[Frontiers in Bioscience 11, 1-14, January 1, 2006]

Immunotherapy as part of a multidisciplinary approach to melanoma treatment

Adam I. Riker [1], Richard Jove [2], and Adil I. Daud [1]

H. Lee Moffitt Cancer Center and Research Institute, [1] Cutaneous Oncology and [2] Molecular Oncology Program, 12902 Magnolia Drive, Tampa, Florida, 33612

TABLE OF CONTENTS

1. ABSTRACT

We have made tremendous advances in the earlier diagnosis and treatment of melanoma. Indeed, the early recognition and surgical management of thin primary cutaneous melanoma of <1.00 mm in Breslow's thickness has resulted in a >97% cure rate (1). For stage III and IV disease, our current treatment options are poor, with response rates well below 20% and only rare long-term responders noted (2-3). This is most clearly highlighted in a recent review by Rosenberg *et al.* who examined the role of immunotherapy in the treatment of patients with stage IV melanoma over a nine-year period (4). Overall, 96% of all patients had metastatic melanoma, utilizing a wide array of vaccine strategies including synthetic peptides, naked DNA, dendritic cells (DC) and recombinant viruses in patients with various advanced malignancies. They found that the overall objective response rate, utilizing conventional oncologic criteria for clinical tumor response, was only 2.6%. (4). By combining the results of the Surgery Branch with the latter trials above, a total of 1,306 vaccine treatments have been given for an overall objective response rate of 3.3%. Such dismal results compel us to take a critical look at our current approach to tumor immunology and immunotherapy, with the intention of improving our current understanding of the complex interactions that occur within the tumor microenvironment. This in turn will provide us with new insight and direction as we design the next generation of vaccines for patients with advanced cancer and in particular melanoma.

2. ADVANCES IN THE MEDICAL MANAGEMENT OF MELANOMA

2.1. IFN-alpha therapy

Interferon-alpha was initially noted to reduce transcription of viral genes in patients with viral infections. Anecdotal evidence suggested it might also produce responses in cancer. The pivotal studies with this cytokine have been conducted by the Eastern Cooperative Oncology Group under the leadership of John Kirkwood (5). The initial trial, E1684, randomized 280 patients to either interferon given at 1800 million units /m^2 over a year (referred to as high-dose interferon) or to observation (6). Median overall survival was significantly prolonged at 5 years from 2.8 years to 3.8 years (p=0.023) and relapse-free survival was also significantly prolonged by about one year. The next trial, ECOG 1690, compared high-dose interferon to low-dose interferon to observation in a similar high risk group of adjuvant patients (7). In this trial, surprisingly, no benefit was seen for survival in either the low-dose or the high-dose interferon arms. This trial caused a great deal of confusion although it was noted at the time that patients relapsing on the observation arm were salvaged with high dose interferon in a significant proportion of cases, thus weakening the overall conclusions.

The next trial, ECOG 1694, compared a vaccine against a ganglioside epitope, GM-2, which had shown promising results in phase I and II clinical trials when

compared to a control interferon arm (8). Again, somewhat surprisingly, this trial was halted by the independent Data Safety and Monitoring Board because the interferon control arm was found to be superior to the vaccine arm. While median survival has not been reported, the risk of dying from melanoma was reduced by 27% in this trial. In addition, a large number of trials have validated this data in Europe, by the EORTC and other multinational groups (9). These trials have utilized low to moderate interferon dosing regimens in comparison to the above mentioned ECOG trials. These trials have shown a reduction in melanoma recurrence but have failed to show a significant prolongation of overall survival.

While this mass of often contradictory data has been debated, a recent analysis helped put the evidence in perspective. Wheatley and colleagues have recently published an extensive single patient meta-analysis looking at all prospective randomized phase III interferon trials (2). They concluded that while the benefit of interferon in reducing recurrence (26%, p=0.001) was unquestionable, the benefit in terms of overall survival was much smaller than expected, and thus utilizing conventional statistical analysis resulted in a failure to achieve statistical significance (15% reduction in risk of death, p=0.06). In summary, the interferon data shows a relatively small degree of benefit for stage III melanoma patients. It should also be noted that Interferon has a tremendous cost associated with its use, in addition to a many possible adverse side effects and toxicities. This added component often makes the discussion to treat patients with Interferon even more difficult.

2.2. High-dose interleukin-2 therapy

It is well established that the use of high-dose intravenous interleukin-2 (IL-2) for the treatment of patients with advanced melanoma and renal cell cancer resulted in an infrequent but durable complete response, consistent with the activation of the host immune system and subsequent tumor eradication. As a result of such compelling clinical responses, IL-2 has become a mainstay of treatment, either alone, or as part of numerous drug, vaccination, and lymphocyte infusion combinations in clinical trials. There is currently a wide spectrum of dosing schedules and regimens for IL-2 therapy, with the current standard used by most oncologists being 600,000 to 720,000 IU/kg/dose, given at 8-hour intervals for 14 planned doses repeated twice over an 8 week period. Although the optimal dosing schedule resulting in the best clinical response is currently unknown, previous data would suggest that the higher dose regimens as well as the number of total doses received correlates best with clinical response. Several groups have begun to look at alternative dosing strategies to achieve an increased drug tolerance and tolerability profile, such as the continuous infusion of IL-2 (18 mIU/m^2/day) over an extended period of 72 hours (10). Although there are no long-term clinical results yet available, this infusion regimen seems to allow for more IL-2 doses to be administered in a sequential fashion with improved tolerability.

One unique immunotherapeutic approach to IL-2 treatment has been the transduction of the IL-2 gene into human melanoma-specific lymphocytes followed by autologous infusion of the gene-modified lymphocytes. This has been shown to result in the autocrine maintenance of the cytotoxic T-cells in the absence of exogenous IL-2 (11). Others have performed the simultaneous transduction of the IL-2 gene and the B7-1 co-stimulatory molecule gene in an attempt to enhance the stimulatory activities of anti-tumor lymphocytes (12). Both of these strategies are still in the experimental stages of development. One of the major immunologic effects of IL-2 on the immune system is to expand the total number of T-lymphocytes (CD4+ and CD8+) and to prevent lymphocyte apoptosis. Additionally, we are beginning to understand a key role of IL-2, which is to provide the appropriate cytokine milieu necessary to overcome tumor-induced immune tolerance. This process is still largely unknown; however, studies are beginning to examine the molecular and genetic mechanisms involved in this complex interaction between the tumor and the host immune response. For the time being, IL-2 therapy remains the primary treatment modality for patients with stage IV melanoma, despite the relatively low overall response rate of about 18% (9% partial response, 8% complete response). One may anticipate that improved vaccination strategies will continue to integrate IL-2 therapy into a combined approach to treatment with a potential synergistic effect to therapy.

2.3. Novel Chemotherapeutic Agents

As we have noted above, standard treatment regimens for metastatic melanoma rely heavily on the atypical alkylating agent dacarbazine. This drug was approved almost 3 decades ago by the Food and Drug Administration, based on data showing responses in 10-20% of patients with melanoma (13). However, dacarbazine has several disadvantages that include its lack of central nervous system penetration and lack of synergy with other chemotherapeutic agents. A novel compound, temozolomide, was developed that was thought to have the advantage of crossing effectively the blood-brain barrier. Temozolomide is an orally bioavailable agent that non-enzymatically converts at physiologic pH to the active drug methyl-thio-imidocarbazole (14). The prodrug, temozolomide, can effectively cross the blood-brain barrier prior to its conversion to the active drug with appreciable CNS activity seen with this agent. A recently published phase III trial by Stupp and colleagues showed that temozolomide significantly improves the survival of patients with glioma when combined with radiation therapy (15). Temozolomide has also been shown to be effective in melanoma patients with known brain metastases and is widely used for this purpose (16). The combination of temozolomide, when combined with a second agent, thalidomide, has shown promising activity in stage IV melanoma patients with CNS involvement (17).

Another disadvantage of current dacarbazine based regimens is the lack of additive and/or synergistic activity with these combinations. For instance, the "Dartmouth" regimen (dacarbazine, BCNU, cisplatin) was shown to be no more effective than dacarbazine alone (18). Furthermore, several such "biochemotherapy" regimens

were also shown to be no better than combination chemotherapy alone (19). In a recent trial, Daud *et al.* showed that the topoisomerase I inhibitor, karenitecin, showed intriguing activity, even in patients with heavily pretreated melanoma (20). While one complete response was reported out of 43 evaluable patients, up to 33% of all patients had stable disease, sometimes for prolonged periods of time, even when they had previously progressed on other therapies. Currently, a trial with this drug combined with a molecularly targeted agent (histone deacetylase inhibitor) is underway.

3. TARGETED THERAPY FOR MELANOMA

There have been many new and exciting compounds that have focused on a "targeted" approach to the immunotherapy of melanoma. Many of the compounds are currently being tested in phase I and II clinical trials and it is clearly too soon to tell if such agents have significant safety and efficacy for advanced melanoma patients. Nonetheless, there has been some reserved optimism for a few of these compounds. We will discuss just a few of these compounds and molecular targets below.

3.1. Denileukin Diftitox (ONTAK)

The expression of the CD25 receptor (a-chain of the trimeric IL-2 receptor) on CD4+ T-cells has been identified as a marker of suppressor cells in murine models (21). These immune lymphocytes have been termed CD4+CD25+ regulatory cells (Tregs), playing a critical role in immune tolerance and the control of autoimmunity. Tregs have been shown to inhibit harmful autoimmune T-cells in a contact-dependent and cytokine-independent mechanism. Consequently, it is hypothesized that Tregs may also impair anti-tumor immune responses that are known to be directed at least in part against auto-antigens expressed by tumor cells. A study by Javia *et al.* analyzed patients with metastatic melanoma undergoing immunizations with known melanoma antigens and found that anergic and functionally suppressive Tregs exist and may play a vital role in modifying the magnitude of the T-cell response to immunization (22). Similar studies have recently extended these findings to isolated CD4+CD25+ tumor infiltrating lymphocytes from non-small cell lung cancer, supporting the potential inhibitory activity these cells may have upon the anti-tumor immune response (23,24). Others have found that Tregs were markedly over-represented in metastatic lymph nodes containing melanoma, with a 2-fold increased frequency compared with both tumor-free lymph nodes and autologous peripheral blood mononuclear cells (25).

Denileukin diftitox (ONTAK) is a recombinant DNA-derived cytotoxic protein composed of the amino acid sequences for diptheria toxin fragments A and B, followed by the sequences for IL-2. Thus, it is a fusion protein designed to direct the cytocidal action of diptheria toxin to cells that express the IL-2 receptor (21). The human IL-2 receptor exists in three forms, low (CD25), intermediate (CD122/132) and high (CD25/CD122/CD132) affinity. *Ex vivo* studies suggest that ONTAK interacts primarily with the high affinity IL-2 receptor on the cell surface and inhibits cellular protein synthesis, resulting in cell death within hours. It has been shown that ONTAK only requires 35-50 high affinity receptors per cell to bind, internalize and cause apoptosis. Although the high affinity IL-2 receptor is found primarily on activated B-cells, T-lymphocytes and macrophages, it also is expressed on malignant cells of hematologic and solid tumor origin, including melanoma (21).

Recent data strongly suggests that Tregs play a critical role in the regulation of the host immune response. The specific regulatory mechanisms are still not entirely clear, however, data suggests that Tregs limit the overall activation of the immune response that involves the innate and cell-mediated arms. It has been hypothesized that the elimination of Tregs prior to the immunotherapy of patients with metastatic disease may eliminate the intrinsic mechanism of immune regulation and result in a more robust and complete activation of the immune system against established and growing tumor. Shimizu *et al.* showed that removal of Tregs was able to abrogate immunological unresponsiveness to syngeneic tumors *in vivo* and *in vitro*, leading to spontaneous development of tumor-specific effector cells as well as tumor non-specific ones (26).. However, the limited removal of Tregs was not sufficient to obtain a lasting complete response of established tumor, and further experiments revealed that cyclophosphamide administration followed 7 days later by active immunotherapy (intradermal injection of tumor cells with BCG) resulted in a complete tumor regression of established tumors (27). Others have shown that the selective and dose-dependent depletion of Tregs in humans with ONTAK resulted in an enhanced anti-tumor immunity, without negatively impacting on the T-helper (CD25-) cells or CD8 and memory T-cells (28). Furthermore, human trials have revealed that the selective depletion of Tregs in patients with cutaneous T-cell lymphoma results in response rates in the 30-40% range (29).

There have been some exciting preliminary reports examining the utility of ONTAK in the treatment of human cancer. Vieweg *et al.* recently presented their data on the enhancement of anti-tumor immunity following depletion of Tregs in patients with renal cell carcinoma (30). A phase I trial was performed in patients who received a single dose of ONTAK followed by vaccination with total tumor RNA-transfected DC. They showed that Tregs expressing the high affinity receptor were selectively eliminated in a dose-dependent manner, without bystander toxicity to CD25- lymphocytes or those T-cells that expressed the intermediate receptor/memory/effector T-cell pool, as evidenced by ELISPOT and proliferation assays (30). Additionally, the depletion of Tregs followed by vaccination with tumor RNA-transfected DC reproducibly led to improved stimulation of tumor specific T-cells when compared to vaccination alone.. This early trial has shed important light upon the role of Tregs in immune regulation and furthermore, the selective depletion of Tregs can result in an enhanced magnitude of the overall cell-mediated immune response to solid tumors as part of a vaccination strategy utilizing DC.

Others have shown that patients with advanced (stage III and IV) ovarian carcinoma can selectively and specifically recruit regulatory T-cells to the tumor cell microenvironment and ascites in response to the tumor-mediated secretion of the chemokine CCL22, which mediates trafficking Tregs to the tumor (31). Importantly, it was shown that the mere accumulation of Tregs at the tumor site predicted a poor survival in individuals with ovarian cancer, highlighting the powerful immunosuppressive effects of the Tregs upon the remaining cells of the immune system. Such depletion of Tregs has important and profound implications in the design of future immunotherapy trials for solid tumor malignancies such as melanoma.

3.2. Protein Kinase Inhibition (Sorafenib, BAY43-9006)

Sorafenib (BAY43-9006) is an orally administered protein kinase inhibitor that is a potent inhibitor of the B-Raf kinase that is frequently mutated in melanoma. Administered alone, sorafenib had relatively little activity in metastatic melanoma, with only one partial response among 20 patients treated on a phase II trial (32). However, when combined with cytotoxic chemotherapy using carboplatin and paclitaxel, a high number of responses were seen with 11 partial responses among 35 melanoma patients treated (31%), plus 19 more patients with stable disease. Interestingly, these results seem far better than what would be expected from treatment with either agent alone. Thus, a phase III trial to examine the contribution of sorafenib to this chemotherapy combination is presently in the planning stages.

3.3. STAT-3 as a novel target for melanoma

Recent studies point to the Signal Transducer and Activator of Transcription (STAT) pathway as a potentially promising new target for melanoma therapy (33). The STAT family of proteins comprises transcription factors that are activated in the cytoplasm by tyrosine phosphorylation in response to cytokine or growth factor engagement of cell surface receptors (34, 35). Upon tyrosine phosphorylation, activated STAT dimers translocate to the nucleus and bind directly to the promoters of genes that regulate fundamental biological processes (34, 35). Some of the genes that are regulated by STAT proteins include those involved in controlling apoptosis, cell cycle progression, angiogenesis and immune responses (36, 37). In contrast to normal cells, where STAT proteins are tightly regulated, tumor cells often harbor persistently activated STAT proteins, leading to continuous deregulation of gene expression (33, 37-39). Among the STAT family of proteins, STAT3 and STAT5 are the most frequently activated in human tumors of diverse origin, including melanoma, breast and prostate cancer, multiple myeloma, head and neck squamous cell carcinoma, leukemias and lymphomas (33, 36, 37, 40, 41). Persistent STAT activation in tumor cells leads to resistance to apoptosis, stimulation of cell cycle progression, enhanced tumor angiogenesis, and immune evasion (33).

In human melanoma cell lines and tumors, STAT3 is the predominant family member that is persistently activated (42). The first studies to establish Stat3 as a promising target for cancer therapy were performed in an immunocompetent, syngeneic mouse model of melanoma. In these studies, gene therapy with a dominant-negative form of Stat3 resulted in tumor regression accompanied by massive tumor cell apoptosis (42). Since only a small fraction of the tumor cells received the STAT3 gene therapy, this massive tumor cell apoptosis led Yu and colleagues to postulate that blocking STAT3 in tumor cells induced a "bystander effect" involving an immune response to tumor cells, including those that did not receive the STAT3 gene therapy (43). Further investigation revealed that STAT3 activation in tumor cells suppresses expression of proinflammatory mediators of immune response (44). Moreover, activated STAT3 signaling in tumor cells leads to production of soluble factors that inhibit dendritic cell (DC) maturation (44). Conversely, blocking STAT3 activity in tumor cells induces expression of proinflammatory cytokines and chemokines that activate DCs and innate immunity, resulting in tumor-specific T-cell responses. Thus, persistent STAT3 activation in tumor cells can mediate immune evasion by blocking the production of inflammatory signals in the immune system. These findings suggest that inhibition of STAT3 signaling in tumor cells may result in more effective antitumor immune responses (33,44).

Small-molecule inhibitors of STAT3 are currently under development for potential treatment of human cancer, including melanoma (33). These inhibitors selectively block dimerization and DNA binding of STAT3, thereby preventing STAT3-mediated gene regulation and its biological consequences (45, 46). These studies have provided proof-of-principle that small molecules can disrupt STAT3 activity directly in tumor cells and provide therapeutic benefit. One of the advantages of targeting STAT3 is that this signaling pathway provides a point of convergence downstream of many cytokine and growth factor receptors that are frequently activated in cancer (33). However, STAT3 inhibitors are still in the research phase and are not yet ready for clinical development. In the mean time, inhibitors of tyrosine kinases that signal through STAT3, such as EGF receptor and Src kinases, may provide therapeutic benefit, at least in part by blocking STAT3 signaling. Due to the ability of STAT3 to promote tumor cell immune evasion, inhibitors of this pathway may prove to be effective in enhancing antitumor immune responses when combined with immunotherapy approaches in melanoma. This is an exciting area of future investigation with inhibitors of tyrosine kinases upstream of STAT3 signaling as well as direct inhibitors of STAT3 protein.

3.4. BCL-2 Antisense Oligonucleotide (Oblimersen, Genasense, Genta)

Antisense oligonucleotides represents a novel, targeted immunotherapeutic approach to treating patients with metastatic melanoma. They are short sequences of synthetic DNA engineered to bind to specific RNA sequences and prevent their translation into proteins. Highly specific inhibitory antisense oligonucleotides can be

made for virtually any known gene, even if it would be very difficult to create a specific pharmacologic inhibitor of the gene product. Millward and colleagues described such an approach using antisense oligonucleotides directed against the anti-apoptotic protein, BCL-2, in an attempt to improve the response to chemotherapy by overcoming the inherent resistance of melanoma cells to apoptosis (47). The first such therapeutic agent is oblimersen, a BCL-2 antisense oligonucleotide, examined in a randomized phase III trial that compared dacarbazine alone to dacarbazine plus oblimersen (47). This large trial involved 771 patients randomized to either dacarbazine alone or the same dose of dacarbazine given the day after a five-day continuous infusion of oblimersen. Dacarbazine alone had an overall objective response rate of 6.8%, compared to the combination of dacarbazine with oblimersen with an overall response rate of 11.7%. Although there was no difference noted in median survival noted, these results provide some evidence that the addition of oblimersen enhanced the anti-melanoma activity of dacarbazine.

3.5. CTLA-4 Blockade

One mechanism of immune regulation is through the CTLA4 protein. This protein becomes expressed on the surface of T cells after activation, and it sends a negative signal back to the cell that eventually shuts off the activated state.. Anti-CTLA4 antibodies are in active clinical development, and results of several different trials have yielded some encouraging results. The human monoclonal anti-CTLA4 antibody, CP-675,206, is an IgG2 antibody with high affinity for CTLA4 on the surface of T cells. Camacho et al. have recently presented the results of a phase I study in 34 patients with stage IV melanoma treated with just a single dose of this antibody (48). There were 3 objective responses, two of which were a complete regression of all disease and one patient with a partial regression. An additional six patients developed a prolonged stabilization of their tumors. There were some transient side effects noted in the treatment groops, however, these results appear to promising.

A second anti-CTLA4 antibody, MDX-010, is currently being tested in both phase I and II clinical trials. Early results have seen some anti-tumor responses, particularly in patients previously treated with vaccines. It appears that a response to therapy is closely associated to the development of autoimmune toxicity. Recently, Hersh and colleagues presented the results of a randomized phase II trial of 76 patients randomized to MDX-010 injections once per month alone (for four months) or combined with dacarbazine (49). Autoimmune-type side effects were again noted, most commonly diarrhea, colitis and dermatitis. A total of two patients had partial responses to the antibody alone and five patients had responses (one complete) to the combination. Several more patients on both arms had stable disease. These results confirm the activity of the MDX-010 antibody as a single agent, and suggest the possibility that combinations of antibody plus chemotherapy, or other immunostimulants, may also be useful.

3.6. Failure of the host immune response to melanoma

There are many potential barriers to overcome in the design and implementation of a successful immunotherapeutic approach to treatment, ranging from tumor cell escape phenomenon to tumor-induced immunosuppression. Tumor cells may lose or down-regulate either the melanoma associated antigens or MHC molecules. Additionally, tumor cells may produce a variety of immunosuppressive factors such as interleukin-10, VEGF and transforming growth factor. These factors create an inherently unfavorable microenvironment that limits the host immune response, in addition to tolerizing the T-cell response to established tumor. Another potential barrier may be due to an intrinsic inefficiency of DC whereby the appropriate co-stimulatory molecules are not being presented on the cell surface. Addition possibilities include tumor-related alterations in T-cell signaling and a skewing of the immune response from a Th1 (immunoactivating) to a Th2 response (immunotolerant).Such complex processes of tumor cell escape and immune tolerance are still not completely understood, adding to the seemingly slow process of developing effective therapies for patients with metastatic melanoma.

4. RECENT ADVANCES IN THE IMMUNOTHERAPY OF MELANOMA

4.1. Tumor cell-based vaccines

Tumor cell-based vaccines are able to stimulate the host immune response through a number of possible different mechanisms. The two major proposed pathways of host immune cell activation are either by the direct migration of the tumor cells to the draining lymph node basin after injection, or possibly by the uptake of apoptotic or necrotic tumor cells by host DC's located within the skin (50). The latter pathway results in subsequent antigenic processing and migration of the DC to the draining lymph node basin. Still others would support the concept that it is essential that intact tumor cells have access to secondary lymphoid organs for the development of a strong and effective anti-tumor response (51, 52). There is also data to support the concept that tumor cells are able to directly prime the immune system upon migration to the lymphoid tissue, albeit with the necessary co-stimulatory molecules residing on the tumor cell surface (53, 54). There are recent data to suggest that multiple such mechanisms may occur simultaneously, in addition to other possible mechanisms that are independent of anti-tumor cytotoxic T-cells (55, 56).

Whether autologous or allogeneic whole tumor cells are being used, presumably they are providing the appropriate antigenic stimulus to the host immune system. However, they may not necessarily stimulate a potent enough signal, and even more importantly, may have tolerized the host immune system. Even though recent data suggest that tumor-cell associated antigens are cross-presented to cytotoxic T-cells 50,000X more efficiently than soluble antigen, it has become clear that this is still an insufficient mechanism for causing tumor regression in most instances (57). Thus, it may not be ultimately important as to whether whole, irradiated tumor cells or tumor cell lysates are being utilized as the appropriate antigenic stimulus for a melanoma vaccine. It seems that the mechanism of tumor cell death, whether apoptotic or

necrotic, has a more important role in guiding the immune response to one of activation or tolerance.

There have been several clinical trials showing that both autologous and allogeneic tumor cell-based vaccines can be given safely with few adverse side effects. The most extensively studied tumor cell-based vaccine is a polyvalent, antigen-rich whole cell vaccine called Canvaxin™ (CancerVax Corp., Carlsbad, CA). Canvaxin is derived from three melanoma cell lines that contain over 20 immunogenic melanoma tumor antigens (58)..One potential advantage of this particular vaccine is the wide array of HLA haplotypes represented, covering over 95% of all melanoma patients and therefore it is not necessary to match patients according to their HLA profile. However, several small, single-institution phase I and II clinical trials of Canvaxin have shown only minimal clinical benefit in most patients (58). The rare complete responder to Canvaxin therapy has prompted the initiation of two multicenter phase III randomized trials of Canvaxin therapy in 1998. In these trials, patients who have undergone complete resection of regional (stage III) or distant (stage IV) metastatic melanoma receive postoperative adjuvant immunotherapy with Canvaxin plus BCG or BCG alone. Recently, it has been reported that the Stage IV melanoma trial has been halted for lack of efficacy; the Stage III melanoma trial is still ongoing.

A second tumor cell-based vaccine that has been well studied since 1988 is Melacine. Melacine is an allogeneic melanoma cell lysate combined with an immunologic adjuvant, DETOX (detoxified Freund's adjuvant), composed of a mixture of detoxified endotoxin, cell wall cytoskeleton and monophosphoryl lipid A. Early clinical trials revealed some promising results, with one complete and three partial responses seen in 25 patients treated with Melacine and DETOX (59). These results prompted the completion of seven open-label phase II trials involving 139 patients with stage III/IV melanoma and a multicenter phase III clinical trial of Melacine versus the Dartmouth regimen (60). The objective response rates for all of the above studies have been between 5 and 10%.

Based largely upon these former results and the clinical results of other phase III trials, the Southwest Oncology Group (SWOG) has completed accrual to a phase III observation controlled trial of Melacine in patients with intermediate thickness (1.5 to 4 mm) or Clark's level IV melanoma lesions and clinically negative regional lymph nodes (T3N0M0, SWOG-9035). The results revealed no evidence of a benefit from Melacine in patients with intermediate thickness melanoma (61). However, on subgroup analysis, it was found that patients who expressed 2 or more of the HLA class I antigens (HLA-A2, A28, B44, B45, C3) had a far superior 5-year relapse free survival than those in the observation arm with the same expression pattern (83% vs. 59%; p=0.0005) (14). Even more striking was the finding that vaccinated patients who expressed HLA-A2 or C3 or both antigens had a statistically significant improvement in overall survival compared to the observation arm patients (62).

Other studies have focused on the utilization of autologous tumor cell vaccines as the foundation for an effective adjuvant treatment strategy. Dillman *et al.* vaccinated 66 melanoma patients (33 with measurable disease at the time of vaccination, 33 who had no evidence of disease) with autologous tumor cell vaccines on a once a week schedule for three weeks followed by monthly injections for an additional 5 months (63). Objective tumor responses were noted in 12% of patients (3/26), with one complete response and two partial responses. At a follow-up of >5 years, the median overall and 5-year survival was 40 months and 39%, respectively, compared to an average response rate of 8.6 months and 10% for patients with metastatic disease without treatment. Among patients who were without evidence of disease when treatment started, the 12 patients whose DTH converted to positive with intradermal vaccination with autologous, irradiated tumor cells had a median survival of 61.4 months with a 5-year survival of 63%, compared to 9.7 months and 0% for the 13 non-converters. This, as well as other studies, has shown an improved survival for patients who convert their DTH test compared to those that do not convert (63-68).

Autologous tumor cells can also be modified with the hapten, dinitrophenol (DNP), in an attempt to increase the immunogenicity of the vaccine. Such an approach has been examined in combination with low dose cyclophosphamide prior to vaccine administration (69, 70). In his update of 83 patients treated with this vaccine strategy, Berd *et al* report a clinical response rate in 11/83 (13%) patients, including 2 complete responses, 4 partial responses, and 5 mixed responses (69). Two additional patients were judged to have stable disease. He also showed that the induction of a DTH response to autologous, unmodified tumor cells was a significant and independent predictor of survival, both in patients with measurable metastases and in the post-surgical adjuvant setting.

Lotem *et al.* describe the use of autologous tumor cells procured from tumor samples, subsequently expanded *in vitro*, conjugated to DNP and given intradermally in combination with bacillus Calmette-Guerin (BCG) (71). The treatment schedule also included low dose cyclophosphamide at various intervals during treatment. A total of 43 patients with resected metastatic melanoma were treated, revealing that both disease free survival and overall survival correlate with the intensity of evolving DTH to unmodified tumor cells. Patients with a DTH reaction of greater than 10 mm had a median disease free survival of 17 months (mean of 35 months) with a mean overall survival of 63 months (median not yet reached). In stark contrast to this, patients with a negative or weak DTH had a median disease free survival of only 9 months with an overall survival of 16 months. It is clear from such studies that both autologous and allogenic tumor cell vaccines exhibit some therapeutic efficacy with few adverse side effects...

4.2. Peptide-Based Vaccines

Recent advances in our understanding of the cellular and immunologic mechanisms that occur within the tumor microenvironment have resulted in the development and administration of experimental peptide vaccines in humans. There are several known melanoma differentiation

antigens known to be involved in the synthesis of melanin and recognized by melanoma-reactive T cells. Such peptide antigens include gp100, MART-1/Melan-A, tyrosinase, TRP-1 and TRP-2, NY-ESO-1 and the MAGE antigens, to name a few. These melanoma/melanocyte antigens represent non-mutated differentiation antigens that are expressed by the majority of melanoma cells and have been readily identified by various T-cell populations of the host immune system in an HLA-restricted fashion. The identification of the genes encoding cancer antigens has been achieved through innovative strategies that have resulted in the discovery and utilization of the peptide epitopes derived from these genes.

Most clinical studies utilizing peptide based-vaccines have been associated with few adverse side effects. Rosenberg *et al.* vaccinated stage IV melanoma patients subcutaneously every three weeks with a modified immunodominant peptide of the gp100 antigen, g209-2M (72). Following two immunizations, 10 of 11 (91%) of patients showed a consistently high level of immunization against the native g209 peptide, but not against the control peptide g280-288.. Clinically, one of nine patients who received the g209 peptide in IFA experienced an objective cancer regression that lasted 4 months. Three of the eleven patients exhibited mixed responses with complete or partial regression of several lesions. All patients in this study however eventually went on to develop progressive disease.

A critical aspect of treating patients with peptide vaccines is the selection of patients for therapy based upon the melanoma antigen expression within the tumor nodule. It is well established that synchronous lesions of patients with metastatic melanoma represent distinct entities in terms of their heterogeneous expression of tumor antigens (73). Data suggest that most tumor cell lines established from fine needle aspiration biopsies of patients with metastatic melanoma exhibit a relatively homogeneous co-expression of MART-1 and tyrosinase, with a much more heterogeneous expression of other tumor antigens, such as gp100, NY-ESO 1 and the MAGE antigens (74). Thus, caution should be applied to utilizing vaccines that are designed to only elicit a cytotoxic T-cell response exclusively against a single tumor antigen, due to the fact that most, if not all, melanoma tumors are heterogeneous in their antigenic profile. Thus, it seems to be the homogeneity of melanoma antigen expression that may dictate whether the proper recognition occurs by the host immune system. Indeed, a recent review from Rosenberg's group analyzing 28 different peptide-based vaccines utilized in stage IV melanoma patients highlights the lack of effectiveness with this approach. A total of 381 patients were treated with 370 patients showing no response, 9 patients showing a partial response and 2 patients with a complete response, for an overall objective response rate of only 2.9% (4).

The vaccination of patients with metastatic melanoma with multiple peptides may possibly overcome such problems of tumor cell antigenic heterogeneity, preventing the development of antigen-loss variants *in vivo.* It would seem to make inherent sense to optimize the induction of clinically relevant immune responses by multi-peptide intradermal vaccination. A recent randomized phase II trial was performed in 26 patients with metastatic melanoma, vaccinating with four melanoma peptides (administered with GM-CSF, Montanide ISA-51 and tetanus helper peptide) or with peptides pulsed onto monocyte-derived DC (75). Although a high level of specific T-cell responses were noted (in 42% of the peripheral blood, 80% of sentinel lymph nodes), only two patients in the GM-CSF arm had a clinical response and only one patient in the DC arm.

Slingluff *et al.* presented their data on the immunological results of a phase II randomized trial of a multi-peptide vaccine for melanoma (76). In this study of 51 patients with resected high-risk melanoma, the vaccination consisted of either 4 (Arm 1) or 12 (Arm 2) peptides derived from the genes for tyrosinase, MAGE and gp100 combined with a tetanus helper peptide, Montanide ISA-51 and GM-CSF. Immunologic responses were analyzed from peripheral blood lymphocytes and in the immunized sentinel nodes, with the T-cell responses examined by IFN-ϒ ELISPOT assay after one *in vitro* sensitization. The data for 31 patients revealed an immune response in 11/14 (79%) patients in arm 1 and in 17/17 patients (100%) in arm 2. Although no clinical follow-up is yet available, this study provides evidence that multiple peptides can be administered safely while maintaining their immunogenicity despite HLA-restricted peptide competition.

Peptide-based vaccines and other immunotherapeutic strategies have convincingly illustrated that an "immunologic response" to therapy is achievable in most cases. However, there is no study to date that has clearly shown a direct correlation between an immunologic response to therapy (immune cell activation) and a clinical response (regression of established tumor). This lack of correlation has been the shortcoming of most trials in terms of explaining why certain patients respond and others do not. Indeed, many peptide based-vaccinations have resulted in a significant increase in the number of lymphocyte precursors reactive against a variety of tumor differentiation antigens, with no concomitant evidence of clinical tumor regression noted in the vast majority of patients treated with immunotherapy.

4.3. Dendritic Cell-Based Vaccines

The dendritic cell plays a central role in immune mediation and represents a potent antigen presenting cell that has the ability to stimulate anti-tumor immune responses in both animals and humans. Studies have shown that the relatively immature DC can effectively "cross-present" tumor-associated antigens to cytotoxic CD8+ T cells, which was not a feature of either macrophages or mature DC (77). It is clear that when relatively immature DC in the skin are triggered to enter afferent lymphatic channels, this migrating pathway also initiates a phenotypic conversion that has profound immunological consequences (78). The matured DC is then capable of forming stable MHC class II-peptide complexes available to activate antigen-specific CD4+ T cells (79-81).

The first published clinical trial of DC vaccination was in 1995 and has since been followed by 98 additional clinical trials describing more than 1,000 DC-based vaccines performed in 15 different countries (82). Twenty-eight trials focused on patients with various advanced stages of melanoma. The safety profile was again noted to be quite remarkable; however, despite the treatment of over 1,000 patients with DC-based vaccines, there still appears to be only limited effectiveness. Most DC trials for melanoma have convincingly shown that both immature and mature DC can be administered to patients safely with few adverse side effects. The autologous DC can then be pulsed in vitro with either whole irradiated, autologous tumor cells or tumor cell lysate. Once the tumor cells are "fed" to the DC in vitro, the apoptotic or necrotic cells are then processed and tumor-specific peptide antigens are then transported to the surface in both an MHC class I- and II-restricted fashion. The administration of DC via various routes of vaccination (intradermal, intranodal and intravenous) is also feasible, although the optimal route of administration remains unknown.

Despite the low overall response rate, it is worth highlighting a select few DC-based trials over the last ten years. One of the most successful DC-based trials for patients with advanced, metastatic melanoma was reported by Nestle et al. (83). He used DC subsequently pulsed with either tumor cell lysate or multiple HLA-matched peptides injected intranodally. This trial involved 16 patients with metastatic melanoma who were immunized on an outpatient basis, utilizing real-time duplex ultrasound to visualize the delivery of the vaccine directly into the lymph node. Overall, 5 of 16 patients experienced an objective response, 2 complete and 3 partial responses. Of particular note was the durability of the clinical responses, with the 2 complete responders remaining free of disease for over 15 months at the time of initial publication.

Chang et al. have recently described a phase I trial of tumor lysate-pulsed DC in the treatment of a variety of advanced cancers (84). There were 14 patients in the trial, eleven with metastatic melanoma, 2 with metastatic colorectal cancer and 1 with neuroblastoma. The trial involved the intradermal administration of autologous tumor lysate-pulsed DC within cohorts of patients receiving 106, 107 and 108 DC. The DC were pulsed with autologous tumor lysate and keyhole limpet hemocyanin (KLH) every 2 weeks for a total of three vaccinations. Due to the known safety and lack of adverse side effects associated with the vaccination, all patients were treated in an outpatient setting and returned home the same day. A total of fourteen patients completed the trial and received all three vaccines every two weeks. Again, an immunologic response to therapy was noted in the majority of treated patients with the local accumulation of CD4+ and CD8+ T-cells at the vaccination sites. Of the 14 patients completing the trial, one patient experienced a partial response and one had a minor response, with 13 of 14 patients ultimately developing progressive disease.

Others have recently reported the results of their pilot, phase I study in 10 patients with stage IV metastatic melanoma (85). All patients were vaccinated with $1x10^7$ DC pulsed with autologous tumor cell lysate in combination with low dose IL-2 for a total of 10 weeks. There were no significant adverse side effects noted (localized skin reaction, mild fever) with 3 patients showing a potential therapeutic effect to therapy (one patient with stable disease, two patients with a mixed response) and seven patients with progression of their disease.

Recently, Schadendorf *et al.* completed a prospective, randomized phase III clinical trial that analyzed the therapeutic effects of an autologous peptide-pulsed DC-based vaccine in patients with stage IV melanoma compared to standard chemotherapy with DTIC alone (86). The results revealed that the overall response in the vaccine group was 3.8% compared to 5.5% in the DTIC group, with no statistically significant differences noted in response, toxicity, overall and progression-free survival between the two groups. The median time to progression was 2.8 months versus 3.2 months, respectively, and the median survival was 11 months for the DTIC arm but only 9 months for the vaccine arm. Although disappointing, several new avenues of DC-based immunotherapy are actively being pursued and in various stages of development, focusing on different ways to enhance the therapeutic efficacy of DC in combination with various immunoadjuvants and other anti-cancer agents.

4.4. Immunoadjuvants in Vaccine Therapy

It has become apparent that in order to increase the overall immunogenicity of the tumor cell vaccine, it will most likely require the aid of an immunologic adjuvant. An adjuvant is an immunopotentiator, that when added to a vaccine, will enhance the immunogenicity of the antigen, with the stimulation of both arms of the immune system. This response can be both humoral and cell mediated, either alone or in combination with a vaccine preparation. Several adjuvants have been utilized in human trials, such as IFA, BCG, and KLH in an attempt to enhance the immunogenicity of such a vaccine. However, we have little knowledge as to the exact mechanisms by which they work, with many questions still unanswered as to their interactions with the tumor cell-based vaccine and how an effective anti-tumor immune response is generated.

As early as 1891, it was the pioneering work of a New York surgeon, William B. Coley, who established the original observation that certain cancer patients who develop concurrent bacterial infections would experience concomitant remissions of their malignant disease. He further performed groundbreaking experimental immunotherapy by vaccinating patients with inoperable sarcoma with the mixed toxins of erysipelas and bacillus prodigiosus (87). Such important historical and early observations have carried over into the development and clinical use of several well-known immunoadjuvants.

A somewhat novel use of an old concept has been the utility of unmethylated CpG motifs, present in most bacteria but not present in the genome of vertebrates. Oligodeoxynucleotides that contain CpG motifs activate

both the innate and adaptive host immune system of vertebrates, providing a powerful "danger signal" with resultant immune cell activation. Cells of the innate immune system, such as DC, macrophages, monocytes, and neutrophils, must be activated in order to trigger the generation of optimal adaptive immune responses. This requires a set of pattern recognition receptors present on such cells that subsequently trigger T-cell activation upon the proper recognition of conserved microbial-specific molecules. The CpG motifs provide a unique signal that initiates a cascade of intracellular events that result in the activation of numerous cells of the immune system, as well as the secretion of antibody molecules and specific cytokines that cause activation and enhancement of host adaptive immunity (88).

The molecular structure of the bacterial DNA is recognized by toll-like receptors (TLR) located on the surface of DC, macrophages and B-cells. Unmethylated CpG dinucleotides in certain base contexts have been extensively analyzed and found to specifically bind to several different TLR, each with a different pattern of cellular expression. It appears that myeloid DC's and monocytes express TLR4, essential for the recognition of lipopolysaccharide from Gram-negative bacteria. Conversely, TLR9 expressed on B-cells and plasmacytoid DC are considered essential for the recognition of viral and intracellular bacterial DNA. It is this specificity of TLR activation that determines the different patterns of immune activation (89). It is now possible to selectively activate TLR9 using specifically synthesized CpG DNA ranging from 8 to 30 bases in length that contain one or more CpG motifs. The use of CpG motifs has been analyzed in a number of experimrntal settings, including their use with donor lymphocytes infusions, peptide-based vaccines, DC-based vaccine and irradiated tumor cells (90-96).

The use of a TLR agonist, such as CpG, appears promising for the development of novel immunoadjuvants in vaccine design. However, this should be approached with some caution for several reasons. First, the expression of the specific TLR9 ligand for CpG is uniquely limited to a small subset of DC in humans called plasmacytoid DC. Indeed, the majority of human DC trials utilize a myeloid-derived, interstitial-like DC, lacking the TLR-9 receptor completely. Thus, although it can be hypothesized that CpG monotherapy may activate plasmacytoid DC *in vivo*, it is unclear if combining CpG as an immunoadjuvant will add to a DC-based vaccine as currently administered. Secondly, recent data suggest that human plasmacytoid DC activated by CpG induce the generation of CD4+CD25+ regulatory T-cells, known to play an important role in the maintenance of immunological tolerance and immune suppressive function (97). Lastly, we do not yet fully understand the interactions of such an immunotherapeutic approach in terms of tumor tolerance and the development of autoimmune phenomenon as a result of such a vaccination strategy.

4.5. DNA Vaccines/Recombinant Viral Vectors

DNA vaccines have been shown to induce long-lasting immunity against infectious agents and protection from tumor outgrowth in several animal models (98-101). Likewise, intramuscular injections of DNA vaccines (composed of naked DNA expression plasmids) into humans have also resulted in the development of an immunologic response (102, 103). It is hypothesized that one mechanism of tumor antigen expression may involve the DNA vaccine introducing the appropriate genes into DC for subsequent processing and presentation to the host immune system. One of the obvious advantages of DNA vaccinations is that they can be administered to patients regardless of HLA-phenotype and without identifying immunogenic epitopes.

In a phase I clinical trial, patients with metastatic melanoma were vaccinated intramuscularly with a plasmid DNA encoding the gp100 melanoma associated antigen (104). The results revealed that none of the patients developed an enhanced T-cell response in response to vaccination, all developing progressive disease. This trial illustrates that vaccination with DNA expressing unaltered "self" antigens alone was not sufficient to induce T-cell immunity. In another phase I clinical trial, stage IV melanoma patients were injected intranodally with a DNA plasmid encoding several tyrosinase epitopes, resulting in tyrosinase-specific T-cell responses in 11 of 26 patients. It was also noted that those patients that developed an immune response had a prolonged survival in 16/26 patients (105). These trials provide some evidence that it is indeed possible to vaccinate with plasmid DNA, with a resultant enhancement in the immune response.

Adenovirus, vaccinia, and poxvirus have all been utilized as vectors of tumor antigen and gene delivery as part of an immunotherapeutic approach to treatment. Although appealing to use for multiple reasons, several viral vectors are known to induce a tremendous antiviral neutralizing antibody response to the first and subsequent vaccinations, severely limiting the effectiveness of this approach. One exception may be the use of fowlpox viral vectors, which seem to not produce neutralizing antibodies. This recombinant viral approach has been tested in the clinics with several different vectors encoding different melanoma associated antigens and genes (4). In over 160 patients treated with various viral vaccines, 157 patients had no evidence of a clinical response to therapy, with two patients developing a partial response and one patient with a complete response. The overall objective response rate for the various viral vaccination strategies was found to be only 1.9% (4).

One such approach may be to enhance tumor vaccines with the addition of several co-stimulatory molecules. Viral vectors expressing a triad of co-stimulatory molecules (B7.1, ICAM-1 and LFA-3, designated TRICOM) have been designed for early phase trials. Kaufman *et al.* have examined the use of a recombinant vaccinia virus expressing the human B7.1 gene in patients with unresectable melanoma (106). This early phase trial is designed primarily to evaluate the toxicity of the vaccine and the ability to generate melanoma-specific immunity. Other similar trials will examine the therapeutic efficacy of recombinant vaccinia/TRICOM, recombinant fowlpox/B7.1 and fowlpox/TRICOM (107, 108).

4.6. T-cell-Based Therapy +/- (Non)Myeloablative Therapy

Non-myeloablative T-cell-based immunotherapy involves the adoptive transfer of highly selective tumor-reactive T-cells directed against over-expressed self-derived differentiation antigens after a non-myeloablative conditioning regimen. Initial results of 13 treated patients with metastatic melanoma have yielded some very exciting results, with 6 of 13 patients exhibiting an objective clinical response and four others demonstrating mixed responses, with significant shrinkage of one or more metastatic deposits (109). In a recent follow-up study of this early trial, cancer regression in patients with refractory metastatic melanoma with large, vascularized tumors was noted in a remarkable 18 of 35 patients (51% response rate), including four patients with a complete regression of all metastatic disease (110). These highly selected patients were treated with the autologous transfer of anti-tumor lymphocytes after lymphodepleting chemotherapy, experiencing an objective clinical response. In analyzing the immunologic response to treatment, it is likely that such results stem from the ability to infuse a large number of fully activated tumor infiltrating lymphocytes with anti-tumor activity into a host that is depleted of regulatory T-cells.

5. WHAT DOES THE FUTURE HOLD FOR PATIENTS WITH MELANOMA?

The successful immunotherapy of patients with metastatic melanoma remains a considerable challenge to researchers and clinicians worldwide. Indeed, the treatment of metastatic melanoma patients is exceedingly difficult, with almost all patients eventually dying of their disease. Novel approaches to therapy are essential and will likely combine several different treatment approaches in an attempt to increase the response rates to treatment. In treating patients with bulky, stage IV melanoma, we may be asking too much from the tolerized host immune system to overcome such barriers as tumor-induced immunosuppression. It may be essential to first perform complete cytoreductive surgery as part of multimodal approach to therapy. Once the bulk of the disease has been surgically removed, immunotherapy will then be utilized to focus on the elimination of microscopic disease. This may be approached through the selective, and possibly combined, use of targeted therapy with the possible addition of other novel adjuvant vaccination strategies. It has become clear that the task of developing of an effective treatment for patients with melanoma will not be easy. There remains numerous challenges ahead in both advancing our understanding of the immunology of the tumor/host immune response as well as developing new immunotherapeutic approaches as our understanding of tumor microenvironment improves.

6. REFERENCES

1. Balch C.M., A.C. Buzaid, S. Soong, M.B. Atkins, N. Cascinelli, D. Coit & I.D. Fleming: New TNM melanoma staging system: Linking biology and natural history to clinical outcomes. *Semin Surg Oncol* 21, 43-52 (2003)

2. Wheatley K., N. Ives, B. Hancock, M. Gore, A. Eggermont & S. Suciu: Does adjuvant interferon-alpha for high-risk melanoma provide a worthwhile benefit? A meta-analysis of the randomised trials. *Cancer Treat Rev* 29, 241-252 (2003)

3. Atallah E & L. Flaherty: Treatment of metastatic malignant melanoma. *Curr Treat Options Oncol* 6, 185-193 (2005)

4. Rosenberg SA, J.C. Yang & N.P. Restifo: Cancer immunotherapy: moving beyond current vaccines. *Nature Medicine* 10, 909-915 (2004)

5. Kirkwood J. Cancer immunotherapy: the interferon-alpha experience. *Semin Oncol* 29, 18-26 (2002)

6. Kirkwood J.M., M.H. Strawderman, M.S. Ernstoff, T.J. Smith, E.C. Borden & R.H. Blum: Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *J Clin Oncol*, 14, 7-17 (1996)

7. Kirkwood J.M., J.G. Ibrahim, V.K. Sondak, J. Richards, L.E. Flaherty, M.S. Ernstoff, T.J. Smith, U. Rao, M. Steele & R.H. Blum: High- and low-dose interferon alfa-2b in high-risk melanoma: first analysis of intergroup trial E1690/S9111/C9190. *J Clin Oncol* 18, 2444-2458 (2000)

8. Kirkwood J.M., J.G. Ibrahim, J.A. Sosman, V.K. Sondak, S.S. Agarwala, M.S. Ernstoff & U. Rao: High-dose interferon alfa-2b significantly prolongs relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIB-III melanoma: results of intergroup trial, E1694. *J Clin Oncol* 19, 2370-2380 (2001)

9. Eggermont AM: The role interferon-alpha in malignant melanoma remains to be defined. *Eur J Cancer* 37, 2147-2153 (2001)

10. Quan W Jr, W. Brick, M. Vinogradov, W.C. Taylor, N. Khan & R. Burgess: Repeated cycles with 72-hour continuous infusion interleukin-2 in kidney cancer and melanoma. *Cancer Biother Radiopharm* 19, 350-354 (2004)

11. Liu K & S.A. Rosenberg: Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of exogenous IL-2 and maintenance of specific antitumor activity. *J Immunol* 167, 6356-6365 (2001)

12. Mazzocchi A, C. Melani, L. Rivoltini, C. Castelli, M. Del Vecchio, C. Lombardo, M.P. Colombo & G. Parmiani: Simultaneous transduction of B7-1 and IL-2 genes into human melanoma cells to be used as vaccine: enhancement of stimulatory activity for autologous and allogeneic lymphocytes. *Cancer Immunol Immunother* 50, 199-211 (2001)

13. Eggermont A.M. & J.M. Kirkwood: Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years*? Eur J Cancer* 40, 1825-1836 (2004)

14. Danson S.J. & M.R. Middleton: Temozolomide: a novel oral alkylating agent. *Expert Rev Anticancer Ther* 1, 13-19 (2001)

15. Stupp R, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer & R.O. Mirimanoff:

Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352, 987-996 (2005)
16. Tarhini A.A. & S.S. Agarwala: Management of brain metastases in patients with melanoma. *Curr Opin Oncol* 16, 161-166 (2004)
17. Hwu W.J., E. Lis, J.H. Menell, K.S. Panageas, L.A. Lamb, J. Merrell, L.J. Williams, S.E. Krown, P.B. Chapman, P.O. Livingston, J.D. Wolchok & A.N. Houghton: Temozolomide plus thalidomide in patients with brain metastases from melanoma. *Cancer* 103, 2590-2597, (2005)
18. Chapman P.B., L.H. Einhorn, M.L. Meyers, S. Saxman, A.N. Destro, K.S. Panageas, C.B. Begg, S.S. Agarwala, L.M. Schuchter, M.S. Ernstoff, A.N. Houghton & J.M. Kirkwood: Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. *J Clin Oncol* 17, 2745-2751 (1999)
19. Atkins M: A prospective randomized phase III trial of concurrent biochemotherapy (BCT) with cisplatin, vinblastine, dacarbazine (CVD), IL-2 and interferon alpha-2b (IFN) versus CVD alone in patients with metastatic melanoma (E3695): An ECOG-coordinated intergroup trial. *ASCO Abstracts* No: 2847, (2003)
20. Daud A, N. Valkov, B. Centeno, J. Derderian, P. Sullivan, P. Munster, P. Urbas, R.C. Deconti, E. Berghorn, Z. Liu, F. Hausheer & D. Sullivan: Phase II trial of karenitecin in patients with malignant melanoma: clinical and translational study. *Clin Cancer Res* 11, 3009-3016 (2005)
21. Shevach E.M.: Regulatory T cells in autoimmunity. *Ann Rev Immunol* 18, 423-449 (2000)
22. Javia L.R. & S.A. Rosenberg: CD4+CD25+ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. *J Immunother* 26, 85-93 (2003)
23. Woo E.Y., C.S. Chu & T.J. Goletz: Regulatory CD4+CD25+ T-cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 61, 4766-4772 (2002)
24. Woo E.Y., H. Yeh & C.S. Chu: Cutting edge: Regulatory T-cells from lung cancer patients directly inhibit autologous T-cell proliferation. *J immunol* 168, 4272-4276 (2002)
25. Viguier M, F. Lemaitre, O. Verola, M. Cho, G. Gorochov, L. Dubertret, H. Bachelez, P. Kourilsky & L. Ferradini: Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 173, 1444-1453 (2004)
26. Shimizu J, S. Yamazaki & S. Sakaguchi: Induction of tumor immunity by removing CD25+CD4+ T-cells: A common basis between tumor immunity and autoimuunity. *J Immunol* 163, 5211-5218 (1999)
27. Ghiringhelli F, N. Larmonier, E. Schmitt, A. Parcellier, D. Cathelin, C. Garrido, B. Chauffert, E. Solary, B. Bonnotte & F. Martin: CD4+CD25+ regulatory T-cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34, 336-344 (2004)
28. Azuma T, T. Takahashi, A. Kunisato, T. Kitamura & H. Hirai: Human CD4+CD25+ regulatory T cells suppress NKT cell functions. *Cancer Research* 63, 4516-4520 (2003)
29. Olsen E, M. Duvic, A. Frankel, Y. Kim, A. Martin, E. Vonderheid, B. Jegasothy, G. Wood, M. Gordon, P. Heald, A. Oseroff, L. Pinter-Brown, G. Bowen, T. Kuzel, D. Fivenson, F. Foss, M. Glode, A. Molina, E. Knobler, S. Stewart, K. Cooper, S. Stevens, F. Craig, J. Reuben, P. Bacha & J. Nichols: Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. *J Clin Oncol* 19, 376-388 (2001)
30. Vieweg J, Z. Su & J. Dannull: Enhancement of antitumor immunity following depletion of CD4+CD25+ regulatory T-cells. Journal of Clinical Oncology, *ASCO Annual Meeting Proceedings (Post-Meeting Edition)* 22, No 14S (2004)
31. Curiel T.J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J.R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M.L. Disis, K.L. Knutson, L. Chen & W. Zou: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10, 942-949 (2004)
32. Ahmad T, R. Marais, L. Pyle, M. James, B. Schwartz, M. Gore & T. Eisen: BAY 43-9006 in patients with advanced melanoma: The Royal Marsden experience. Journal of Clinical Oncology, *ASCO Annual Meeting Proceedings (Post-Meeting Edition)* 22, No 14S (2004)
33. Yu H. & R. Jove: The STATS of cancer - New molecular targets come of age. *Nature Rev Cancer* 4, 97-105 (2004)
34. Darnell J.E.: STATs and gene regulation. *Science* 277, 1630-1635 (1997)
35. Darnell J.E.: Transcription factors as targets for cancer therapy. *Nature Rev Cancer* 2, 740-749 (2002)
36. Bromberg J. & J.E. Darnell: The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19, 2468-2473 (2000)
37. Bowman T., R. Garcia, J. Turkson & R. Jove: STATs in oncogenesis. *Oncogene* 19, 2474-2488 (2000)
38. Yu C.L., D.J. Meyer, G.S. Campbell, A.C. Larner, C. Carter-Su, J. Schwartz & R. Jove: Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 269, 81-83 (1995)
39. Bromberg J.F., M.H. Wrzeszczynska, G. Devgan, Y. Zhao, R.G. Pestell, C. Albanese & J.E. Darnell: STAT-3 as an oncogene. *Cell* 98, 295-303 (1999)
40. Grandis J.R., S.D. Drenning, A. Chakraborty, M.Y. Zhou, Q. Zeng, A.S. Pitt & D.J. Tweardy: Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth *In vitro*. *J Clin Invest* 102, 1385-1392 (1998)
41. Catlett-Falcone R., T.H. Landowski, M.M. Oshiro, J. Turkson, A. Levitzki, R. Savino, G. Ciliberto, L. Moscinski, J.L. Fernandez-Luna, G. Nunez, W.S. Dalton & R. Jove: Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells *Immunity* 10, 105-115 (1999)
42. Niu G., T. Bowman, M. Huang, S. Shivers, D. Reintgen, A. Daud, A. Chang, A. Kraker, R. Jove & H. Yu:_Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. *Oncogene* 21, 7001-7010 (2002)

43. Niu G., K.H. Shain, M. Huang, R. Ravi, A. Bedi, W.S. Dalton, R. Jove & H. Yu: Overexpression of a dominant-negative signal transducer and activator of transcription 3 variant in tumor cells leads to production of soluble factors that induce apoptosis and cell cycle arrest. *Cancer Res* 61, 3276-3280 (1999)
44. Wang T., G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, W. Dalton, R. Jove, D. Pardoll & H. Yu: Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med* 10, 48-54 (2004)
45. Turkson J., D. Ryan, J.S. Kim, Y. Zhang, Z. Chen, E. Haura, A. Laudano, S. Sebti, A.D. Hamilton & R. Jove: Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation *J Biol Chem* 276, 45443-45455 (2001)
46. Turkson J., J.S. Kim, S. Zhang, J. Yuan, M. Huang, M. Glenn, E. Haura, S. Sebti, A.D. Hamilton & R. Jove: Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. *Mol Cancer Ther* 3, 261-269 (2004)
47. Millward M.J., A.Y. Bedikian, R.M. Conry, M.E. Gore, H.E. Pehamberger, W. Sterry, A.C. Pavlick, R.C. Deconti, D. Gordon & L.M. Itri: Randomized multinational phase 3 trial of dacarbazine (DTIC) with or without Bcl-2 antisense (oblimersen sodium) in patients (pts) with advanced malignant melanoma (MM): Analysis of long-term survival. *Journal of Clinical Oncology, (Post-Meeting Edition)* 22, No 14S (2004)
48. Camacho L.H., A. Ribas, J.A. Glaspy, G. Lopez-Berestein, J.M. Reuben, C. Parker, E. Seja, B. Comin-Anduix, C. Bulanhagui & J. Gomez-Navarro: Phase 1 clinical trial of anti-CTLA4 human monoclonal antibody CP-675,206 in patients (pts) with advanced solid malignancies. Journal of Clinical Oncology, (Post-Meeting Edition) 22, No 14S (2004)
49. Hersh E.M., J. Weber, J. Powderly, M. Yellin, K. Kahn, A. Pavlick, W. Samlowski, G. Nichol & S. O'Day: A phase II, randomized multi-center study of MDX-010 alone or in combination with dacarbazine (DTIC) in stage IV metastatic malignant melanoma. *Journal of Clinical Oncology, (Post-Meeting Edition).* 22, No 14S (2004)
50. Ward S., D. Casey, M.C. Labarthe, M. Whelan, A. Dalgleish, H. Pandha & S. Todryk: Immunotherapeutic potential of whole tumour cells. *Cancer Immunol Immunother* 51, 351-357 (2002)
51. Ochsenbein A.F., P. Klenerman, U. Karrer, B. Ludewig, M. Pericin, H. Hengartner & R.M. Zinkernagel: Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc Natl Acad Sci USA* 96, 2233-2238 (1999)
52. Ochsenbein A.F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner & R.M. Zinkernagel: Roles of tumor localization, second signals and cross priming in cytotoxic T-cell induction. *Nature* 411, 1058-1064 (2001)
53. Ostrand-Rosenberg S., B.A. Pulaski, V.K. Clements, L. Qi, M.R. Pipeling & L.A. Hanyok: Cell-based vaccines for the stimulation of immunity to metastatic tumors. *Immunol Rev* 70, 101-121 (1999)
54. Tepper R.I. & J.J. Mule: Experimental and clinical studies of cytokine gene-modified tumor cells. *J Exp Med* 194, 823-827 (1994)
55. van Elsas A., R.P. Sutmuller, A.A. Hurwitz, J. Ziskin, J. Villasenor, J.P. Medema, W.W. Overwijk, N.P. Restifo, C.J. Melief, R. Offringa & J.P. Allison: Elucidating the autoimmune and anti-tumor mechanisms of a treatment based on cytotoxic T-lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. *J Exp Med* 194, 481-486 (2001)
56. Wolkers M.C., G. Stoetter, F.A. Vyth-Dreese & T.N. Schumacher: Redundancy of direct priming and cross-priming in tumor-specific CD8+ T-cell responses. *J Immunol* 167, 3577-3581 (2001)
57. Li M, G.M. Davey, R.M. Sutherland, C. Kurts, A.M. Lew, C. Hirst, F.R. Carbone, & W.R. Heath. Cell-associated ovalalbumin is cross-presented much more efficiently than soluble ovalalbumin *in vivo. J Immunol* 166, 6099-7003 (2001)
58. Hsueh E.C. & D.L. Morton: Antigen-based immunotherapy of melanoma: Canvaxin therapeutic polyvalent cancer vaccine. *Semin Cancer Biol* 13, 401-407 (2003)
59. Elliot G.T., R.A. Mcleod, J. Perez & K.B. Von Eschen: Interim results of a phase II multicenter clinical trial evaluating the activity of a therapeutic allogeneic melanoma vaccine in the treatment of disseminated malignant melanoma. *Semin Surg Oncol* 2, 41-53 (1993)
60. Mitchell M.S. & K.B. von Eschen: Phase III trial of Melacine melanoma theraccine versus combination chemotherapy in the treatment of stage IV melanoma. *Proc Am Soc Clin Oncol* 16, 494a (1997)
61. Sondak V.K., P.Y. Liu, R.J. Tuthill, R.A. Kempf, J.M. Unger, J.A. Sosman, J.A. Thompson, G.R. Weiss, B.G. Redman, J.G. Jakowatz, R.D. Noyes & L.E. Flaherty: Adjuvant immunotherapy of resected, intermediate-thickness node-negative melanoma with an allogeneic tumor vaccine. I. Overall results of a randomized trial of SWOG. *J Clin Oncol* 20, 2058-2066 (2002)
62. Sondak V.K. & J.A.Sosman: Results of clinical trials with an allogeneic melanoma tumor cell lysate vaccine: (Melacine). *Semin Can Biol* 13, 409-415 (2003)
63. Dillman R.O., C. deLeon, L.D. Beutel, N.M. Barth, L.S. Schwartzberg, L.E. Spitler, D.H. Garfield, A.A. O'Conner & S.K. Nayak: Short-term autologous tumor cell lines for the active specific immunotherapy of patients with metastatic melanoma. *Critical Rev Oncol Hematol* 39, 115-123 (2001)
64. Dillman R.O., L.D. Beutel, N.M. Barth, C. deLeon, L.D. Beutel, A.A. O'Conner, C. DePriest & S.K. Nayak: Irradiated cells from autologous tumor cell lines as patient-specific vaccine therapy in 125 patients with metastatic cancer: Induction of delayed-type hypersensitivity to autologous tumor is associated with improved survival. *Cancer Biother Radiopharm* 17, 51-66 (2002)
65. Hoover H.C., M. Surdyke, R.B. Dangel, L.C. Peters & M.G. Hanna: Delayed cutaneous hypersensitivity to autologous tumor cells in colorectal cancer patients immunized with an autologous tumor cell-BCG vaccine. *Cancer Res* 44, 1671-1676 (1984)
66. Bystryn J.C., R. Oratz, D. Roses, M. Harris, M. Henn & R. Lew: Relationship between immune response to melanoma vaccine immunization and clinical outcome in stage II malignant melanoma. *Cancer* 69, 1157-1164 (1992)

67. Barth A, D.S. Hoon, L.J. Foshag, A. Nizze, E. Famatiga, E. Okun & D.L. Morton: Polyvalent melanoma cell vaccine induces a delayed-type hypersensitivity and *in vitro* cellular immune response. *Cancer Res* 54, 3342-3345 (1994)
68. Miller K, G. Abeles, R. Oratz, A. Zeleniuch-Jacquotte, J. Cui, D.F. Roses, M.N. Harris & J.C. Bystryn: Improved survival of patients with melanoma with an antibody response to immunization to a polyvalent melanoma vaccine. *Cancer* 75, 495-502 (1995)
69. Berd D, H.C. Maguire, L.M. Schuchter, R. Hamilton, W.W. Hauck, T. Sato & M.J. Mastrangelo: Autologous hapten modified melanoma vaccine as a post surgical adjuvant treatment after resection of nodal metastases. *J Clin Oncol* 15, 2359-2370 (1997)
70. Berd D, T. Sato, H. Cohn, H.C. Maguire & M.J. Mastrangelo: Treatment of metastatic melanoma with autologous hapten-modified melanoma vaccine: Regression of pulmonary metastases. *Int J Cancer* 94, 531-539 (2001)
71. Lotem M, T. Peretz, O. Drize, Z. Gimmon, D. Ad El, R. Weitzen, H. Goldberg, I. Ben David, D. Prus, T. Hamburger & E. Shiloni: Autologous cell vaccine as a post operative adjuvant treatment for high-risk melanoma patients (AJCC III and IV). *Br J Cancer* 86, 1534-1539 (2002)
72. Rosenberg S.A., J.C. Yang, D.J. Schwartzentruber, P. Hwu, F.M. Marincola, S.L. Topalian, N.P. Restifo, M.E. Dudley, S.L. Schwarz, P.J. Spiess, J.R. Wunderlich, M.R. Parkhurst, Y. Kawakami, C.A. Seipp, J.H. Einhorn & D.E. White: Immunologic and therapeutic evaluation of a synthetic tumor associated peptide vaccine for the treatment of patients with metastatic melanoma. *Nature Med* 4, 321-327 (1998)
73. Riker A.I., M. Panelli, U.S. Kammula, G. Ohnmacht, E. Wang, A. Abati, S.A. Rosenberg & F.M. Marincola: Threshold levels of gene expression of the melanoma antigen, gp100, correlates with tumor cell recognition by cytotoxic T-lymphocytes. *Int J Cancer* 86, 818-826 (2000)
74. Riker A.I., M. Panelli, U.S. Kammula, E. Wang, J. Wunderlich, A. Abati, P. Fetsch, S.A. Rosenberg & F.M. Marincola: Development and characterization of melanoma cell lines established by fine needle aspiration biopsy: Advances in the monitoring of patients with metastatic melanoma. *Can Detect and Prev* 23, 387-396 (1999)
75. Slingluff C.L., G.R. Petroni, G.V. Yamshchikov, D.L. Barnd, S. Eastham, H. Galavotti, J.W. Patterson, D.H. Deacon, S. Hibbitts, D. Teates, P.Y. Neese, W.W. Grosh, K.A. Chianese-Bullock, E.M. Woodson, C.J. Wiernasz, P. Merrill, J. Gibson, M. Ross & V.H. Engelhard VH: Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in granulocyte-macrophage colony-stimulating factor in adjuvant or pulsed on dendritic cells. *J Clin Oncol* 21, 4016-4026 (2003)
76. Slingluff C.L., G.R. Petroni, K.A. Bullock, E. Bissonnette, S. Hibbitts, C. Murphy, N. Anderson, W.W. Grosh, P.Y. Neese & R. Fink: Immunological results of a phase II randomized trial of multipeptide vaccines for melanoma. Journal of Clinical Oncology, *2004 ASCO Annual Meeting Proceedings (Post-Meeting Edition*) 22, No 14S (2004)
77. Albert M.L., B. Sauter & N. Bhardwaj: Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392, 26-29 (1998)
78. Albert M.L., S.F. Pearce, L.M. Francisco & B. Sauter: Immature dendritic cells phagocytose apoptotic cells via CD36, and cross-present antigens to cytotoxic T-lymphocytes. *J Exp Med* 188, 1359-1368 (1998)
79. Randolph G.J., S. Beaulieu, R.M. Steinman & W.A. Muller: Differentiation of moncytes into dendritic cells in a model that mimics entry of cells into afferent lymphatics. *Science* 282, 480-483 (1998)
80. Pierre P, S.J. Turley, E. Gatti, M. Hull, J. Meltzer, L. Mirza, K. Inaba, R.M. Steinman & I. Mellman: Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* 388, 787-792 (1997)
81. Cella M, A. Engering, V. Pinet, J. Pieters & A. Lanzavecchia: Inflammatory stimuli induce accumulation of M HC class II transport in mouse dendritic cells. *Nature* 388, 782-787 (1997)
82. Ridgway D: The first 1,000 dendritic cell vaccines. *Cancer Invest* 21, 876-886 (2003)
83. Nestle F.O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg & D. Schadendorf: Vaccination of melanoma patients with peptide-or tumor lysate-pulsed dendritic cells. *Nat Med* 4, 328-332 (1998)
84. Chang A.E., B.G. Redman, J. Whitfield, B.J. Nickoloff, T. Braun, P. Lee, J. Geiger & J.J. Mule: A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. Clinical. *Clin Canc Res* 8, 1021-1032 (2002)
85. Nagayama H, K. Sato, M. Morishita, K. Uchimaru, N. Oyaizu, T. Inazawa, T. Yamasaki, M. Enomoto, T. Nakaoka, T. Nakamura, T. Maekawa, A. Yamamoto, S. Shimada, T. Saida, Y. Kawakami, S. Asano, K. Tani, T.A. Takahashi & N. Yamashita: Results of a phase I clinical study using autologous tumor lysate-pulsed monocyte-derived mature dendritic cell vaccinations for stage IV malignant melanoma patients combined with low dose interleukin-2. *Melanoma Research* 13, 521-530 (2003)
86. Schadendorf D, F.O. Nestle, E.B. Broecker, A. Enk, S. Grabbe, S. Ugurel, L. Edler, G. Schuler & DeCOG-DC Study Group: Dacarbacine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) as first-line treatment of patients with metastatic melanoma: Results of a prospective-randomized phase III study. *Journal of Clinical Oncology, ASCO Annual Meeting Proceedings (Post-Meeting Edition)* 22, No 14S (2004)
87. Coley W.B.:The treatment of inoperable sarcoma with the mixed toxins of erysipelas and bacillus prodigiosus. *JAMA* 7, 389-395 (1898)
88. Krieg A.M.: CpG motifs in bacterial DNA: the active ingredients in BCG and CFA? *Nature Medicine* 9, 831-835 (2003)
89. Krieg A.M.: CpG motifs in bacterial DNA and their immune effects. *Annu. Rev Immunol* 20, 709-760 (2002)
90. Blazar B.R., A.M. Krieg & P.A. Taylor: Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potent stimulators of antileukemia responses in naïve and bone marrow transplant recipients. *Blood* 98, 1217-1225 (2001)

91. Heckelsmiller K, S. Beck, K. Rall, B. Sipos, A. Schlamp, E. Tuma, S. Rothenfusser, S. Endres & G. Hartmann: Combined dendritic cell- and CpG oligodeoxynucleotide- based immune therapy cures large murine tumors that resist chemotherapy. *Eur J Immunol* 32, 3235-3245 (2002)
92. Liu H.M., S.E. Newbrough, S.K. Bhatia, C.E. Dahle, A.M. Krieg & G.J. Weiner: Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor. *Blood* 92, 3730-3736 (1998)
93. Sandler A.D., H. Chihara, G. Kobayashi, X. Zhu, M.A. Miller, D.L. Scott & A.M. Krieg: CpG oligodeoxynucleotides enhance the tumor antigen-specific immune response of a granulocyte-macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. *Cancer Res* 63, 394-399 (2003)
94. Davila H.L. & E. Celis: Repeated administration of cytosine-phosphorothiolated guanine- containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. *J Immunol* 165, 539-547 (2000)
95. Kawarada Y, R. Ganss, N. Garbi, T. Sacher, B. Arnold & G.J. Hammerling: NK- and CD8+ T-cell mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides. *J Immunol* 167, 5247-5253 (2001)
96. van Ojik H.H., L. Bevaart, C.E. Dahle, A. Bakker, M.J. Jansen, M.J. van Vugt, J.G. van de Winkel & G.J. Weiner: CpG-A and B oligodeoxynucleotides enhance the efficacy of antibody therapy by activating different effector cell populations. Cancer Res 63, 5595-5600 (2003)
97. Moseman E.A., X. Liang, A.J. Dawson, A. Panoskaltsis-Mortari, A.M. Krieg, Y.J. Liu, B.R. Blazar & W. Chen: Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol* 173, 4433-4442 (2004)
98. Anderson R, X.M. Gao, A. Papakonstantinopoulou, N. Fairweather, M. Roberts & G. Dougan: Immunization of mice with DNA encoding fragment C of tetanus toxin. *Vaccine* 15, 827-829 (1997)
99. Caselli E, M. Betti, M.P. Grossi, P.G. Balboni, C. Rossi, C. Boarini, A. Cafaro, G. Barbanti-Brodano, B. Ensoli & A. Caputo: DNA Immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type tat. *Journal of Immunology* 162, 5631-5638 (1999)
100. Pilon S.A., M.P. Piechocki & W.Z. Wei: Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. *J Immunol* 167, 3201-3206 (2001)
101. Piechocki M.P., S.A. Pilon & W.Z. Wei: Complementary antitumor immunity induced by plasmid DNA encoding secreted and cytoplasmic human ErbB-2. *J Immuno* 167, 3367-3374 (2001)
102. Tang D, M. Devit & S.A. Johnston: Genetic immunization is a simple method for eliciting an immune response. *Nature* 356, 152-54 (1992)
103. Ulmer J.B., J.J. Donnelly, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Deck, C.M. DeWitt & A. Friedman: Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259, 1745-1749 (1993)
104. Rosenberg S.A., J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, K.E. Morton, S.A. Mavroukakis & D.E. White: Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen. *Hum Gene Ther* 14, 709-714 (2003)
105. Tagawa S.T., P. Lee, J. Snively, W. Boswell, S. Ounpraseuth, S. Lee, B. Hickingbottom, J. Smith, D. Johnson & J.S. Weber: Phase I study of intranodal delivery of a plasmid DNA vaccine for patients with Stage IV melanoma. *Cancer* 98, 144-154 (2003)
106. Kaufman H.L., W. Conkright, J. Divito, H. Horig, R. Kaleya & D. Lee: A phase I trial of intra-lesional rV-B7.1 vaccine in the treatment of malignant melanoma. *Hum Gene Ther* 11, 1065-1082 (2000)
107. Kaufman H.L., G. DeRaffele, J. Divito, H. Horig, D. Lee & D. Panicali: A phase I trial of intra-lesional rV-TRICOM vaccine in the treatment of malignant melanoma. *Hum Gene Ther* 12, 1459-1480 (2001)
108. Kaufman H.L., K. Cheung, Z. Haskall, H. Horig, C. Hesdorffer & D. Panicali: Intra-lesional rF-B7.1 versus rF-TRICOM vaccine in the treatment of metastatic cancer. *Hum Gene Ther* 14, 803-827 (2003)
109. Dudley M.E., J.R. Wunderlich, P.F. Robbins, J.C. Yang, D.J. Schwartzentruber, S.L. Topalian, R.M. Sherry, N.P. Restifo, A.M. Hubicki, M.R. Robinson, M. Raffeld, P. Duray, C.A. Seipp, L.J. Freezer, K.E. Morton, S.A. Mavroukakis, D.E. White & S.A. Rosenberg. Cancer Regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298, 850-854 (2002)
110. Rosenberg S.A., M.E. Dudley: Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. *Proc Natl Acad Sci* 101, 14639-14645 (2004)

Key Words: Melanoma, Cancer, Tumor, Treatment, Immunotherapy, Targeted Therapy, Immunoadjuvant, Interferon, Interleukin-2, Dendritic Cells, Review

Send correspondence to: Adam I. Riker M.D., F.A.C.S., H. Lee Moffitt Cancer Center and Research Institute, SRB Room 22043, 12902 Magnolia Drive, Tampa, Florida, 33612; Tel: 813-745-1085, Fax: 813-745-1797 E-mail: rikerai@moffitt.usf.edu

http://www.bioscience.org/current/vol11.htm