltrating lymphocytes (TIL) in patients treated with this combination, because they could express other coinhibitory ligand-receptors, which could participate in the immune suppressive action of the tumor microenvironment. In fact, recent studies showed that concurrent blockade of other exhaustion/coinhibitory molecules (TIM3 and LAG3) and PD-1 can result in synergistic antitumor activity (51). The characterization of the immune infiltrate will be the basis of the immunoscore/immunoprofiling evaluation in melanoma and other cancers. Finally, the TIL and peripheral blood lymphocyte CTLA-4 expression increases in response to nivolumab treatment (51), and PD-1 expression similarly is induced in T cells after CTLA-4 blockade. These could also be important for sequencing of CTLA-4- and PD-1–blocking antibodies.

Clearly, all markers potentially useful for treatment with ipilimumab or anti-PD-1/PD-L1 as single agents may also be useful for the combined therapy approach. However, ongoing and planned clinical trials of monotherapy and combinatorial antibody treatment present an opportunity to identify new biomarkers unique to the combination.

Combinations with Chemotherapy or Targeted Inhibitors

The hypothesis that chemotherapy-induced release of tumor antigens might amplify the antitumor activity of ipilimumab was tested in an international, multicenter phase III trial (NCT00324155) that compared dacarbazine plus ipilimumab 10 mg/kg with dacarbazine plus placebo. Although this was the first study that showed the efficacy of a possible combination in patients with metastatic melanoma, it is unclear if this result may have been due to ipilimumab alone instead of the combination of dacarbazine plus ipilimumab. Other ongoing trials are also exploring other combinations with temozolomide, fotemustine, paclitaxel, and carboplatin. Future studies will evaluate the best chemotherapeutic drug to combine with ipilimumab. This again highlights the need for biomarkers to understand if this is the correct chemotherapy agent to combine with ipilimumab.

Two studies combining ipilimumab with vemurafenib in either sequential or concurrent schedules are presently ongoing (NCT01400451 and NCT01673854). These trials will test if targeted BRAF inhibition together with CTLA-4 blockade is safe and will result in clinical benefit, which compares favorably when either of these agents is used as a monotherapy. It has been proposed that RAF inhibitors may provide additive or synergistic benefit in combination with immunotherapy mediated because of increased tumor antigen release. Whereas synergy will be assessed comparing clinical response rates, immune monitoring during the trial will determine if there is in fact greater T-cell activation and development of antigen-specific responses in combination therapy–treated patients compared with monotherapy controls. Most studies to date have generally supported the view that RAF inhibitors are unlikely to impair the immune

system or prevent the generation of antitumor immunity (63–65). Several studies have provided evidence that RAF inhibitors may enhance immune activation. Boni and colleagues found that melanoma cells increased expression of tumor-associated antigens after treatment with RAF inhibitor *in vitro* (66). Wilmott and colleagues observed that patients treated systemically with vemurafenib or dabrafenib had an increase in intratumoral $CD8^+$ T-cell infiltration (67). Koya and colleagues found evidence of enhanced T-cell antitumor activity for adoptively transferred T cells in the presence of vemurafenib (68). Finally, Callahan and colleagues reported that the RAF inhibitor BMS908662 potentiated T-cell activation *in vitro* and *in vivo*, leading to enhanced antigen-specific T-cell expansion and antitumor activity in combination with CTLA-4 blockade in mouse models (69). In contrast, only 1 group has reported that PLX4720 can have a negative effect on antitumor immune responses (70). RAF inhibitors also seem to impact the PD-1 pathway. A recent study by Jiang and colleagues reports that PD-L1 expression is upregulated in melanoma cells that have developed resistance to RAF inhibitors (71).

Although the combination of immunotherapy with radiotherapy has also recently received much attention after a report of abscopal responses seen in an ipilimumab-treated patient with melanoma after radiotherapy treatment, trials are just beginning, which will investigate if this approach is truly synergistic in multiple malignancies along with melanoma (72). As part of a trial being conducted by the Ludwig Institute (New York, NY), we will be investigating if the combination results in the generation of *de novo* antitumor responses after radiation treatment.

Placing Biomarkers of the Tumor Microenvironment in Context Immune Score and Gene Signature to Predict Outcome and Response

The adaptive immune response influences the behavior of human tumors. In fact, characterization of tumor-infiltrating immune cells in large cohorts of human colorectal cancers by gene expression profiling and *in situ* has suggested that TH1-adaptive immunity has a beneficial effect on clinical outcome (73). Tissue microarrays show that tumors from patients without recurrence had higher immune cell densities (CD3, CD8, granzyme B, and CD45RO) within the central tumor region and invasive margins (CT and IM) than did those from patients whose tumors had recurred. For all the markers of the combined analysis of central tumor plus invasive margin regions, it was shown that coordinated adaptive immune reaction, more so than tumor invasion, predicts clinical outcome. Collectively, the immunologic data (type, density, and location of immune cells within the tumor samples) were found to be a better predictor of patient survival than the histopathology (73).

The concept of "immune contexture" represents the combination of immune variables associating the nature, density, functional orientation, and distribution of immune cells within the tumor of a natural *in situ* immune reaction (74, 75). A pioneer in this field was Alistair Cochrane, who observed that some regional lymph nodes close to melanoma and breast cancer are immune suppressed and that the degree of immune suppression correlates directly with the closeness of the node to the tumor (76). Subsequently, he showed that sentinel lymph nodes from melanoma and breast cancer (77) have reduced paracortical activity compared with the nonsentinel lymph nodes, with a reduction of the paracortical area and the interdigitating dendritic cell (IDC) area and the proportion of IDCs that express long dendritic processes. These findings suggested that in nodes proximal to the tumor or partly replaced by tumor (such as the sentinel lymph nodes), IDCs are reduced and lack the complex dendrites that characterize active antigen presentation. This implied the existence of nodal immune suppression due to an influence of the tumor, mediated in part by melanoma-derived materials.

Histopathologic analysis of tumors reveals the presence of infiltration of inflammatory and lymphocytic cells (Fig. 2). Such cells are not randomly distributed but can be organized within dense infiltrates and localized in specific parts of the tumor (78, 79). Tumor-infiltrating immune cells are heterogeneous between tumor types and are different from patient to patient. The immune infiltrate may include macrophages, dendritic cells, natural killer (NK) cells, naïve and memory lymphocytes, B cells, and T lymphocytes (which includes various subsets of T cells including regulatory and effector T cells; Fig. 3).

Tumor-infiltrating immune cells have been studied in many cancers. In colorectal cancer, histopathologic analysis showed that such tumors are often infiltrated by a variable degree of inflammatory and lymphocytic cells. A closer evaluation shows that these immune cells are not distributed randomly but seem to be organized in more or less dense infiltrates in the center of the tumoral zone, at the invasive margin of tumoral nests, and in lymphoid islets adjacent to the tumor. An immune-classification of colorectal cancer was proposed on the basis of a simple immune score, quantifying the density and location of immune-cells within the tumor (78). The prognostic significance of the immune score ("immunoscore") based on the evaluation of CD45RO-CT/IM and CD8-CT/IM was compared with that of the standard staging criteria using the American Joint Committee on Cancer (AJCC)/International Union against Cancer (UICC) tumor-node-metastasis (TNM) staging system (AJCC/UICC-TNM). This immune-classification has a prognostic value that is superior to the AJCC/UICC-TNM classification, and tumor invasion was shown to be, in fact, statistically dependent on the host–immune reaction (78, 80). Moreover, in colorectal cancer, the presence of specific chemokines (CX3CL1, CXCL10, and CXCL9) correlate with high densities of T-cell subpopulations within specific tumor regions, and their high expression is associated with prolonged disease-free survival (78, 79).

Similarly, for other malignancies, a correlation between lymphocytic infiltration and survival has been shown (78, 80). These findings, though a revision of the current

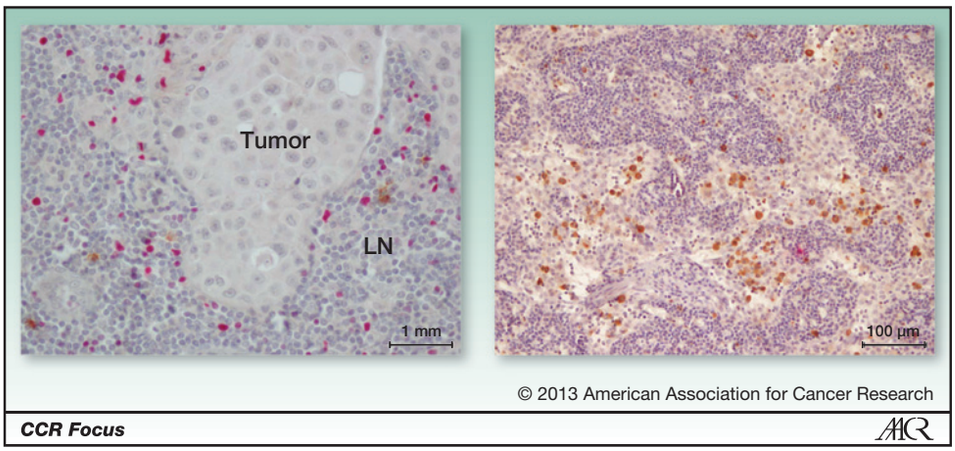

Figure 2. IHC staining with anti-FoxP3 and anti-granzyme B. Two opposite cases of lymph node (LN) in different patients with melanoma. Left, a metastatic lymph node with an high expression of FoxP3$^+$ cells (red) at margin of melanoma and very low expression of GB+ cells (brown). Granzyme B is specific for CTL and NK cells. Right, a nonpathologic lymph node with high expression of granzyme B and low expression of FoxP3. The metastatic lymph node showed an increase of the immunosuppressive cells compared with the negative lymph node.

indicators of clinical outcome, may help to better identify the high-risk patients who would benefit from adjuvant therapy.

The immunoscore and associated gene signatures represent a new challenge and opportunity in the field of immunotherapy biomarkers. As discussed earlier, several findings support the hypothesis that cancer development is influenced by the host–immune system. The evaluation of systemic and local immunologic biomarkers could offer useful prognostic information and facilitate clinical decisions about the need for systemic treatment (78).

Conclusions

Immunotherapies are now showing the ability to mediate durable remissions in a variety of cancers and have been proven to extend OS in melanoma. Despite much enthusiasm for the approach, only a subset of patients benefit from the most advanced therapies and potentially serious side effects can sometimes accompany therapy. Predictive and pharmacodynamics biomarkers are therefore vital to further efforts by not only informing clinical decision-making but also by identifying new targets for immunomodulation. Careful histologic and genomic analysis of the tumor and microenvironment are

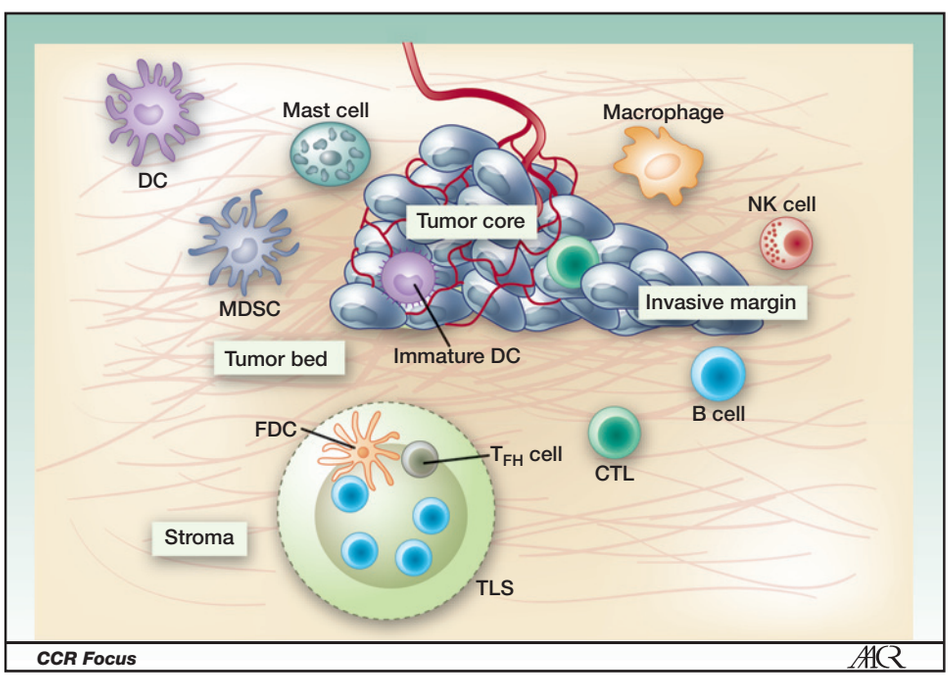

Figure 3. The immune-contexture. The concept of "immune contexture" is represented by the presence, into the tumor microenvironment, of different immune variables associating the type, density, and distribution of immune cells. FDC, follicular dendritic cell; TLS, tertiary lymphoid structures; T_{FH}, T follicular helper cell. Adapted by permission from Macmillan Publishers Ltd. (81), copyright 2012.

providing additional substantive evidence for the importance of host immunity in determining clinical outcomes. Integration of biomarkers for individual therapies with predictive information from pretreatment tumor may allow for more precise and optimal application of immunomodulation in a greater proportion of patients with cancer.

Disclosure of Potential Conflicts of Interest

P.A. Ascierto has honoraria from Speakers Bureau of Bristol Myers Squibb, Merck Sharp & Dohme, and Roche-Genentech and is a consultant/advisory board member of Bristol Myers Squibb, Merck Sharp & Dohme, Roche-Genentech, GlaxoSmithKline, Amgen, Celgene, Medimmune, and Novartis. M. Kalos has ownership interest (including patents) in patents and potential royalties associated with licensing and commercialization of CART19 technology and is a consultant/advisory board member of Adaptive Biotechnologies. M.K. Callahan has a commercial research grant from BMS and is a consultant/advisory board member of GSK. J.D. Wolchok has commercial research grants from Bristol-Myers Squibb and Novartis Genetics Foundation and is a consultant/advisory board member of Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other author.

Authors' Contributions

Conception and design: P.A. Ascierto, M. Kalos, J.D. Wolchok
Development of methodology: P.A. Ascierto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.A. Ascierto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.A. Ascierto
Writing, review, and/or revision of the manuscript: P.A. Ascierto, M. Kalos, D.A. Schaer, M.K. Callahan, J.D. Wolchok
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.A. Ascierto
Study supervision: P.A. Ascierto, J.D. Wolchok

Acknowledgments

The authors thank Drs. Marilena Capone and Gerardo Botti from Istituto Nazionale Tumori of Naples, Italy for providing us IHC photos.

Received November 23, 2012; revised January 7, 2013; accepted January 10, 2013; published online March 4, 2013.

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Biomarkers for Immunostimulatory Monoclonal Antibodies in Combination Strategies for Melanoma and Other Tumor Types

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Clin Cancer Res 2013;19:1009-1020.