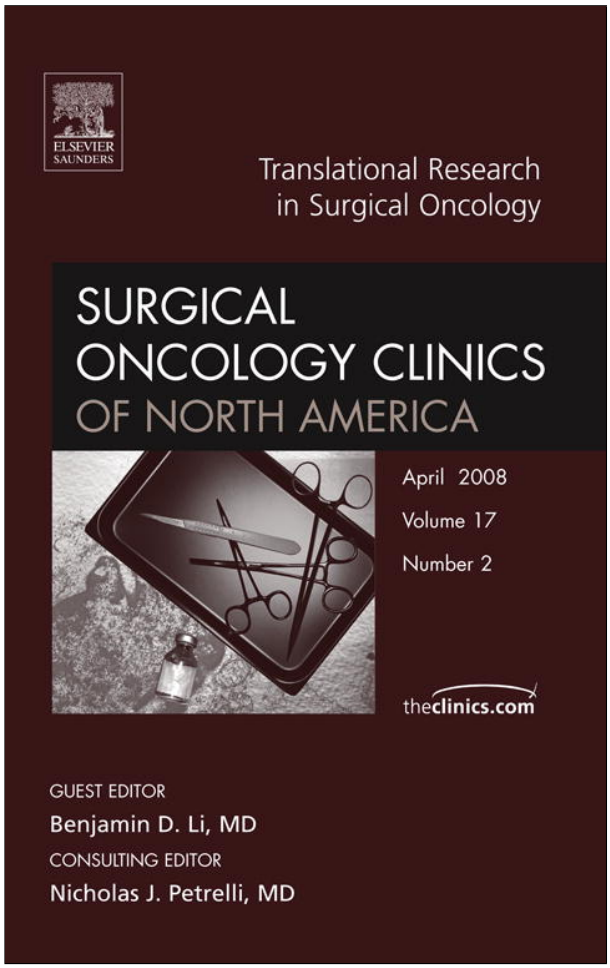

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# Translational Research in Melanoma

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Current treatment of malignant melanoma exemplifies not only the need for translational research but also many of the frustrating challenges of moving from the bench to the bedside. Melanoma remains somewhat unique among solid tumors in that its treatment, at early stage or advanced disease, primarily is surgical. Although adjuncts may play a role in some situations, radiation is of limited benefit and, more importantly, chemotherapy has been uniformly disappointing in adjuvant and metastatic settings. This leaves clinicians with few viable options for reducing the chance of recurrence after surgery and for treating unresectable disease. This fact is even more sobering when considering the rate at which malignant melanoma is increasing in the United States and worldwide. It is with this cost in mind that there has been a fervent attempt to identify novel approaches to melanoma therapy and rapidly translate these therapeutic approaches to clinical use.

Unfortunately, there are several obstacles to translational research in melanoma. Animal models of melanoma are limited in their translatability to the human model, particularly regarding carcinogenesis. Despite the widespread use of the murine B16 melanoma cell line in preclinical studies, it is a model for transplantable tumors, which differ inherently from spontaneously arising melanomas, limiting translatability, particularly when studying immunotherapy. Further, although there is a wealth of promising preclinical data focusing on the unique relationship between melanoma and the immune system, immunotherapy has yet to be translated to the clinical setting successfully. There even has yet to be a randomized trial demonstrating an overall survival benefit to melanoma vaccines. Areas of research that are making great strides in other solid tumors, such as tumor markers or genomic analysis, are hampered in melanoma not only by differences in

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biology but also by the limited availability of fresh primary tumor specimens for study.

Notwithstanding these challenges, recent studies have provided much-needed insight into melanoma biology, prompting exciting areas of clinical research into the prevention and treatment of melanoma, including targeted therapies and new approaches to immunotherapy.

## Chemoprevention of melanoma

Although the majority of translational research in melanoma focuses on therapy, increasing knowledge of the genetic and molecular events responsible for melanoma development has prompted a search for strategies to prevent melanoma. UV radiation likely is the primary initiating event in most melanomas, causing direct DNA damage through signature mutations (C→T and CC→TT) that have been documented in genes associated with melanocytes transformation, such as the oncogenes NRAS or BRAF or the tumor-suppressor gene CDKN2A [1–4]. Beyond malignant transformation, UV radiation also causes damage to nonmelanocytic cells (keratinocytes and dendritic cells [DCs]). This can lead to the loss of local control and suppression of the local immune response, contributing to the subsequent formation and proliferation of melanoma [1,5]. Strategies to preventing melanoma address some or all of the events related to UV radiation, including inducing apoptosis of DNA-damaged melanocytes or nonmelanocytic cells, enhancement of DNA repair, and enhancement of local immune responses [6].

The design and implementation of chemoprevention strategies face several unique challenges compared with therapeutic strategies. First, the agents of choice must have little to no toxicity; otherwise, there is poor compliance [7]. The use of a single agent may not be ideal; combinations of agents with a broad spectrum of anticarcinogenic mechanisms likely are needed given the heterogeneity of the carcinogenesis process. Finally, translating chemoprevention from bench to bedside is challenging (for reasons discussed previously). Animal models of UV-induced melanoma do not mimic human melanoma development completely, although the newly described transgenic hepatocyte growth factor/scatter factor mouse model seems most characteristic of human melanoma [8]. The ultimate goal, to demonstrate the ability of these interventions to prevent melanoma in prospective randomized trials, will be extremely costly, as it will require large populations to be studied for long periods of time. Alternatively, researchers will need to identify and validate surrogate endpoint biomarkers [9].

Despite these challenges, several agents have emerged as candidate chemoprevention agents in melanoma, some of which are summarized in Table 1. Several of these agents occur naturally. Curcumin is the major yellow pigment extracted from turmeric, a spice used commonly in India and Southeast Asia. Resveratrol is found in grapes, mulberries, and peanuts.

Table 1
Agents proposed for chemoprevention of melanoma

| Agent | Mechanism | Potential | Side effects |
|---|---|---|---|
| Statins | Inhibition of protein prenylation, preventing post-translational modification of the Ras superfamily, diminishing their oncogenicity | Strong preclinical data. Epidemiologic studies fail to demonstrate a significant decrease in melanoma among statin users. | Small risk for myositis but otherwise good side-effect profile, including cardiovascular benefit |
| Curcumin | Multiple, including antioxidant, inhibition of tyrosine kinases, COX and lipoxygenase inhibition, suppression of NF-κB | Good preclinical data in B16 melanoma and UV-induced carcinogenesis. Limited absorption and rapid metabolism are limiting factors. | Topical: yellow staining of skin<br>Oral: excellent tolerance |
| Reservatrol | Scavenging of free radicals, suppression of NF-κB, COX inhibition | Topical administration shows several mechanisms of action in preclinical data. Oral administration still being studied | Minimal |
| Epigallocatechin-3-gallate | Protection against UV-induced COX activity and prostaglandin production. Free radical scavenger. Increase in IL-12 and augmented immune response. | Topical administration inhibits tumor formation in mice. No strong evidence in melanoma, although people have used green tea for chemoprevention of other cancers for many years. | Little to no side effects |
| Silymarin | Scavenging of free radicals, suppression of NF-κB, COX inhibition. Inactivation of PI3K-Akt and MAPK signaling. | Most data in nonmelanoma skin cancers. Limited preclinical data for melanoma. | Excellent tolerance |
| Sorafenib | Inhibition of Raf kinase and receptor tyrosine kinases leading to apoptosis of melanoma cells | Promising data on treatment of melanoma. Associated pathways are appealing targets for chemoprevention but limited data. | Significant for chemoprevention, including rash and hypertension. |
| COX-2 inhibitors | COX inhibition, prevention of UV-induced prostaglandin production | Promising mouse data. Case control studies suggest a decreased incidence of melanoma in patients on a COX inhibitor. | Significant cardiac risk precluding use |
| Retinoids | Induction of apoptosis by binding of RAR or RXR in nuclei and interference of transcription factors controlling differentiation and proliferation | Shown effective in nonmelanoma skin cancers. Limited data on melanoma; however,<br>because melanoma is relatively apoptosis resistant, there is promise. | Need careful monitoring for hypercholesterolemia, hypertriglyceridemia, increased LFTs, myalgias/arthralgias, and dry skin and mucous membranes. |

Epigallocatechin-3-gallate is the major constituent of green tea. As understanding of melanocyte biology and carcinogenesis improves, more agents likely will be identified as potential instigators. A dedicated effort by basic scientists and clinicians will be necessary to move forward and potentially prevent the development of melanoma in high-risk populations.

## Targeted therapies in melanoma

### *Inhibitors of signal transduction*

Recent studies have shed light on the cumulative genetic and molecular events that result in neoplasia. Transforming mutations in oncogenes and loss of heterozygosity of tumor suppressor genes have been a major focus of target molecular therapies. Transforming mutations in oncogenes can have an impact on pathways that regulate cellular proliferation, differentiation, cell cycle control, and apoptosis. Two such pathways have garnered significant interest in the field of melanoma biology: the RAS– mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide kinase-3 (PI3K)/AkT pathway.

The MAPK signal transduction pathway has been of significant interest since the discovery of frequent mutations of BRAF kinase. MAPK signaling begins when receptor tyrosine kinases bind with their ligand, which transmits activation signals via the RAS GTPase on the cell inner surface (Fig. 1). Once activated, RAS can bind several effector proteins, the best

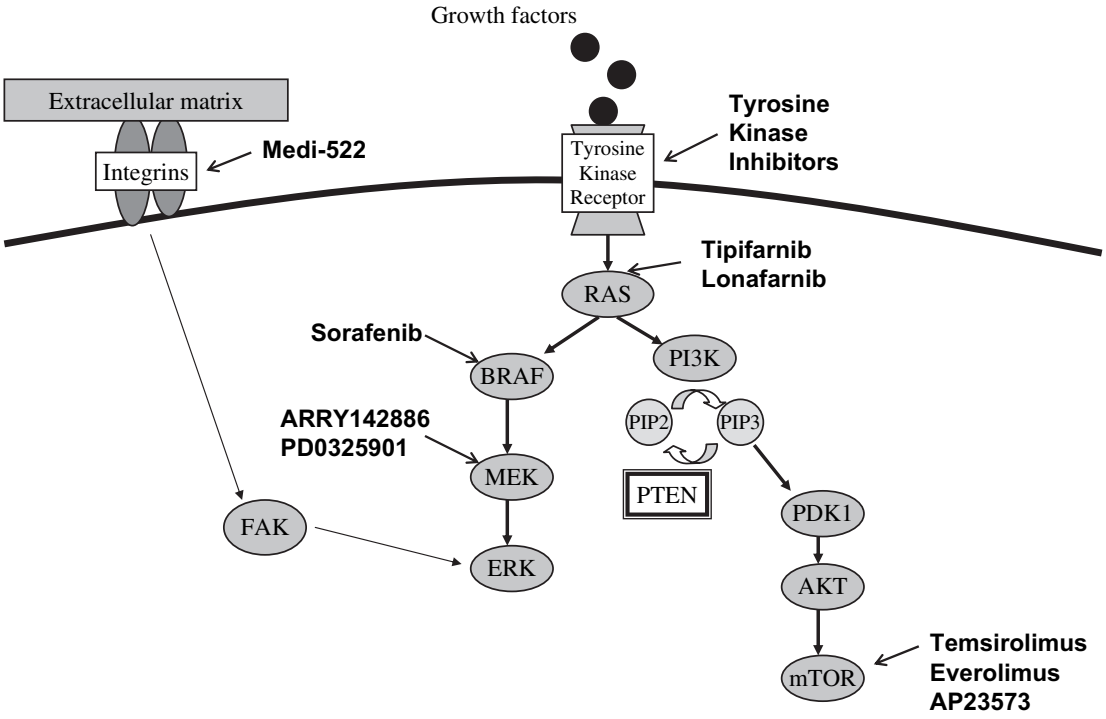


Fig. 1. The MAPK signal transduction pathway.

characterized of which are RAF and PI3K. The RAF family of kinases (A-RAF, BRAF, and C-RAF) links RAS and the MAPK pathway. Once activated, RAF phosphorylates and activates MAPK/eukaryotic protein kinase (EPK) kinase, which then activates extracellular signal-regulated kinase (ERK). ERK also can be activated when integrins on the cell surface adhere to the extracellular matrix, activating focal adhesion kinase. ERK relays multiple proliferative or survival signals through phosphorylation of a variety of targets in the cytoplasm and nucleus. Aberrant activation of this pathway results in immortalization, growth factor–independent growth, ability to invade and metastasize, and avoidance of apoptosis. Therapeutic targeting of this pathway is approached from many angles. Inhibitors of RAS-MAPK signal transduction include farnesyl transferase inhibitors that interfere with the translocation of RAS to the cell membrane. Direct inhibitors of RAF, such as sorafenib (BAY 43-9006), or of MEK, such as ARRY142886/AZD6244 and PD0325901, are alternative inhibitory mechanisms.

Sorafenib is a RAF tyrosine kinase inhibitor that inhibits the MAPK pathway. It targets the ATP-binding site of the kinase and, at low concentrations, inhibits wild-type and mutant BRAF and other tyrosine kinase receptors, including vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3, c-kit, and platelet-derived growth factor receptor β (PDGFR-β). It is Food and Drug Administration approved in the treatment of advanced renal cell cancer, with the most common toxicities diarrhea, rash, and hand-foot syndrome [10]. As a single agent, sorafenib has not shown significant activity in metastatic melanoma, but in combination with chemotherapy, it is associated with an improvement in response rate and progression-free survival. Based on these responses, an Eastern Cooperative Oncology Group (ECOG) phase III trial was initiated to assess carboplatin and paclitaxel with or without sorafenib. Sorafenib also is being examined in combination with other targeted agents. Other inhibitors that target mutant BRAF specifically also are in clinical trial as are agents that inhibit other targets within the MAPK pathway (Table 2).

Another pathway involved in cell survival is the PI3K pathway, which is altered in a variety of human tumors. After activation through RAS, phosphorylation of PIP2 to PIP3 occurs. This ultimately leads to the activation of Akt (protein kinase B). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) serves to negatively regulate the PI3K pathway by dephosphorylating PIP3. Akt, once activated, phosphorylates several targets that control cell survival, proliferation, and invasion. At least 13 substrates for Akt are recognized and can be divided into two main subgroups: regulator of apoptosis and regulators of cell growth, protein synthesis, and cell cycle regulation. The PI3K pathway represents another set of targets for therapeutic intervention. A popular target is the mammalian target of rapamycin (mTOR). mTOR is a serine-threonine kinase that functions downstream of Akt. Members of the TOR subfamily are inhibited by rapamycin, a macrolide antibiotic with immunosuppressive properties.

Table 2
Targeted therapies in clinical trials for melanoma

| Agent | Company | Targets | Mechanism of action |
|---|---|---|---|
| 17-AAG | | HSP90 | Disrupts HSP 90 complexes |
| Aprinocarsen (Affinitak, ISIS 3521) | ISIS (Carlsbad, CA) | PKC-α | Antisense oligonucleotide |
| ARRY-1422886 | Array BioPharma (Boulder, CO) | MEK | Tyrosine kinase inhibitor |
| AZD2171 | AstraZeneca (Wilmington, DE) | VEGFR, PDGFR, c-KIT, FGFR | Tyroskine kinase inhibitor |
| AZD6244 | AstraZeneca (Wilmington, DE) | MEK | Tyrosine kinase inhibitor |
| Bevacizumab (Avastin) | Genentech (San Francisco, CA) | VEGF | Monoclonal antibody |
| Bortezomib (PS-341, Velcade) | Millenium (Cambridge, MA) | Cell cycle regulatory proteins | Proteasome inhibitor |
| Cilengitide (EMD 121974) | | Integrin | Angiogenesis inhibitor |
| CNTO 95 | Centocor (Horsham, PA) | αV integrin | Monoclonal antibody |
| Everolimus (RAD001) | Novartis (Basel, Switzerland) | PI3K/AkT/PTEN | mTOR inhibitor |
| Imatinib (STI-571, Gleevec) | Novartis (Basel, Switzerland) | c-KIT | Tyrosine kinase inhibitor |
| Marimastat (BB2516) | | Matrix metalloproteinases (MMPs) | MMP inhibitor |
| MEDI-522 (Abergrin, Vitaxin) | MedImmune (Gaithersburg, MD) | αVβ3 integrin | Monoclonal antibody |
| Oblimersen | | Bcl-2 | Antisense oligonucleotide |
| PD0325901 (CI-1040) | Pfizer (New York, NY) | MEK | Tyrosine kinase inhibitor |
| PI-88 | Progen (Queensland, Australia) | VEGF, FGF 1, FGF 2 | Growth factor inhibitor |
| RAF-265 (Chir 265) | Novartis (Basel, Switzerland) | BRAF, CRAF, VEGFR | Tyrosine kinase inhibitor |
| Sorafenib (Bay 43-9006, Nexavar) | Bayer (West Haven, CT) and Onyx (Richmond, CA) | BRAF, CRAF, PDGFR-β, VEGFR, FGFR, c-KIT, FLT-3, RET | Tyrosine kinase inhibitor |
| Semaxanib (SU5416) | | VEGFR-1 | Tyrosine kinase inhibitor |
| Sunitinib (SU011248, Sutent) | Pfizer (New York, NY) | VEGFR, PDGFR, FLT-3, c-KIT, FGFR 1, RET | Tyrosine kinase inhibitor |
| Temsirolimus (CCI-779) | Wyeth-Ayerst (Madison, NJ) | PI3K/AkT/PTEN | mTOR inhibitor |
| Tibifarnib (R115777, Zarnestra) | Johnson & Johnson (New Brunswick, NJ) | RAS | Farnesyltransferase inhibitor |
| UCN-01 | | PDK-1, Chk1 kinase | PKC-α/β/γ inhibitor |

*Abbreviations*: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HSP, heat shock protein; PDGFR, platelet derived growth factor receptor; PKC, protein kinase C; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Temsirolimus (CCI-799) is an ester derivative of rapamycin, which inhibits mTOR kinase activity, which is responsible for the translation of proteins required for progression through the cell cycle. Preclinical investigation of CCI-779 demonstrates growth inhibition in multiple solid tumor lines and animal models. In phase I clinical trials, dose-limiting toxicities included myelosuppression, diarrhea, stomatitis, fatigue, dermatitis, and hyperlipidemia [11]. As a single agent, temsirolimus did not have enough activity to warrant further testing as a single agent [12]. Several ongoing studies are examining its potential when combined with sorafenib, bevacizumab, or other targeted agents. Several other mTOR inhibitors, such as everolimus (Novartis) and AP-23573 (Ariad Pharmaceuticals, Cambridge, Massachusetts), are in phase I and II trials currently open to accrual.

### *Antiapoptotic therapies*

One of the consequences of constitutive MAPK and PI3K/AkT signal transduction is the deregulation of apoptosis and tissue homeostasis. Drug resistance in melanoma also is attributed partially to overexpression of Bcl-2, an antiapoptotic protein that locks the release of cytochrome C [13]. Cells transfected with Bcl-2 demonstrate multidrug-resistant phenotype multiple tumor lines. Reciprocal targeted inactivation of Bcl-2 augments chemotherapeutic responses in in vivo models [14].

Oblimersen sodium is an 18-base phosphorothioate antisense oligonucleotide that binds the first six codons of the Bcl-2 mRNA open reading frame and mediates RNA cleavage by RNase H, thus degrading the message. In xenotransplantation models for human melanoma, down-regulation of Bcl-2 concentration has been observed with the administration of oblimersen and dacarbazine (DTIC) [15]. A phase I/II clinical trial was performed in patients who had metastatic melanoma. Toxicities included fever, liver function abnormalities, rash, and lymphopenia. One complete response and two partial responses were observed [16]. A phase III trial in 771 patients was performed in which randomized patients received DTIC alone or DTIC plus oblimersen given by intravenous infusion. Overall survival was not statistically significant between the two groups (9.1 months for combination group versus 7.9 months for DTIC alone), but the overall response rate favored the combination arm (11.7% versus 6.8%, $P = .019$).

Another target of apoptosis is nuclear factor κB (NF-κB). Its constitutive activation is associated with impaired apoptosis in a variety of tumor lines. In healthy individuals, NF-κB regulates the expression of genes involved in normal immunologic responses. Persistent activation of NF-κB, however, inhibits apoptosis and promotes proliferation leading to hyperplasia. Boronic acid–-erived compounds inhibit the proteosome pathway, which helps to control NF-κB degradation. In the animal models, a combination of a proteosome inhibitor, bortezomib, and temozolimide significantly reduced tumor growth [17]. A phase II study of bortezomib in 27 patients was

terminated because of lack of clinical responses [18]. Six patients achieved stable disease although four of the six patients were removed from the study for significant toxicities. Median time to disease progression was 1.5 months; however, median overall survival was 14.5 months. Although bortezomib was not effective as a single agent, a phase II trial of combination carboplatin and paclitaxel with bortezomib is ongoing, based on the previous animal models.

*Antiangiogenic therapy*

Neoangiogenesis is crucial for continued neoplastic proliferation and metastasis. The development of new vessels is a key step for neoplastic progression. Several proangiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (FGF), and transforming growth factor-beta, are produced by tumor cells. Endothelial cells express a family of tyrosine receptor kinases that bind VEGF with high affinity and stimulate several signaling pathways that induce endothelial cell mitosis, migration, and neoangiogenesis [19]. These growth factors serve as potential targets for molecular therapies.

Bevacizumab (Avastin) is a recombinant humanized monoclonal antibody to VEGF that has demonstrated efficacy in patients who have colorectal cancer and improves response and survival when combined with chemotherapy [20]. A phase II randomized trial of bevacizumab with or without low-dose interferon in patients who had stage IV melanoma demonstrated two responses (one complete and one partial) and four prolonged stabilizations of disease [21]. There was no observed added benefit of the low-dose interferon. Additional trials in melanoma are examining bevacizumab in combination with chemotherapy, high-dose interferon, or other targeted therapies. Another agent targeting VEGF activity is SU5416, an inhibitor of VEGFR-1 tyrosine kinase. Although only 1 of 31 patients in a phase II trial in pretreated stage IV melanoma patients had a response, MRI showed a significant decrease in tumor perfusion. There may be a role for SU5416 in combination with chemotherapy.

Thalidomide is a synthetic glutamic-acid derivative first manufactured in the 1950s as a sedative but was associated with phocomelia and withdrawn from the market. Judah Folkman first elucidated the effect of thalidomide on angiogenesis, showing that thalidomide inhibits basic FGF–induced angiogenesis. Despite the subsequent intensive study of thalidomide as an antiangiogenic agent, the precise mechanism of its activity remains unknown [22]. In addition to its antiangiogenic properties, thalidomide has immunomodulating properties. It costimulates T cells that have been activated partially by the T-cell receptor (TCR) and inhibits monocyte-derived tumor necrosis factor $\alpha$ [23,24]. Thalidomide has been studied as a single agent or in combination with temozolimide in patients who have metastatic melanoma. No objective responses were seen in a phase II clinical trial of

14 patients who had metastatic melanoma [25], but more favorable results were seen in a study of stage IV melanoma patients who had brain metastases using thalidomide in combination with temozolimide. Three of 15 patients who had evaluable disease demonstrated complete or partial responses. Minor responses or stable disease was observed in seven additional patients [26]. Unfortunately, and despite initial encouraging results, the therapy was associated with significant adverse events, most notably a high rate of thromboembolic events [27].

Not long after the recognition of the antiangiogenic properties of thalidomide, efforts were made to synthesize thalidomide analogs that had fewer side effects than the parent compound. Immunomodulatory drugs (IMiDs) are compounds based on the thalidomide structural backbone. Second- and third-generation IMiDs, including CC 5013 (Revlimid) and CC 4047 (Actimid), exert similar antiangiogenic activity with varying levels of immunomodulating activity. A large phase III trial of CC 5013 versus placebo as second-line therapy in patients who had advanced melanoma was terminated secondary to inactivity of the study drug.

Other targets of antiangiogenic therapies are integrins. The integrin αVβ3 can act as the vitronectin receptor, and it seems to play a critical role in melanoma growth and further metastasis. This integrin is specific for tumor-associated vasculature and is required for melanoma cell survival. Integrin αVβ3 is up-regulated by VEGF and β-FGF. It is expressed on a large percentage of cancers, including melanoma, but not by normal melanocytes. Its expression in melanoma primary lesions increases as they progress from the horizontal to vertical growth phases, and tumors from stage IV melanoma patients seem to express the integrin more intensely [28]. MEDI-522 is a humanized form a murine monoclonal antibody to integrin αVβ3. A phase II study comparing MEDI-522 (8 mg/kg per week) with or without DTIC (1000 mg/m$^2$ once every 3 weeks) in patients who had metastatic melanoma demonstrated no objective responses in the single therapy arm. The combination with DTIC demonstrated a 13% response rate compared with no responses for the single agent [29]. Without a DTIC alone arm, it is impossible to draw any conclusions, and there were two deaths possibly attributed to MEDI-522, which raises safety concerns.

## Immunotherapy of melanoma

For several decades, vaccines have represented the holy grail of melanoma therapy, a quest driven by frustration with standard chemotherapy and clear evidence of the immune system's ability to recognize and eradicate melanoma. Immunotherapy represents the ideal therapeutic, a natural response that can be initiated in an outpatient setting, has minimal side effects, and has long-lasting memory. Conceptually, there are three criteria necessary for the effective generation of an antitumor response, and they seem within our grasp: the generation of sufficient number of cells with highly

avid recognition of tumor antigens, appropriate homing of these cells to the tumor targets, and appropriate activation of these cells. Thus, a plethora of vaccines has been developed to optimize the generation of tumor avid lymphocytes, delivered with various types of adjuvants to increase the immunogenicity.

Despite multiple phase I and II trials showing promise (Table 3), phase III trials have been disappointing (Table 4). There are, unfortunately, many hurdles to successful translation of vaccine therapies from preclinical studies to clinical use. One difficulty is the need to balance immunogenicity with feasibility. Approaches that use peptides or allogeneic cells are advantageous in that the treatment easily is standardized and distributed, but these preparations seem to be less immunogenic. Using autologous tumor allows for a broader array of relevant antigens and can generate more powerful immune responses, but the approach is limited to patients who have harvestable tumor. Another problem is finding appropriate surrogates for phase I and II trials. There are limitations to the ability to measure and compare immune responses generated by vaccines accurately, and current surrogate endpoints, such as immunologic assays, tumor infiltration by effector cells, or even partial clinical responses, do not necessarily predict which approaches most likely will result in improved survival. It becomes difficult, therefore, to know which phase I and II data are most worthy of proceeding to prospective, randomized trials. Over the years, several vaccine strategies have been examined with variable results.

### *Peptide vaccines*

Growing melanoma-specific T-cell clones in vitro has allowed investigators to identify the major histocompatibility complex (MHC)-restricted peptide antigens they recognize [30]. Immunogenic peptides can arise from the genetic mutations that originally led to malignant transformation; they can be from proteins originally expressed on germ cells (which lack MHC molecules so the antigens are silent) but now are expressed openly on cancer cells; or they may be from proteins shared by cancer cells and normal cells. A major benefit of using peptide vaccines is the ability to standardize, mass produce, and test them effectively. As the immunogenicity of peptide antigens alone is weak, the peptides often are delivered to patients along with an immune adjuvant meant to induce inflammation and improve the immunogenicity, pushing the immune process toward immunity rather than tolerance. BCG and DETOX (detoxified Freund's adjuvant, composed of monophosphoryl lipid A and a purified mycobacterial cell-wall skeleton) are examples of adjuvants meant to cause a nonspecific inflammatory response that increases the likelihood of recognition of the administered peptide.

Unfortunately, peptide vaccines have several drawbacks when translated to clinical use. Even if the peptides are recognized, melanoma cells easily can escape recognition through antigenic modulation. Because a T cell's

Table 3
Clinical trials of vaccines in melanoma

| Author | Vaccine (adjuvant) |
|---|---|
| Single peptide vaccines | |
| Rosenberg [121] | gp100 (IFA, IL-2) |
| Cormier [122] | MART-1 (IFA) |
| Rosenberg [123] | gp100 (IFA, IL-12 or GM-CSF) |
| Powell [124] | gp100 (IFA) |
| Speiser [125] | MART-1 (IFA, CpG 7909) |
| Wang [126] | MART-1 (IFA) |
| Marchand [127] | MAGE-3 |
| Scheibenbogen [128] | Tyrosinase (GM-CSF) |
| Jager [129] | NY-ESO-1 (GM-CSF) |
| Khong [130] | NY-ESO-1 (IFA) |
| Phan [131] | gp100 (anti-CTLA4 mAb) |
| Ganglioside vaccines | |
| Guthmann [132] | GM3 (Neisseria meningitides outer membrane protein complex, Montanide ISA 51) |
| Livingston [133] | GM2 (BCG) |
| Chapman [134] | GM2 (KLH/QS-21) |
| Multipeptide or protein vaccines | |
| Chianese-Bullock [34] | 12, including MAGE-A1, MAGE-A10, gp100 (GM-CSF and Montanide ISA-51) |
| Pullarkat [35] | MART-1, gp100, tyrosinase (IFA, SD-9427 [progenipoietin]) |
| Weber [36] | gp100, tyrosinase (IFA, GM-CSF) |
| Lee [37] | gp100, tyrosinase (IFA, IL-12) |
| Slingluff [38] | gp-100, tyrosinase (THP, IL-2) |
| Atzpodien [39] | MART-1, gp100, tyrosinase (GM-CSF) |
| Sanderson [40] | gp100, MART-1, tyrosinase (Montanide ISA 51, anti-CTLA-4 mAb) |
| Marchand [41] | MAGE-3 protein (MPL + QS21) |
| Davis [42] | NY-ESO-1 protein (ISCOMATRIX) |
| Allogeneic tumor cells | |
| Belli [135] | IL-12 gene-modified allogeneic cells |
| Maio [136] | IL-4 or IL-2 gene-modified allogeneic cells |
| Das Gupta [137] | IL-2 gene-modified allogeneic cells |
| Morton [138,139] | Canvaxin (BCG) |
| Morton [77] | Canvaxin (BCG) |
| Chan [84] | Canvaxin (BCG) |
| Cassel [140] | Viral Oncolysate using Newcastle Disease Virus |
| Wallack [141] | VMO |
| Hersey [142] | VMCL |
| Autologous tumor cells | |
| Mahvi [143] | GM-CSF gene-modified autologous tumor cells |
| Kosomoto [144] | GM-CSF gene-modified autologous cells |
| Stingl [145] | IL-2 gene-modified autologous tumor cells |
| Moiseyenko [146] | Tag7/PGRP-s gene-modified autologous tumor cells |
| Soiffer [147] | GM-CSF gene-modified autologous tumor cells |
| Schreiber [148] | IL-2 gene-modified autologous tumor cells |
| Moller [149] | IL-7 gene-modified autologous tumor cells |
| Sun [150] | IL-12 gene-modified autologous tumor cells |
| Abdel-Wahab [151] | IFN-γ gene-modified autologous tumor cells |
| Veelken [152] | Autologous tumor cells with IL-2 secreting fibroblasts |
| Nawrocki [153] | Autologous tumor cells with allogeneic melanoma cells secreting IL-6 and SIL6R |
| Berd [74] | M-Vax |

*Abbreviations*: BCG, Bacillus Calmette-Guerin; GM-CSF, granulocyte macrophage colony stimulating factor; IFA, incomplete Freund's adjuvant; IFN, interferon; IL, interleukin; KLH, keyhole limpet hemocyanin; VMCL, vaccinia melanoma cell lysates; VMO, vaccinia melanoma oncosylate.

Table 4
Phase III randomized studies of melanoma vaccines

| Investigator | Study population | Treatment arms | N | Results |
|---|---|---|---|---|
| Mitchell | Stage III | High-dose interferon<br>versus<br>Melacine + low-dose interferon | 604 | No difference in survival [114] |
| Morton | Stage III | Canvaxin + BCG<br>versus<br>placebo + BCG | 1118 | Discontinued secondary to no effect at interim analysis |
| Morton | Stage IV resected | Canvaxin + BCG<br>versus<br>placebo + BCG | 670 | No significant difference in survival [115] |
| ECOG 4697 (group A) | Stage III or stage IV resected<br>HLA-A2+ | GM-CSF + peptide vaccine<br>versus<br>GM-CSF + placebo<br>versus<br>peptide vaccine + placebo<br>versus<br>placebo + placebo | 800 for group A and B | Closed to accrual<br>Results pending |
| ECOG 4697 (group B) | Stage III or stage IV resected<br>HLA-A2- | GM-CSF<br>versus<br>placebo | | Closed to accrual<br>Results pending |
| EORTC 18961 | T3-T4N0M0 | GM2-KLH/QS-21<br>versus<br>observation | 1350 | Closed to accrual<br>Results pending |
| Oncophage/antigenics | Stage IV | HSPPC-96<br>versus<br>physician's choice | 322 | No significant survival benefit in M1a patients [116] |

| | | | | |
|---|---|---|---|---|
| MEDAREX | Stage III or IV HLA-A*0201+ | MDX-010 (anti–CTLA-4 mAb) alone<br>Versus<br>MDX-1379 (gp100 vaccine) alone<br>versus<br>MDX-010 and MDX-1379 | 750 | Accruing patients |
| Sondak/Southwest Oncology Group | Stage IB, IIA | Melacine<br>versus<br>observation | 689 | No difference in survival [81] |
| Kirkwood/ECOG | Stage III | High-dose interferon<br>versus<br>GM2-KLH/QS-21 vaccine | 880 | Improved survival with HDI [117] |
| Wallack | Stage II | VMO<br>versus<br>placebo | 250 | No difference in survival [118] |
| Hersey | Stage IIB and III | VMCL<br>versus<br>placebo | | No difference in survival [119] |
| Voit | Stage III | NDV-lysate | 17 | No difference in survival [120] |

*Abbreviations*: BCG, Bacillus Calmette-Guerin; GM-CSF, granulocyte macrophage colony stimulating factor; HSPPC, heat shock protein peptide complex; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; VMCL, vaccinia melanoma cell lysates; VMO, vaccinia melanoma oncosylate.

recognition of an antigen depends on the presentation of that antigen on a specific MHC molecule, only in patients who have a specific human lymphocyte antigen (HLA) phenotype can a given peptide induce an immune response. For example, MART-1/Melan-A is a well-defined protein antigen expressed by 80% of melanomas. This peptide binds to HLA-A2, which is expressed by only approximately 45% of whites. Therefore, only 36% of patients (80% of 45%) possibly benefit from a vaccine comprised of MART-1/Melan-A [31]. Today, many vaccine trials are limited to patients who are HLA-A2 positive.

With these limitations in mind, newer approaches to increasing the immunogenicity of peptide vaccines include modifying the peptides by substituting amino acids (heteroclitic peptides) that increase the affinity of peptide binding [32,33], using multiple peptides [34–40], using entire proteins [41,42], or using anti-idiotype antibodies [43–52]. One of the most promising approaches to peptide vaccination seems to be the combination of tumor peptide antigens with heat shock proteins (HSPs). HSPs are highly conserved intracellular chaperone molecules that carry potentially immunogenic peptides. They are produced by cells in response to stress and when complexed with peptides, they readily are taken up by DCs for presentation to naïve T-cells [53–55]. The combination of tumor peptides and autologous HSPs results in cross-presentation with MHC-class I presentation of exogenous peptide by APCs, ultimately eliciting tumor-specific immunity [56–58]. HSP 70, HSP 90, and HSP 96 seem to play an important role in processing of antigen before being taken up by antigen presenting cells [59–61]. HSP-peptide complexes can be generated by fusing individual peptides to HSPs [60] or readily can be purified from individual tumors for use as a therapy [61,62]. In the latter case, it is conceivable that these complexes may represent the total set of processed peptides from a population of tumor cells, although using HSP purified from individual tumors has the same limitations as other approaches requiring autologous tumor. Autologous HSP peptide complex-96 (HSPCC-96 [Oncophage]) has been evaluated in several clinical trials that have demonstrated evidence of immune responses and objective responses [63–66], although a phase III trial of patients who had metastatic melanoma treated by Oncophage or physician's choice failed to demonstrate a clear benefit to the vaccine [67]. There is some suggestion of a survival benefit in M1a disease, and a second phase III trial for M1a and M1b disease is planned.

### *Cellular vaccines*

Accepting the inherent limitations of using a limited number of peptides, tremendous focus has been placed on using melanoma cells as the antigenic source. The use of autologous tumor theoretically ensures that all biologically relevant antigens are presented to the immune system. Autologous cellular vaccines, however, are a veritable poster child for the obstacles facing

immunotherapists. Although preclinical studies show these vaccines to be the most immunogenic, early attempts to use irradiated autologous tumor cells as vaccines had little success owing to the poor immunogenicity of native tumor cells themselves [68,69]. With the addition of adjuvants, the results improved, albeit modestly [70–72]. Markedly improved results were seen when Berd and colleagues [73] conjugated the hapten ditnitrophenyl to proteins on autologous tumor cells to increase the immunogenicity. A total of 77 patients who had clinically evident nodal metastases were given the vaccine (known as M-Vax) with BCG, in the adjuvant setting after lymphadenectomy. The investigators reported more favorable than expected 5-year relapse-free and overall survival rates (45% and 58%, respectively) [74,75]. An initial randomized trial was attempted; however, there were considerable difficulties with specimen transportation and vaccine manufacturing issues, illustrating the difficulties of doing large trials with autologous vaccines. A new randomized trial was initiated for stage III melanoma comparing M-Vax to high-dose interferon-$\alpha$, using a lower dose of M-Vax, which requires a smaller amount of a patient's tumor tissue to create the vaccine, but also had difficulties.

Beyond the technical complexities inherent in procuring tumor and preparing a vaccine, another inherent problem with autologous cellular vaccines is that they are limited to individuals who had sufficient tumor for preparation of a vaccine. Therefore, clinical trials, and ultimately the clinical use of autologous cellular vaccines, must be restricted to patients who have bulky nodal or resectable distant metastatic disease. Such patients have a poor overall prognosis and likely have significant residual tumor burden, making them less-than-ideal candidates for any immunotherapeutic approach. For this reason, many investigators have sought an alternative strategy. Given that melanoma-associated antigens are common among a large number of patients, it is reasonable to expect that an allogeneic vaccine, prepared from cultured tumor cell lines, could stimulate a relevant antitumor immune response [76,77]. Allogeneic vaccines can be standardized, preserved, and distributed in a manner akin to peptide vaccines. Unfortunately, this approach also has seen difficulty in clinical translation.

Melacine consists of a lysate of two homogenized melanoma cell lines combined with the adjuvant DETOX. Initial phase I and phase II trials demonstrated a clinical response to Melacine [78], but a phase III trial comparing Melacine with combination chemotherapy in patients who had metastatic melanoma demonstrated no statistically significant difference in median survival duration between the two groups [79]. As Melacine was statistically equivalent to chemotherapy with much less toxicity, it was approved in Canada as a treatment for advanced melanoma [80]. A prospective randomized trial evaluating Melacine in the adjuvant setting for patients who had intermediate-thickness, node-negative melanoma also failed to demonstrate a survival benefit to the vaccine [81]. Retrospective analysis, however, demonstrated a relationship between success of the vaccine and HLA alleles. In their

initial work, Mitchell and colleagues [80] reported a strong association between patient HLA phenotype and evidence of clinical benefit from Melacine, specifically in patients who expressed at least two of the following five alleles: HLA-A2, A28, B44, B45, and C3. In the randomized trial, the 81 patients in the vaccine arm expressing two or more of these alleles had a better disease-free survival than the 70 patients in the observation arm who had two or more of these alleles (4-year disease-free survival rate 87% versus 64%, $P = .0001$). The specific alleles contributing the major component of this effect were HLA-A2 and C3. In the vaccine arm, patients positive for HLA-A2 or HLA-C3 had a significantly better 4-year disease-free survival rate than patients in the observation arm or among patients given the vaccine but negative for both alleles. A follow-up phase III clinical trial to examine Melacine in patients expressing HLA antigens HLA-A2 or HLA-C3 (approximately 59% of the study patients in the original trial) was planned but, unfortunately, further development of the vaccine was abandoned.

Canvaxin is an allogeneic vaccine composed of three viable, irradiated, melanoma cell lines chosen for their high content of immunogenic melanoma- and tumor-associated antigens [82,83]. In a phase II study of patients who had metastatic melanoma, the median survival of treated patients was 23 months compared with 7.5 months for historical controls [77]. A more significant survival advantage was seen in patients who underwent resection of clinically detectable disease before vaccination [84]. In a case-control study of 88 patients who had stage IV melanoma and who had complete resection of metastases followed by Canvaxin who were matched to 88 controls having surgery only, the 5-year survival was 40% for Canvaxin; this was compared with 13% for the control group [85]. A similar approach was used in a study to evaluate the use of Canvaxin in patients who had American Joint Committee on Cancer stage III melanoma. Canvaxin was given as adjuvant therapy to 283 patients who underwent lymphadenectomy for palpable nodal disease. Compared to historical controls, the 5-year survival rate increased from 39% to 53%, and the median survival rate increased from 35.1 months to 90 months [77,83]. Unfortunately, two subsequent randomized trials failed to demonstrate any benefit of Canvaxin plus BCG compared with placebo plus BCG in patients who had stage III melanoma and in patients who had stage IV melanoma who have undergone surgical resection.

### *Dendritic cell vaccines*

DCs are a unique system of cells that induce, sustain, and regulate immune responses. DCs express a variety of molecules at various stages of maturation, allowing them to capture antigens, process them, and then present them to naïve T cells. DCs can prime T cells to class I and class II MHC-restricted antigens and are the most potent cells for the initiation of T-cell–mediated immunity [86,87]. Most of the vaccine therapies (described previously) depend on DCs to take up tumor-associated antigens and present

Table 5
Dendritic cell vaccines in clinical trial in melanoma

| Author | Dendritic cell type (antigens) |
|---|---|
| Nestle [154] | Immature DCs (MART-1, gp100, MAGE-3, tyrosinase) |
| Panelli [155] | Immature DCs (MART-1, gp100) |
| Mackensen [156] | Mature DCs (MAGE-1, MAGE 3, MART-1, gp100, tyrosinase) |
| Toungouz [157] | Immature DCs (MAGE-A1, MAGE-A3) |
| Gajewski [158] | Immature DCs (MAGE-3, MART-1) |
| Thurner [159,160] | Mature DCs (MAGE-3) |
| Schuler-Therner [161] | Mature DCs (MAGE-3) |
| Bancherau [162] | Mature DCs (MART-1, gp100, tyrosinase, MAGE-3) |
| Lau [163] | Immature DC (gp100, tyrosinase) |
| Hersey [164] | Immature DCs (MAGE-3.A2, tyrosinase, gp100, MART-1) |
| Lotze [165] | Immature DCs (MART-1, tyrosinase, gp100) |
| de Vries [166] | Mature DCs (gp100, tyrosinase) |
| Butterfield [167,168] | Mature DCs (MART-1) |
| Chakraborty [169] | Immature DCs pulsed with tumor lysate |
| Chang [170] | Immature DCs pulsed with tumor lysate |
| Nestle [154] | Immature DCs pulsed with tumor lysate |
| Griffioen [171] | Immature DCs pulsed with tumor lysate |
| Dillman [89] | Immature DCs pulsed with tumor cells |

them to T cells to generate an immune response. DC vaccines attempt to bypass this step by delivering to the patient DCs already expressing tumor antigens. One approach to DC vaccines is to load exogenous peptides onto the empty MHC class I molecules. This approach is limited, however, to known tumor antigens and to patients who have a given HLA type. In addition, responses are limited to cytotoxic T cells. Another approach is to expose immature DCs to unfractionated tumor material. This allows for antigen expression on MHC class I and class II epitopes and the diversification of immune responses. This approach is attractive in that immune responses can be generated without the need for the molecular characterization of tumor specific antigens. One potential drawback is that these methods may induce potentially toxic autoimmune responses to unknown antigens. Early clinical trials have demonstrated the ability of DC-based vaccination to generate objective tumor responses in melanoma-bearing patients (Table 5). Alternative DC vaccine approaches being examined include the use of tumor RNA; transduction of DCs with retroviruses, poxviruses, or adenoviruses encoding specific antigens; or fusing tumor cells and DCs together [88,89].

### *Anti–cytotoxic T-lymphocyte–associated protein 4 monoclonal antibodies*

One of the most promising areas of translational research in melanoma is direct immune modulation and the blockade of cytotoxic T-lymphocyte–associated antigenn 4 (CTLA-4). Although much of the research on immunotherapy to date has focused on generating tumor-specific effector cells,

only recently has it begun to focus on tolerance and the inherent controls on the immune system that dampen this response. Regulatory T cells play an important role in down-regulating responses to antigens through several mechanisms, including the expression of CTLA-4. CTLA-4 is a cell surface molecule that binds to the B7 family of TCRs. It is up-regulated in activated cells and serves as an immunologic'brake.'' The role of CTLA-4 initially was suggested by the severe autoimmunity and lymphoproliferative disorders seen in knockout mice. Ultimately, it was shown in a series of preclinical studies that the blockade of CTLA-4, primarily through the use of monoclonal antibodies, could augment an antitumor immune response [90]. Based on these findings, different antibodies blocking CTLA-4 have been developed and moved into clinical trials.

MDX-010 is a humanized anti–CTLA-4 monoclonal antibody. A phase II study of 56 patients who had progressive stage IV melanoma, using two different dosing schedules, resulted in an overall objective response rate of 13% [91]. When combined with interleukin (IL)-2, a phase I/II study showed an overall response rate of 22%. Ticilimumab (CP-675206) is another human anti–CTLA-4 monoclonal antibody undergoing testing [92]. The use of anti–CTLA-4 monoclonal antibodies may be limited by grade III/IV autoimmune toxicities, including severe autoimmune colitis. Multiple phase III studies are ongoing to explore the safety and efficacy of CTLA-4 inhibition further in metastatic and adjuvant settings and alone or in combination with vaccines, other immune modulators, or chemotherapy.

### *Adoptive immunotherapy*

Passive immunotherapy involves delivering to the host components of the immune system that previously were sensitized to host tumor antigens. These may include antibodies [93,94] or nonspecific lymphoid cells activated in vitro by exposure to high concentrations of IL-2, known as lymphokine-activated killer (LAK) cells [95]. Unfortunately, neither of these approaches has had tremendous success in melanoma. A clinical trial of LAK cells plus IL-2 versus IL-2 alone in stage IV melanoma demonstrated no significant difference in response rates between the two treatments [96]. A more fruitful approach has been to use tumor-specific effector cells for adoptive immunotherapy. Tumor-reactive T cells are more efficient than LAK cells in mediating tumor regression. The T cells in patients who have cancer, however, often are functionally impaired [97]. In vitro culture of these T cells can restore their effector function. Although this approach is more complex and expensive, it shows tremendous potential in the clinical setting.

Initial approaches to adoptive cellular immunotherapy involved purification of tumor infiltrating lymphocytes (TIL) from metastatic foci, ex vivo expansion in the presence of high-dose IL-2, and infusion of TIL back into the patient. In human studies, approximately 30% of TIL from patients who have melanoma exhibit specific cytolytic reactivity [98]. When adoptive

immunotherapy is performed with TIL, objective responses are seen in 10% to 30% of patients [99,100]. Several mechanisms are being explored to improve the effectiveness of the T cells and their ability to traffic and survive at the tumor site. These include activating the cells in the presence of monoclonal antibodies (anti-CD3 or anti-CD28) or irradiated tumor cells, developing methods for isolating and selecting the T-cell subsets most responsible for antitumor reactivity, and genetically engineering the T cells [101,102]. Another promising approach has been to lymphodeplete patients using cyclophosphamide and fludarabine before adoptive transfer of T cells [103].

One drawback to adoptive cellular immunotherapy is that harvesting TIL from metastatic foci requires that patients have procurable stage IV disease. This limits the clinical settings in which the effect of this approach might be clinically relevant. Dreno and colleagues [104,105] generated T cells from invaded lymph nodes instead of from metastatic lesions for infusion back into patients who had stage III melanoma. There was no difference in either disease-free survival or overall survival in patients receiving adoptive immunotherapy plus IL-2 compared with patients receiving IL-2 only. One explanation might be that compared with TIL, lymph nodes contain only a small percentage of T cells that are tumor specific. One method to overcominge this drawback is to give patients a vaccine, then excise the vaccine-draining lymph nodes and use these lymphocytes for adoptive cellular immunotherapy [106–109]. In clinical trials by Chang and colleagues [110,111], patients who had melanoma or renal cell carcinoma received intradermal inoculations of autologous tumor vaccines with BCG. Seven to 10 days later, vaccine-draining lymph nodes were removed, expanded, and delivered back to patients; durable tumor responses were seen in some patients [112]. More recent efforts have focused on developing lymphocytes that are completely independent of preexisting antitumor T cells. Retroviral gene transduction into peripheral blood mononuclear cells may be one method of achieving this. Peripheral blood mononuclear cells from patients were tranduced with the gene for the TCR-$\alpha$ and -$\beta$ chains against the MART-1 melanoma antigen. Seventeen patients received a nonmyeloablative chemotherapy regimen with fludarabine and cyclophosphamide followed by the administration of the transduced T cells and IL-2. Two patients had complete responses [113]. These encouraging results have spawned investigations into other TCRs, such as p53.

## Summary

Recent advancements in the fields of molecular biology and immunology have provided a window of opportunity to provide true multimodality treatment for melanoma. As stand-alone therapies or in combination with chemotherapy, the addition of targeted molecular therapies or immunotherapies ultimately may serve an important role in disease control. There are many challenges, however, still faced in moving these promising therapies toward clinical use. To date, the road to improved systemic therapy in melanoma

is paved with a plethora of approaches that seemed promising but ultimately failed to deliver. Better preclinical models that reflect human melanoma carcinogenesis and the immune response/tolerance to melanoma more accurately are needed. Similarly, superior assays of response for phase I and II trials that may predict the likelihood of clinical success more accurately desperately are needed. In order to understand better why promising preclinical data fail to succeed in clinical evaluation, research must shift from the development of new drugs or methods that generate an immune response to the other side of the equation: how melanoma cells develop resistance or avoid immune recognition. The current concept of translational research also must be re-evaluated. The occasional complete or partial response in stage IV disease may not guarantee clinical success nor does a lack of response against measurable metastatic disease mean that a treatment might not eradicate microscopic disease in the adjuvant setting. It is imperative to redesign the methodology by which new therapies are moved from preclinical studies through clinical testing and ultimately into clinical use, not only to avoid wasting valuable resources on therapies unlikely to succeed but also to avoid giving up on other therapies too soon.

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