Targeted Therapies to Improve Tumor Immunotherapy

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Abstract

Durable tumor regression and potential cures of metastatic solid cancers can be achieved by a variety of cellular immunotherapy strategies, including cytokine therapy, dendritic cell–based vaccines, and immune-activating antibodies, when used in so-called immune-sensitive cancers such as melanoma and renal cell carcinoma. However, these immunotherapy strategies have very low tumor response rates, usually in the order of 5% to 10% of treated patients. We propose that the antitumor activity of adequately stimulated tumor antigen–specific T cells is limited by local factors within the tumor milieu and that pharmacologic modulation of this milieu may overcome tumor resistance to immunotherapy. By understanding the mechanisms of cancer cell immune escape, it may be possible to design rational combinatorial approaches of novel therapies able to target immunosuppressive or antiapoptotic molecules in an attempt to reverse resistance to immune system control. We term this mode of treatment “immunosensitization.” Ideal candidates for immunosensitizing drugs would be targeted drugs that block key oncogenic mechanisms in cancer cells resulting in a proapoptotic cancer cell milieu and at the same time do not negatively interfere with critical lymphocyte functions.

One of the hallmarks of cancer responses to cellular immunotherapy is that they are extremely long lived, frequently measured in years. However, objective responses (as opposed to the more common claim of mixed responses or disease stabilization with immunotherapy) are rare, usually noted in a minority of patients, in the range of 5% to 10% (1, 2). The improved understanding of the oncogenic events in cancer and the development of multiple new highly targeted drugs that block or neutralize oncogenic factors open the door for potential novel combinatorial approaches with immunotherapy. In this article, we postulate that targeted agents against key immunosuppressive, oncogenic, or antiapoptotic factors in cancer cells can sensitize cancers to immunotherapy. We restrict the definition of tumor immunotherapy to mean to activate cellular cytotoxic antitumor immune responses, mostly mediated by CD⁸⁺ CTLs and natural killer (NK) cells. This is opposed to therapy mediated by antibodies such as rituximab, trastuzumab, or bevacizumab, which target surface or secreted proteins in cancer cells but are not designed to induce adaptive immune responses to the cancer. We suggest that optimal combinations of these drugs and cellular immunotherapy will result in improved antitumor responses as targeted drugs fight immune resistance and allow the durable responses seen with immunotherapy to take hold.

Limiting Steps for Effective Tumor Immunotherapy

The multiple advances in understanding the immunobiology of T-cell responses to cancer have not translated into widespread clinical utility (1). Cytokine-based therapies, dendritic cell (DC)-based vaccines, and treatment with immune-activating antibodies, particularly with blocking antibodies to the CTLA-4 associated protein 4 (CTLA4), have a ceiling of tumor responses in a subset of so-called immune-sensitive tumors (mainly melanoma and renal cell carcinoma) in the range of 5% to 10% of treated patients (1, 2). However, enthusiasm for tumor immunotherapy is maintained by the realization that most tumor responses are durable, leading to rare chances of cure in patients with widely metastatic solid tumors. That is the basis for the Food and Drug Administration licensing of the use of high-dose interleukin (IL)-2 in metastatic melanoma and renal cell carcinoma (3), and consistent with the findings emerging from ongoing clinical studies developing DC-based vaccines (2, 4) and anti-CTLA4 antibodies (5, 6). Therefore, although we have the proof of concept about the effectiveness of tumor immunotherapy, major improvements are needed in many key aspects of this type of therapy.

There are many factors that influence tumor response to immunotherapy. Neoplastic cells survive and proliferate in an often hostile environment. Under constant selection pressure, cells with a survival advantage replicate, thus ensuring the tumor “evolves” to suit its environment. Tumor cells that develop mechanisms to evade the immune system allow the cancer to become immunoresistant in a process known as cancer immunoediting (7). Within this context, the ability of immune system cells to attack cancers is limited. Figure 1 depicts a hypothetical model of tumor immunotherapy
highlighting the key steps in generating an effective antitumor immune response. At each step, the immune system or the tumor may limit or prevent the effective elimination of cancer cells by T cells. Obviously, this is a simplified model given the complexity of the immune response and the multiple cell subsets involved. However, we focus on the key interactions leading to an adaptive immune response to cancer to help describe what we believe are the key limitations to current immunotherapy.

**T-cell activation by antigen-presenting cells.** The first step in generating an effective antitumor immune response leads to the licensing of CD8+ CTLs, which, through the T-cell receptor, specifically recognize their cognate antigen presented by tumor cells through class I molecules. Most tumor antigens are self-antigens (i.e., antigen normally expressed on healthy cells and shared by cancer cells). T cells recognizing these self-antigens would cause autoimmunity; self-reactive T cells are subjected to negative selection in the thymus and are only detectable in very low levels in the periphery. In addition, tumor antigens are frequently down-regulated by cancer cells and do not themselves lead to activation of tumor antigens—specific CTL (8). For these antigens to be recognized, they need to be presented by DC to CD4+ T helper cells or cross-presented (a process in which exogenous antigen is presented in MHC class I) by DC to CD8+ CTL (9) in an interaction modulated by costimulatory and coinhibitory molecules (10). The development of methods to generate DC ex vivo allowed their testing as a powerful means to activate the immune system to cancer self-antigens (11). The clinical results to date are suboptimal (1) and highlight the need to address other variables involved in immune-mediated tumor regression other than the use of powerful vaccines.

**Costimulatory and coinhibitory molecules.** Antigen presentation by MHC recognized by T-cell receptors on T lymphocytes is not enough to activate an adaptive immune response; it critically requires the delivery of costimulatory signals provided by B7 molecules (CD80 and CD86) recognized by their positive receptor CD28 on T cells. The coinhibitory molecule CTLA4 (CD152) has a pivotal role in dampening immune responses to self-antigens by competing with CD28 (12). Blocking antibodies to CTLA4 are in clinical development and have shown durable immune-mediated response rates in the order 5% to 20% in patients with metastatic melanoma (5, 6, 13, 14). Several other costimulatory and coinhibitory targets are amenable to intervention, and specific antibodies against CD40, CD80, CD134 (OX40), CD137 (41BB), programmed death 1, and others are being tested (10). Therefore, several off-the-shelf potent immunostimulating agents are entering clinical testing for cancer, making it feasible the future study of combinatorial approaches.

**T-cell expansion and tumor targeting.** After CD8+ CTLs are activated against cells expressing tumor antigens, they need to overcome homeostatic mechanisms that limit immune activation and expansion. They then traffic to tumors. The immune system has multiple breaks limiting this activation process, including the expression of CTLA4 by activated T cells leading to interference in establishing lasting interactions between activated T cells and target cells expressing their cognate antigen (15).

**Recognition of cancer cells.** Activated tumor antigen–specific CD8+ cells that have traveled to the tumor have to then recognize their cognate antigen presented by tumor cells and (4) overcome tumor-induced local negative immune regulators; and (5) the tumor cells need to be sensitive to the lytic effects of the immune effector cells, delivered either through perforin/granzyme or by death receptor ligation (Fas, TRAIL, and TNF). GzB, granzyme B; Treg, Regulatory cell; VEGF, vascular endothelial growth factor; PGE2, prostaglandin E2; TGF-β, transforming growth factor-β; TAP, transporter associated with antigen processing; PDT, programmed death 1.

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**Fig. 1.** Model of CTL response to melanoma. There are several steps required for the induction of an effective antitumor immune response: (1) antigen-presenting cells need to activate CD8+ tumor-specific effector cells overcoming the limitations of targeting tumor self-antigens; (2) CD8+ cells need to overcome homeostatic mechanisms that limit immune activation, and then they need to expand and traffic to tumor targets; (3) CD8+ cells have to recognize antigen presented by tumor cells and (4) overcome tumor-induced local negative immune regulators; and (5) the tumor cells need to be sensitive to the lytic effects of the immune effector cells, delivered either through perforin/granzyme or by death receptor ligation (Fas, TRAIL, and TNF). GzB, granzyme B; Treg, Regulatory cell; VEGF, vascular endothelial growth factor; PGE2, prostaglandin E2; TGF-β, transforming growth factor-β; TAP, transporter associated with antigen processing; PDT, programmed death 1.
Immunosuppressive tumor milieu. Local immunosuppressive factors within the tumor milieu include secreted molecules, such as transforming growth factor-β, IL-10, prostaglandin E2, and vascular endothelial growth factor (VEGF). These factors are produced either by the tumor cells themselves or by surrounding host stromal cells on tumor cell signaling. The suppressive tumor environment is further maintained by immunosuppressive cells, such as T regulatory cells, and indolamine 2,3-dioxygenase (IDO)-producing plasmacytoid DC (17, 18). These are professional immune suppressor cells that are frequently found inside tumors and provide means for tumor escape from activated lymphocytes.

Escape from immune effector cell killing of target cells. Immune effector cells (either CD8+ CTL or NK cells) kill their targets by four defined mechanisms: the release of perforin and granzymes (see ref. 19 for review) and the engagement of the Fas, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and TNF death receptors (see ref. 20 for review). A simplified schematic of this process is shown in Fig. 2. On entry into target cells facilitated by perforin, granzyme B directly cleaves Bid (a proapoptotic Bcl-2 family member) resulting in activation of caspase-3 and other downstream effector caspases, leading to apoptotic cell death. Engagement of the surface Fas, TRAIL, or TNF death receptors by their secreted or cell surface–anchored ligands presented on immune effector cells leads to the activation of caspase-8 within the target cell and activation of the extrinsic pathway of apoptosis. Caspase-8 also activates the intrinsic (also called “mitochondrial”) apoptosis pathway (see ref. 21 for review), which depends heavily on the balance between proapoptotic and antiapoptotic molecules, particularly of the Bcl-2 family.

Intracellular effectors of cytotoxicity and apoptotic cell death. The fate of a cell reflects the dynamic interplay between apoptosis and survival; the Bcl-2 family is critical in maintaining this balance. The family is classified into three groups: the “antiapoptotic,” “proapoptotic,” and “BH3-only” proteins (see ref. 22 for further review). The proapoptotic Bcl-2 family members include Bcl-2, Bcl-XL, Bcl-w, Bcl-2A1, Mc11, and Boo, which promote cell survival by antagonizing apoptotic signals. Overexpression of these molecules protects cells from apoptosis and mediates cell survival (23). The proapoptotic Bcl-2 family members include Bax, Bak, Box, and Bcl-XL. Overexpression of these molecules has been shown to promote apoptosis (23). These molecules are directly responsible for initiating apoptosis in the mitochondrial pathway and are considered important sensors of cellular stress (such as DNA damage). The BH3-only subfamily is proapoptotic but is classified separately due to its mechanism of action. It includes Bad, Bik, Bid, Hrk, Bim, Noxa, Puma, and Bmf. The primary role of these molecules is the activation of the proapoptotic family members.

Cancer resistance to apoptosis. Given the critical role of proapoptotic and antiapoptotic proteins, it is not surprising that altered expression of these proteins in cancerous cells often allows them to resist apoptosis. Fas and TRAIL receptors are down-regulated or undetectable in many cancers, obviously leading to resistance to death receptor–induced apoptosis in most cases (24). The nuclear factor-κB is a transcription factor with constitutive expression in many cancers (25) and is responsible for the transcription of many antiapoptotic genes, including the FLICE inhibitory protein (FLIP). FLIP is expressed at high levels in many cancers, where it competes with caspase-8 for binding to the death-inducing signaling complex. Expression of FLIP in cancer cells renders them insensitive to Fas ligand or TRAIL. Bax or Bak expression is significantly reduced in many cancers and confers worse prognosis (26). Low expression of Apaf-1 (a proapoptotic molecule), probably through methylation, is associated with chemoresistance (27). Equally, down-regulation of the death signaling molecule Fas-associated protein with death domain and caspase-8 has been shown in several cancers (28, 29). As expected, up-regulation of inhibitors of apoptosis (antiapoptotic molecules that inhibit the effector caspases) is overexpressed in tumor cells (30, 31).

Therapeutics with Immunopotentiating Profile

With an understanding of the potential role of the cellular immune system in detecting and killing cancer cells, and insight into how cancer cells avoid death from the immune system, pharmacologic interventions may be designed to sensitize cancer cells to immune attack. Table 1 provides the features that we feel would make up an ideal immunosensitizing drug. It should potentiate the immune effector cell recognition of cancer cells and shift the balance in the cancer cell toward a proapoptotic milieu. Of particular importance, potential immunosensitizing drugs must not be cytotoxic against nor inhibit critical functions of immune cells.

Decreasing tumor-induced immune suppression. Secreted proteins released by tumors that lead to immune escape can be targets to monoclonal antibody blockade. For example, the anti–VEGF antibody bevacizumab, in addition to its effect on tumor vasculature, may decrease VEGF–induced inhibition of DC and T-cell function (32). Monoclonal antibodies to transforming growth factor-β and IL-10 are also potential means to reverse immune escape induced by tumor-released molecules. In addition, anti-inflammatory drugs such as aspirin and other nonsteroidal anti-inflammatory drugs, including the specific cyclooxygenase-2 inhibitor celecoxib, could be means to inhibit cyclooxygenase-2 that leads to prostaglandin E2 production (33).

Depletion of immunosuppressive cells. T regulatory cells, myeloid suppressor cells, and IDO-positive plasmacytoid DC have emerged as major immunosuppressive cells reported to be implicated in escape of tumors from immune control (34, 35). Depletion of CD4/CD25/FoxP3-positive natural T regulatory cells can be achieved in mice using anti-CD25 antibodies, which leads to improvement in responses to immunostimulating approaches such as CTLA4 blockade (36). There is much interest in developing means to deplete human T regulatory cells, although no compelling approach has yet been developed. The available antibodies to human CD25 approved by the Food and Drug Administration are immunosuppressive as opposed to immunostimulating, and there are mixed reports of an understanding of the potential role of the cellular immune system in detecting and killing cancer cells, and insight into how cancer cells avoid death from the immune system, pharmacologic interventions may be designed to sensitize cancer cells to immune attack. Table 1 provides the features that we feel would make up an ideal immunosensitizing drug. It should potentiate the immune effector cell recognition of cancer cells and shift the balance in the cancer cell toward a proapoptotic milieu. Of particular importance, potential immunosensitizing drugs must not be cytotoxic against nor inhibit critical functions of immune cells.

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Increase activating ligands for immune effector cells. Drugs that mediate epigenetic mechanisms, such as the demethylating
agents and the histone deacetylase inhibitors, have multiple effects on gene transcription. They tend to promote a pro-apoptotic phenotype, can up-regulate MHC and tumor antigen expression, induce “stress” molecules recognizable by NK-activating receptors such as NKG2D, and increase expression of surface death receptors (38). The ability of this classes of novel agents to increase the surface expression of antigen-presenting MHC molecules (HLA molecules in humans) and at the same time increase expression of tumor antigens, such as the MAGE family of cancer-testis antigens, may allow these agents to overcome a major mechanism of tumor escape to cellular immunotherapy (16).

Decreased inhibitory ligands for immune effector cells. Peptides derived from proteasomal degradation are required for assembly of MHC molecules in the endoplasmic reticulum (39). This would suggest that ubiquitin ligase inhibitors and proteasome inhibitors may decrease the pool of peptides able to support assembly of surface MHC class I molecules. Increased expression of surface MHC class I allows cancer cells to escape from CD8+ T-cell control, but at the same time, they become more sensitive to NK cell recognition and killing. NK cells are effector cells of the innate immune system, which sense the loss of surface MHC molecules (frequent after viral infections and in cancer cells) or the presence of foreign MHC molecules (as occurs after organ transplants), which trigger these potent cytotoxic cells. This possibility has been tested in an experimental model of melanoma, suggesting that the proteasome inhibitor bortezomib increased TNF-α-mediated killing by immune cells (40).

Direct modulation of apoptotic pathways. Death receptors are on the outer surface of cells and therefore accessible to monoclonal antibodies. TRAIL receptors seem to be preferentially expressed by cancer cells (and activated lymphocytes), whereas Fas and TNF-α receptors are expressed more promiscuously. A soluble TRAIL ligand construct and TRAIL receptor-activating antibodies are in clinical development for the treatment of cancer. These approaches may be viewed as bypassing the need for immune effector cells but would be unlikely to lead to sustained tumor regression without chronic dosing. It is possible that such modulation of TRAIL receptors may synergize with immunotherapy and other targeted therapy approaches. Histone deacetylase inhibitors have been shown to up-regulate the TRAIL death receptor DR5, leading to sensitization of cancer cells to TRAIL-induced death (41, 42), and therefore would be logical drugs to use in combination with

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**Table 1. Ideal attributes of targeted immunosensitizing drugs**

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<thead>
<tr>
<th>Effects on tumor cells</th>
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<td>Specifically target a key oncogenic pathway</td>
<td>Increase death receptor expression</td>
<td>Increase proapoptotic molecules</td>
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<tr>
<td>Increase expression of tumor antigen or NK-activating receptors</td>
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<th>Effects on immune system cells</th>
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<tr>
<td>No cytotoxic effects on immune system cells</td>
<td>No interference with T-cell receptor, NK receptor, or costimulatory signal transduction pathways</td>
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both TRAIL-engaging reagents and immune effector cells. Furthermore, the demethylating agent decitabine has been shown to sensitize choroidal melanoma cell lines to the cytotoxic effects of IFNs by inducing the expression of proapoptotic and growth-inhibitory genes (43).

The antiapoptotic members of the Bcl-2 family are potential targets for immunosensitizing drugs. Bcl-2 is up-regulated in many cancers (44), and inactivating its function may result in a proapoptotic cancer cell milieu facilitating the cytotoxic effect of immune cells. Small-molecule inhibitors of Bcl-2 family members (45) have obvious pharmacologic advantages over Bcl-2 antisense oligonucleotides (46) and may be logical compounds to be used in combination with immunotherapy. Other proteins in the apoptotic pathways also represent potential targets for immunosensitizing agents. An inhibitor of apoptosis inhibitor sensitized cells to TRAIL (47), making it a reasonable drug to combine with immunotherapy. Apoptotic pathways may also be targeted by altering transcription of regulators of apoptosis. Inhibition of nuclear factor-κB in melanoma sensitizes cells to TRAIL-induced (but not Fas or TNF-α) apoptosis (48). An additional approach would be the use of the proteasome inhibitor bortezomib, which is known to inhibit nuclear factor-κB and down-regulate the antiapoptotic molecule FLIP (49).

**Alteration of Oncogenic Pathways Leading to Immune Sensitization**

Many interventions that damage cancer cells lead to a proapoptotic milieu. Carefully sequenced treatment with cytotoxic drugs or radiation therapy leading to DNA damage stimulates DNA stress pathways, which can lead to immune sensitization (50, 51). In cells that have functional p53, DNA damage is sensed by p53, resulting in increased transcription of death receptors (52). Although the adverse effects of standard chemotherapy agents and radiation therapy, which are non-specific genotoxins, on immune system cells make combinations of chemotherapy and immunotherapy a rather poor match, when adequately sequenced there may be situations when cytotoxic therapy can be efficiently delivered to improve on the antitumor activity of immunotherapy (51, 53).

Novel targeted therapies that disrupt dominant oncogenic signals in cancer cells may result in increased sensitivity to immune responses. There is a lot of interest in finding ways to combine tyrosine kinase inhibitors, such as sorafenib and sunitinib, with immunostimulating cytokines in the treatment of renal cell carcinoma. A careful assessment of the effects on the tumor, tumor microenvironment, and the immune system will be required to define optimal combinations. Several clinical trials are ongoing where these multitargeted tyrosine kinase inhibitors are tested in combination with immunostimulating agents, such as IL-2, IL-21, anti-CTLA4 antibodies, and poxvirus vaccines, in patients with metastatic renal cell carcinoma and melanoma.

Inhibition of constitutively activated signal transduction pathways in cancer cells also has the potential to decrease tumor escape from immunotherapy. For example, loss of the tumor suppressor PTEN in glioblastoma cells leads to immune escape in part by increased translation of the immune suppressor surface receptor B7-H1 (CD274, also known as programmed death ligand 1). Treatment of these cells with an Akt inhibitor (which blocked the Akt tyrosine kinase constitutively activated in PTEN-deficient cells) reversed immune escape of glioblastoma cells (54). However, in addition to its key role in oncogenesis, the phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin signaling pathway is key to many lymphocyte functions. If targeted drugs blocking this signal transduction pathway are to be used as immune sensitizers, they will need to be carefully scheduled or will need to show a high therapeutic window to have the required effect on cancer cells without suppressing lymphocyte functioning.

The signal transducer and activator of transcription (STAT) proteins transmit cytoplasmic signals from polypeptide cytokines and growth factors that have receptors with intrinsic or associated tyrosine kinase activity. They have become promising molecular targets for cancer because they are persistently activated in many cancers due to oncogene signaling through STATs, especially STAT-3 and STAT-5 (55). These two STAT proteins have key roles in regulating cell proliferation, resistance to apoptosis, and sustained angiogenesis (55). In addition, STAT-3 has an important role in the immune system. Inhibiting STAT-3 in immune system cells increased DC, T-cell, and NK cell function (56). Small-molecule inhibitors are being developed to block STAT-3 and would therefore have the potential of simultaneously attacking cancer cells and increasing immune system function (56).

**Issues About the Testing of Targeted Therapy-Immunotherapy Combinatorial Approaches**

Despite the promise, there are several practical limitations to the combined testing of new agents in preclinical or clinical development and tumor immunotherapy approaches. Preclinical testing of a potentially immunosensitizing pharmacologic agent and immunotherapy is best done in fully immunocompetent animal models. *Ex vivo* cell culture systems cannot fully recapitulate a functional immune system and may provide biased results, where potential adverse effects of the drug on immune system cells may not be detected. However, implantable murine tumors may not be an adequate model for human cancer because the oncogenic events that drive the cancers and the relation between the cancer cells and the tumor milieu may not be the same as in spontaneously arising tumors. A notable example is the frequently used B16 murine melanoma, which does not seem to have a constitutively active mitogen-activated protein kinase pathway, a marked difference with human melanomas where mutually exclusive activating mutations on Nras of Braf are prevalent. The use of human tumor xenografts requires implantation into severe immunodeficient mice, obviously devoid of an immune system. Regeneration of a human immune system in these mice is technically challenging, and current approaches may not provide a fully functional immune response to test immune sensitization approaches.

Another important limitation to the testing of the immune sensitization combinatorial approach is access to adequate compounds. It is widely known that intellectual property issues limit the availability of emerging new drugs, and the testing of combination regimens is frequently explicitly prohibited by the manufacturers of the novel agents. Because the combination is only relevant if there is a fully functioning immune system, the concept frequently needs to be tested in a living animal. The doses required for treatment in animal models are much higher
than the amount of agent that would be sufficient for in vitro testing, so the immune sensitization combinatorial approach is unlikely to be tested unless the manufacturer provides enough agent and explicit permission for combined agent testing. Alternatives such as using a published chemical structure to synthesize the agent de novo are likely to be limited by the high costs of producing large amounts for testing in animal models. Therefore, this approach usually requires a material transfer agreement with the manufacturer of the agent, with its resulting delay and limitations for combination testing. Testing in the clinic is also subjected to practical limitations. The same issues related to the intellectual property of a combination described for the preclinical testing apply to the clinic. Even when permission is obtained to test a new combination for which there is strong scientific rationale, the combined testing may be delayed until the safety, toxicity, and antitumor activity of each agent has been thoroughly tested independently.

Despite all of these limitations, it is likely that in the next several years the number of articles describing combinations of novel targeted agents with established or new immunotherapy approaches will continue to increase. This will be greatly facilitated once the novel agents have proven their benefit in certain cancers and become standard of care therapies, as exemplified by the ongoing clinical testing of multitargeted tyrosine kinase inhibitors and cytokines. At some point, there may be a need for prioritization of the combinations. It is clear that combinations successfully tested in animal models for which there is mechanistic understanding of how they potentiate each approach will be likely to be tested first. In particular, pharmacologic means to affect tumor escape from cellular immunotherapy would be a high priority for clinical testing.

Conclusions

Improvements in our understanding of the antiapoptotic and immune escape mechanisms activated by oncogenic events, and the ability to specifically alter them with targeted drugs, will result in novel approaches allowing rational combinations of drugs with immunostimulating effects. These combinations would bring together the elegance of targeted therapy for cancer with the ability of the immune system to lead to long-term regressions in some patients with metastatic cancers.

Disclosure of Potential Conflicts of Interest

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