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Chemocentric Chemoimmunotherapy: A New Concept in Melanoma Immunotherapy

Paul Howell[1], Soroosh Radfar[2], Yixiang Wang[3] and Hung Khong[1]
[1]*Mitchell Cancer Institute, University of South Alabama, Mobile*
[2]*Dana-Farber Cancer Institute, Boston*
[3]*Central Laboratory, Peking University School and Hospital of Stomatology, Beijing*
[1,2]*United States of America*
[3]*China*

1. Introduction

According to the American Cancer Society, 68,130 new cases of melanoma and 8,700 deaths were predicted for 2010 (Jemal et al., 2010). Although melanoma comprises only 5% of all skin cancers, it is by far the deadliest, responsible for 75% of skin cancer-related deaths, and its incidence is steadily increasing worldwide. Primary cutaneous melanoma has an excellent outcome with early surgical intervention, but regional and distant metastatic disease have a much more dismal prognosis. Current five-year survival rates equal 91% for all disease sites, including 98% for local disease, 62% for regional disease (including regional lymph node involvement), and only 15% for those with distant metastasis (median survival is only 7.5 months). Unfortunately, for those patients who will develop locally advanced and metastatic melanoma, current treatment options are limited in scope and effectiveness; thus, the overall poor mortality rates for these patients emphasize the need for better treatment modalities, methods of relapse prevention and earlier recognition of pre-neoplastic and neoplastic cells (Atallah & Flaherty, 2006; Balch et al., 2001a, 2001b; Blesa et al., 2011).

Currently, there are no curative therapies for metastatic melanoma. Until March 25, 2011, the U.S. Food and Drug Administration (FDA) had only approved two drugs for the treatment of metastatic melanoma, the DNA alkylating agent dacarbazine and high-dose Interleukin-2 (IL-2). Dacarbazine alkylates and crosslinks DNA in all phases of the cell cycle, impairing DNA function, inducing cell-cycle arrest and apoptosis (Bajetta et al., 2002), and is a common chemotherapeutic option for multiple cancers (Seam et al., 2009; Yang & Chapman, 2009). IL-2 activates endogenous anti-tumor-reactive T cells and natural killer (NK) cells. Notably, circulating NK cells from stage IV melanoma patients were recently shown to exhibit a unique phenotypic and functional character toward melanoma cells, and this unique character was altered in patients after treatment with chemotherapy (Fregni et al., 2011). Dacarbazine induces objective tumor regression in 15-20% of metastatic melanoma patients with a median survival of only 8-9 months and a 2-3% complete response rate, and single agent bolus IL-2, while providing a 20-30% objective response rate, creates significant long-term response in only 5% of patients treated, is fraught with toxicity and costly. Combination low-dose IL-2 and chemotherapy and single-agent high-dose IL-2 produce a

similarly low complete response (Atkins et al., 2000; Tsao et al., 2004). Other cytotoxic compounds have resulted in poor response rates and significant adverse effects, including temozolomide, cisplatin, carboplatin, vinca alkaloids, taxanes, and nitrosoureas (Bafaloukos et al., 2002; Bajetta et al., 2002; Blesa et al., 2011; Khayat et al., 2002; Middleton et al., 2000). Notable agents of promise currently undergoing testing are anti-CD40, anti-PD-1L, 1-MT (1-methyl-D-tryptophan), and the BRAF-inhibitor PLX4032 (Bollag et al., 2010; Comin-Anduix; Flaherty et al., 2010; Fonsatti et al., 2010). On March 25, 2011, the U. S. Food and Drug Administration approved ipilimumab (Yervoy™, Bristol-Myers Squibb Company, New York, NY, USA), a monoclonal antibody against the inhibitory lymphocyte receptor, CTLA4, as an injection for the treatment of unresectable or metastatic melanoma (Hodi et al., 2010).

Advanced melanomas are infamously resistant to chemotherapy, principally due to the presence of inherent primary anti-apoptotic and acquired cellular chemoresistance mechanisms (Serrone & Hersey, 1999). Of note, a recent study by Rosner et al. (Rosner et al., 2011) described a means to circumvent such apoptosis resistance. Without activating the apoptosis signaling cascade or utilizing other apoptotic nucleases, recombinant deoxyribonuclease I (DNaseI), engineered with a nuclear localization signal and mutated actin-binding site to avoid entering its inactive bound form, was effective in inducing apoptosis in an apoptosis-resistant melanoma cell model with 70-100% killing efficiency, emphasizing its potential application in many of the known apoptosis-resistant cancers. Surgical resection of primary and known metastatic lesions is still the most effective means of improving overall survival of melanoma patients, though palliative care is standard for patients with metastases after first-line dacarbazine or other physician-preferred therapy, as survival is very rare (Rass et al., 2008; Tagawa et al., 2006; Yang et al., 2006). Surgery in partnership with radiation is standard for locoregional control, while additional chemical-, cell- or antibody- based therapies, or some combination thereof, are commonly attempted for systemic eradication of tumor cells in the blood or at unspecified sites in the body to combat current disease and prevent relapse and metastatic implantation. Melanoma has attracted a lot of interest from immunologists due to a heightened frequency over other solid tumors to display fast, spontaneous, complete tumor regression associated with a specific cellular immune response (Kadison & Morton, 2003). Chemoimmunotherapy, tumor vaccines and other chemotherapeutic combinations have shown improvement in objective response rates for melanoma patients, but they have not yet been able to show significant survival benefits (Bajetta et al., 2006; Eton et al., 2002; Kaufmann et al., 2005). Indeed, a recent meta-analysis of 18 studies comprising 2,625 participants revealed improved objective response rates in metastatic melanoma patients treated with chemoimmunotherapy compared with chemotherapy, but no improvement was noted in overall survival benefit (Sasse et al., 2007). Additionally, hematological and non-hematological toxicities were greatest in those undergoing chemoimmunotherapy.

While the historic chemoimmunotherapeutic approach has been to sensitize tumor cells to immunotherapy with administration of chemotherapeutic compounds, we have described a high degree of chemosensitization with pre-treatment of non-tumor-Ag-specific CD4+ T cells (Radfar et al., 2009), our termed 'chemocentric chemoimmunotherapy' strategy. This technique may provide a basis for development of novel methods of selectively reducing the chemoresistance of virtually all tumor cell types, given the lack of requirement for tumor-Ag-specific T cells. We will present an overview of conventional chemoimmunotherapy development for melanoma and examine the functional significance of chemocentric chemoimmunotherapy for melanoma and other cancers.

2. Background

It has long been part of the clinical canon that chemotherapy drugs negatively affect immunological systems, inhibiting anti-tumor immune activity by a variety of related mechanisms. However, the intricacies of the chemo-immuno interaction provide areas of opportunity for utilizing chemo drugs to enhance the immune response. These cytotoxic drugs tend to target dividing lymphocytes, necessary for development of an immune response, but they also deplete CD4+/CD25+ regulatory T cells (T reg), potentially enhancing the response. Again, conversely, lymphodepletion triggers homeostatic T cell reconstitution which proceeds to create T cells recognizing tumor as self. Opportunities arise during the depletion of T reg and the heightened formation of T cells, allowing an avenue to both infuse the patient with tumor-reactive T cells which are now not impeded by T reg and the potential to create within the patient a large quantity of T cells reactive to some tumor antigen(s).

Conventional cancer chemotherapy is capable of inducing the death of immunologically sensitive tumor cells by utilizing the host innate and adaptive immune responses, e.g. through the induction of immunoregulatory cytokines (Bracci et al., 2007), and some of these drugs can alter cellular pathways of immune suppression and tolerance in a dose- and time-dependent manner, e.g. inducing homeostatic proliferation (Bracci et al., 2007; Proietti et al., 1998), or modulate the extracellular release of certain proteins upon cell death, e.g. HMGB1 release and subsequent tumor survival/metastasis-promoting inflammation is inhibited by oxaliplatin (Dong et al., 2007). Additionally, some chemo drugs can modulate the expression of tumor antigens and antigen processing/presentation machinery on tumor cells. For example, 5-Fluorouracil (5-FU) has been shown to induce carcinoembryonic antigen (CEA) expression in colon and breast cancer cells (Correale et al., 2003) while 5-aza-2′-deoxycytidine can induce the expression of cancer testis antigens and the cell surface MHC class I complex in melanoma and other cancers (Adair & Hogan, 2009; Coral et al., 2002; Fonsatti et al., 2007; Natsume et al., 2008). Notably, direct intralesional injection of the MHC class I complex, which allows tumor immune evasion when defective in numerous cancers (Lampen & van Hall, 2011; Maleno et al., 2011), via high-dose Allovectin-7® (Vical Inc., San Diego, CA, USA), a cationic lipid-formulated bicistronic plasmid encoding MHC-I components β-2 microglobulin and HLA-B7, in 127 recurrent or previously unresponsive to chemotherapy stage III and IV melanoma patients tested for efficacy in a recent phase II dose escalation study produced an objective response of 11.8% with median duration of response of 13.8 months (Bedikian et al., 2010). Tissue from two responding patients was noted for the absence of melanoma upon histological analysis, and the therapy was well tolerated.

Drugs of complementary activity and dissimilar toxicities are routinely combined to enhance individual anti-tumor effects. In the 1960s, effective combination antibiotic therapy for tuberculosis influenced the combination treatment strategy of acute lymphocytic leukemia and lymphoma. A number of significant combination chemotherapeutic regimens were developed in the 1980s, including CVD (cisplatin, vinblastine, and dacarbazine) and the Dartmouth regimen (cisplatin, carmustine, dacarbazine, and tamoxifen) (Del Prete et al., 1984; Legha et al., 1989). Notably, according to two independent clinical trials of the 1990s, CVD and Dartmouth were not shown to significantly increase overall survival relative to dacarbazine alone (Buzaid et al., 1993; Chapman et al., 1999). Although dacarbazine and cisplatin-based combination chemotherapy/biochemotherapy regimens combining IFN

and/or IL-2 are now recommended by the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for Melanoma for stage III and IV melanomas, promising increases in response rates notwithstanding (Riker & Howell, 2010a, 2010b), significant improvement in long-term responses has yet to be seen.

Early efforts to combine chemotherapy with immunomodulators, particularly cancer vaccines, were discounted given the understood detrimental myelosuppressive properties of many chemotherapeutic compounds (Emens, 2008). This suppression was purported to be short-lived and reversible, as the replenishing of leukocytes following chemotherapy would show, by Harris et al. in 1976 (Harris et al., 1976). As reviewed by Sinkovics and Horvath (Sinkovics & Horvath, 2006), around this time, the M.D. Anderson Hospital Melanoma-Sarcoma Service submitted multiple grant applications to the NCI for combination chemoimmunotherapeutic intervention strategies for patients with metastatic melanomas and sarcomas. Despite NCI rejection, clinical trials began, utilizing the findings of Lindenmann and Klein (Lindenmann & Klein, 1967) from nearly a decade earlier which showed an acquired immunity to tumor formation in mice injected with the cell culture lysates of oncolytic myxo- or paramyxovirus-infected tumor cells. Sinkovics et al. further adapted this strategy for the post-chemotherapy administration of cultured tumor cell lysates infected with oncolytic, attenuated PR8 influenza A virus. PR8 viral oncosylates, the immunotherapeutic suspension, were comprised of UV-irradiated, endotoxin-free tumor cell cultures with low levels of live PR8 virus. 'Tumor-specific active immunization' with viral oncosylates two-to-three weeks post-chemotherapy resulted in heightened rates of remission with moderate, occasionally significant increases in survival.

Early phase clinical trials have been able to show response rates of up to 50% for chemoimmunotherapies (Atkins et al., 2003; Eton et al., 2002; Keilholz et al., 1997; Rosenberg et al., 1999); however, the duration of responses has been limited in most cases to less than a year. Importantly, systemic toxicities and adverse side effects tend to be heightened with chemoimmunotherapeutic interventions in relation to chemotherapy or immunotherapy alone. Furthermore, many of the most common chemotherapies were not inherently designed to take into account the entire tumor-host microenvironment and the effects of such therapies in conjunction with locally and systemically active immunological mechanisms. Designing treatment course strategy according to the character of the tumor-host microenvironment milieu is vital for its long-term efficacy (Formenti, 2010; Pages et al., 2009; Zitvogel et al., 2008). Today, thirty years after the first modern chemoimmunotherapy clinical trials were initiated, the impasse that is melanoma chemoresistance has continued to stymie significant, clinically-applicable advancement in this therapeutic field. Given the recurring roadblocks of current treatment strategies, traditional methods of chemotherapy and immunotherapy use are being radically adjusted to achieve a better overall clinical outcome.

3. Conventional chemoimmunotherapy for the treatment of melanoma

Immunotherapy can play an important role in the treatment of cancer, and combinations of chemotherapy and immunotherapy have been investigated in many clinical trials. Although some phase III trials have shown mixed results regarding duration of survival, meta-analyses have found no improvement in overall survival (Allen et al., 1998; Ives et al., 2007; Keilholz et al., 1998; Sasse et al., 2007). Chemoimmunotherapy, or biochemotherapy, has been extensively studied in melanoma. Conventional chemoimmunotherapeutic strategies

include two major categories. The first category uses cytokines such as IL-2 and/or IFN-α in combination with chemotherapy. This experimental strategy was not supported by strong preclinical evidence and has not been shown to be superior to chemotherapy alone (Atkins et al., 2003; Eton et al., 2002; Keilholz et al., 1997, 2005; Rosenberg et al., 1999). The second category is a strategy that we have termed 'immunocentric chemoimmunotherapy'. In this approach, the primary or central focus is on immunotherapy, with chemotherapy playing a peripheral role in immunomodulation. Using this approach, melanoma patients have been successfully treated with non-myeloablative, lymphodepleting chemotherapy and adoptive cell transfer with tumor-Ag specific lymphocytes and high dose IL-2 (Dudley et al., 2002, 2005). Although this strategy is promising, it is not widely applicable to non-melanoma cancers due to the difficulty in generating large numbers of tumor-Ag specific tumor-infiltrating lymphocytes. To date, there have been no studies using chemoimmunotherapy that have shown a significant improvement in overall survival (Atkins et al., 2003, 2008; Bajetta et al., 2006; Eton et al., 2002; Kielholz et al., 1997, 1998, 2005; Middleton et al., 2007; Punt et al., 2006; Ridolofi et al., 2002; Rosenberg et al., 1999), and the development of immunotherapy for the treatment of a broad range of cancer types is still lacking. Given the proven capacity of many already developed and thoroughly tested, well characterized chemotherapeutic agents to have significant, specific and intricate influence over global and localized immune response, adjustments to the multitudes of chemoimmunotherapeutic strategic fronts are currently being made in anticipation of a clinically relevant breakthrough.

3.1 Cytokines and chemotherapy

Activated T cells amplify cytokines that are essential for an effective immune response (Szabo et al., 2003). These include IL-2, TNF-α, IL-21 (by activated CD4+ T cells), GM-CSF, IFN-γ, and other as yet unidentified cytokines. In addition to their immunomodulatory activities, some cytokines can also have direct effects on tumor cells and/or tumor vasculature. TNF-α is known to induce hemorrhagic necrosis in tumors (Carswell et al., 1975; Old, 1985) and has also been shown to induce apoptotic and necrotic tumor cell death *in vitro* (Helson et al., 1975; Laster et al., 1988; Rubin et al., 1988; Sugarman et al., 1985). The hemorrhagic necrotic effect of TNF-α has been shown to be more potent when used in combination with chemotherapy in a rat sarcoma model (Manusama et al., 1996). However, in these studies, TNF-α had no direct activity against tumor cell lines *in vitro* and demonstrated no synergy with chemotherapy, acting instead on the tumor vasculature (van Horssen et al., 2006; Watanabe et al., 1988). The IFNs can exert direct effects on the proliferation, differentiation, and apoptosis of tumor cells (Chawla-Sarkar et al., 2001, 2003; Detjen et al., 2001; Gooch et al, 2000; Pfeffer et al., 1998). However, the response to the IFNs varies considerably, depending on the tumor histology, and resistance to the IFNs has been reported in several tumor types (Burke et al., 1999; Harvat & Jetten, 1996; Kaplan et al., 1998; Phan-Bich et al., 1997).

Several early-phase trials of cytokines combined with cisplatin-based therapies have shown an overall response rate of ~50%, though such responses are of very short duration and often complicated by a high degree of systemic toxicity and adverse side effects. Over the past 15 years, numerous cisplatin-based cytokine combination regimens have been assessed (Atkins et al., 2002, 2003; Atzpodien et al., 2002; Buzaid et al., 1993; Flaherty et al., 2001; Legha et al., 1989, 1998; McDermott et al., 2000; Pejcic et al., 2010; Rosenberg et al., 1999,

2011; Thompson et al., 1997). Notably, in a phase II trial from 1997, Thompson et al. describe the development and utility of an outpatient chemoimmunotherapy treatment regimen of monthly cycles of intravenous cisplatin, carmustine, dacarbazine, and tamoxifen plus self-administered subcutaneous recombinant IL-2 and IFN-α for 32 metastatic melanoma patients, 30 assessed for clinical response (Thompson et al., 1997). After three months, they note a complete response rate of 13% and a 30% partial response, and toxicity was primarily restricted to minor fever and nausea with only a few instances of hospitalization for toxicity management.

Rosenberg et al. (Rosenberg et al., 1999) reported in 1999 on their phase II clinical trial testing tamoxifen, cisplatin, and dacarbazine in 52 metastatic melanoma patients, while the 50 patients in the other arm received the same therapy followed by IL-2 and IFN-α2b. The chemoimmunotherapy arm exhibited 44% total response rate while the chemotherapy arm was 27%; however, of the seven complete responders, only three received the cytokines, indicating no significant impact of cytokines on complete response, and median duration of response was less than six months. Even more disappointing, most of the toxicities were reported for the chemoimmunotherapy group. Median duration of survival was 10.7 months for those receiving cytokines compared to 15.8 months for those without. A regimen of concurrent treatment with CVD chemotherapy, IL-2 and low-dose IFN-α has shown to be better tolerated and more practical than sequential treatment of CVD followed by IL-2/IFN-α (Atkins et al., 2002; Eton et al., 2002; Legha et al., 1998), though randomized phase III trials of this CVD/IL-2/IFN-α combination show no improvement in response or survival over CVD and modified CVD chemotherapy regimens alone (Atkins et al., 2003; Keilholz et al., 2005). In regard to pre-treatment of a chemoimmunotherapeutic regimen with chemotherapy, Punt et al. (Punt et al., 2006) reported on a randomized, phase II clinical trial of metastatic melanoma patients receiving either dacarbazine, cisplatin, IFN-α and IL-2 or the same combination after an initial treatment of two cycles of dacarbazine, finding no difference between the two groups in terms of median overall survival.

O'Day et al. (O'Day et al., 2009) treated 133 chemotherapy-naïve metastatic melanoma patients with CVD, decrescendo IL-2, and IFN-α2b with granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine support. Maintenance biotherapy with low-dose IL-2 and GM-CSF followed by intermittent pulses of decrescendo IL-2 over 12 months was provided for those without disease progression. The overall response rate was 44% with 8% complete response and 36% partial response. Stable disease was noted for 29% of patients; median progression-free survival was 9 months, and median survival was 13.5 months with 12-month and 24-month survival rates at 57% and 23%, respectively, providing evidence of above average long-term survival rates for these patients. Further randomized trials need to be carried out.

A recent multicenter phase II chemoimmunotherapy trial of the Dermatologic Cooperative Oncology Group (DeCOG) testing the multi-kinase inhibitor sorafenib in combination with pegylated-IFN-α2b (PEG-IFN-α2b) in 55 patients with metastatic melanoma resulted in two patients with partial response and 14 with stable disease (29.1% disease control rate in intent-to-treat population) (Egberts et al., 2011). Median progression-free survival was 2.5 months, and toxicities were primarily hematological, including one treatment-related bleeding complication leading to death. A smaller study of 22 metastatic melanoma patients receiving a regimen of CVD with PEG-IFN-α2b, subcutaneous IL-2 and oral 13-cis-retinoic acid, with maintenance biotherapy for those with clinical response after six courses,

provided 12 objective responses with seven maintenance-receiving patients having stable disease after six months (Recchia et al., 2008). Median progression-free and overall survivals were 23.3 and 45.7 months, respectively, with mostly hematological toxicities and 21% of patients exhibiting grade 2 autoimmune reactions.

3.2 Immunocentric chemoimmunotherapy

Another conventional strategy is one that we have termed 'immunocentric chemoimmunotherapy'. This strategy is an immunotherapy-focused approach which uses chemotherapy as an immunomodulator to enhance the effect of cancer vaccines or adoptive cell transfer therapy. Based on this approach, recent studies using chemotherapy to prepare the host before the infusion of *ex vivo* activated, melanoma Ag-specific tumor-infiltrating lymphocytes and high dose IL-2 have resulted in impressive response rates.

3.2.1 Cancer vaccines

Cancer vaccines are promising for their abilities to elicit both humoral (antibody) and cellular (T cell) immune responses (Finn, 2008; Schoenfeld et al., 2010) specifically against tumor cells, as well as for their minimal side effects and the potential for development of immunologic memory allowing for a long-term, durable response to treatment without the need for continuous therapy (Yannelli & Wroblewski, 2004). The presence of cell surface antigens and receptors, capable of activating a myriad of intracellular biochemical pathways, makes possible the development of cancer vaccines with highly stringent criteria for target tumor cell selection and may assist in the focused delivery and activity of chemotherapeutic agents. The recent FDA-approval of sipuleucel-T (Provenge®; Dendreon Inc., Seattle, WA, USA), the first personalized therapeutic cancer vaccine, for the treatment of advanced prostate cancer, has opened the doors for novel vaccine development for other cancers, contributing to the heightened interest in targeted immune-based study of cancer systems.

Nistico et al. (Nistico et al., 2009) recently reported on their phase I/II pilot study testing 36 HLA-A2+ disease-free, stage II to IV melanoma patients with standard-dose dacarbazine administered one day before vaccination with HLA-A2 restricted melanoma antigen A (melan-A/MART-1) and gp100 melanoma peptide vaccination compared with vaccination alone. Dacarbazine significantly increased the numbers of peptide-specific CD8+ effector memory T cells which recognize and lyse HLA-A2+/melan-A+ tumor cells, indicating the enhancement of effective CD8+ T cell recognition of vaccine peptides after treatment with a chemotherapy drug in patients with melanoma. Another more recent pilot clinical trial (Palermo et al., 2010) by the Nistico group examined the role of dacarbazine again as pre-treatment to this vaccine therapy, noting a progressive widening of the T cell receptor repertoire diversity with concomitant high avidity and tumor reactivity in melan-A–specific T cell clones of patients treated with this chemoimmunotherapy and a trend toward longer survival. Alternatively, patients receiving vaccine alone exhibited a tendency to narrowing the T cell receptor repertoire diversity and a decrease of tumor lytic activity in one patient.

3.2.2 Adoptive cell transfer

One of the most exciting avenues for melanoma therapy is adoptive cell transfer of tumor-reactive T cells harvested from the tumor itself, expanded and activated *ex vivo* using various methods (e.g., high concentrations of IL-2) and then transferred back into the

patient. The success of adoptive cell transfer depends on the specificity of T cells for the tumor and their ability to survive and proliferate in the environment. Notably, intratumoral injection of toll-like receptor agonists were found to enhance the potency of activated adoptive cell transfer against a murine model with established subcutaneous melanoma tumors, acting to enhance IFN-γ production and subsequent killing of the now more immunogenic tumor cells by adoptively transferred T cells (Amos et al., 2011). Metastatic melanoma patients have benefited in certain settings with adoptive cell transfer of tumor-infiltrating lymphocytes in combination with chemotherapeutic lymphodepletion, with an impressive objective response rate of 50-70% (Dudley & Rosenberg, 2007; Gattinoni et al., 2006; Hershkovitz et al., 2010; Khattar et al., 2009; Rosenberg et al., 2008, 2011; Rosenberg & Dudley, 2009). Given the promising results of adoptive cell transfer strategies in melanoma and other cancers, many notable contributions are being made toward a more discrete understanding of this field.

During expansion of tumor-infiltrating lymphocytes for infusion, CD8+ T cells are driven to differentiate into effector cells, losing key costimulatory molecules such as CD28 and CD27. Costimulation with CD137/4-1BB was recently shown to significantly increase lymphocyte survival during melanoma adoptive cell transfer and improve the anti-tumor response (Hernandez-Chacon et al., 2011). Inozume et al. (Inozume et al., 2010) reported on the recovery of function of PD-1 cell surface receptor protein on CD8+ T cells in melanoma digests after *ex vivo* conditioning with IL-2, leading to the production of much more tumor-specific IFN-γ compared with PD-1$^-$ T cells and the proposal of PD-1 functional operation or presence being a capable biomarker for selecting tumor-specific lymphocytes from melanomas. As a common practice in adoptive cell transfer prior to infusion of these lymphocytes, patient lymphodepletion of T reg and other lymphocytes in competition for cytokines allows for a much more potent response to activated tumor-specific T cells (Dudley et al., 2002, Wrzesiniski et al., 2010). Adoptive cell transfer with autologous tumor-infiltrating lymphocytes, conditioned with cyclophosphamide and fludarabine, and IL-2 tested in 50 metastatic melanoma patients selected to receive either 2-Gy (non-myeloablative) or 12-Gy (myeloablative) of total body irradiation resulted in response rates of 49%, 52% and 72% for patients not receiving radiation and those receiving either 2- or 12-Gy, respectively (Dudley et al., 2008). The authors note the possibility for increased tumor-infiltrating lymphocyte activity, proliferation, and/or persistence in the body and at the tumor site as a result of rising cytokine availability following irradiation, having seen a significant increase of IL-7 and IL-15 in the serum following the myeloablative regimen. This trial is part of three whose combined and complete results were recently published by Rosenberg et al. (Rosenberg et al. 2011), providing impressive objective response and overall survival rates. In a panel of 93 heavily pretreated metastatic melanoma patients (86% with visceral metastases and 95% recurring after prior therapy), they found adoptive cell transfer of autologous lymphocytes plus high-dose IL-2, administered within one day following a lymphodepletion regimen of cyclophosphamide and fludarabine or either 2- or 12-Gy of total body irradiation, to produce 52 objective responses (56%) with 20 (22%) complete responses, 19 of which lasted a span of three to seven years.

Other clinical studies have utilized genetically engineered peripheral T cells in place of tumor-infiltrating lymphocytes. Due to limitations in generating tumor-specific T cells for adoptive cell transfer, Morgan et al. (Morgan et al., 2006) reported on the specific conference of tumor recognition by autologous lymphocytes from peripheral blood of 15 metastatic melanoma patients using a retrovirus that encodes a T cell receptor recognizing a tumor

antigen. Adoptive cell transfer of these transduced cells after lymphodepletion resulted in durable engraftment of more than 10% of peripheral blood lymphocyte levels for over two months after infusion. In two patients demonstrating objective regression of metastatic melanoma lesions, circulating engineered cells were maintained at high levels at one year after infusion.

Such viral T cell receptor engineering is currently being studied for advanced melanoma and general (Chinnasamy et al., 2010) cancer therapy by Dr. Steven A. Rosenberg's group and colleagues in The National Cancer Institute's Surgery Branch (Rosenberg & Dudley, 2009). Indeed, they have recently described the selective delivery of IL-12, noted for its limited immunostimulatory clinical application due to toxicity, into the tumor microenvironment upon tumor-Ag recognition by T cell receptors on genetically engineered lymphocytes, enhancing *in vivo* melanoma tumor regression without toxicity (Zhang et al., 2011). Another recent report from this group describes adoptive cell transfer of autologous T cells with a T cell receptor against the NY-ESO-1 cancer/testis antigen, expressed in ~25% of patients with melanoma and other epithelial cancers, resulting in an objective response in five of 11 patients with NY-ESO-1-expressing tumors, with two complete responses of greater than one year (Robbins et al., 2011).

3.3 Cyclophosphamide for melanoma immunocentric chemoimmunotherapy

Cyclophosphamide has been an invaluable chemotherapeutic agent in studying immunocentric methodology and application. Numerous reports have shown the synergistic capabilities of cyclophosphamide in combination with immunotherapeutics, describing its ability to induce cytokine expression, type I IFNs and homeostatic proliferation of B and T cells and to deplete the T reg population from the tumor site, increasing the possibility of CD8+ T cell-mediated targeting and destruction of tumor cells (Berd et al., 1986, 1990, 2004; Bracci et al., 2007; Dummer et al., 2002; Ercolini et al., 2005; Ghiringhelli et al., 2004; Livingston et al., 1987, 1994; Maine & Mule, 2002; North, 1982; Nowak et al., 2006; Proietti et al., 1998; Schiavoni et al., 2000; Turk & Parker, 1982; Zitvogel et al., 2008). An important study in 2005 showed non-myeloablative lymphodepleting chemotherapy with cyclophosphamide and fludarabine conditioning of tumor-infiltrating lymphocytes followed by infusion and subsequent high-dose IL-2 treatment to produce a 51% overall response rate in 35 metastatic melanoma patients previously having received IL-2 therapy, all but one being refractory to IL-2 (Dudley et al., 2005).

A surge of recent studies have expounded on the role of cyclophosphamide in T reg modulation and immune therapies that can take advantage of the T reg depleted tumor environment. Combination cyclophosphamide and an agonist antibody against the co-stimulatory CD4+ Foxp3+ T reg cell receptor OX40 has recently been shown to induce apoptosis of the tumor T reg population and induce the influx of CD8+ T cells resulting in a potent anti-tumor response in an *in vivo* B16 melanoma model (Hirschhorn-Cymerman et al., 2009). Furthermore, Kohlmeyer et al. (Kohlmeyer et al., 2009) reported chemotherapeutic preconditioning with cyclophosphamide prior to adoptive cell transfer and viral vaccination followed by adjuvant peritumoral injections of immunostimulatory nucleic acids to be a highly effective chemoimmunotherapeutic regimen, resulting in complete regression of primary and lung metastatic lesions in the normally adoptive cell transfer-resistant genetically engineered Hgf-Cdk4R24C metastatic melanoma mouse model.

In following the combination of chemotherapy and adoptive cell transfer with immune-enhancing cytokines, such as IL-2 (Dudley et al., 2002, 2005; Dudley & Rosenberg, 2007;

Mihalyo et al., 2004), and the recent characterization of IL-21 with adoptive cell transfer by Hinrichs et al. (Hinrichs et al., 2008), another study describes the boosting of immunocentric therapeutic efficacy with IL-21 (Petersen et al., 2010), an immune-enhancing cytokine that, unlike IL-2, does not support proliferation of activated T reg nor activation-induced cell death (Spolski & Leonard, 2008; Waldmann, 2006). As collective data would indicate, IL-21 likely enhances the T reg-depleting capabilities of cyclophosphamide by abrogating T reg development, via the inhibition of IL-2 secretion, and function (Elsaesser et al., 2009; Fantini et al., 2007; Hinrichs et al., 2008; Peluso et al., 2007; Piao et al., 2008; Yi et al., 2009). Mice with established tumors, subsequently treated with cyclophosphamide and adoptive cell transfer and/or daily injections of IL-21, exhibited better early tumor growth inhibition up to five days after the last (day 7) IL-21 injection and increased circulating tumor-specific T cells with the IL-21 administration. The authors note the potential for continued tumor growth stunting with continued IL-21 injection as well as the importance of timing considerations for this particular design (Skak et al., 2009). A similar study by Salem et al. (Salem et al., 2007) showed a more pronounced effect using peptide vaccination and naïve T cells, possibly a more effective option than pre-primed cells for adoptive cell transfer (Hinrichs et al., 2008).

4. Chemocentric chemoimmunotherapy

We have developed a third strategy for combining chemo- and immunotherapy, termed 'chemocentric chemoimmunotherapy' (Radfar et al., 2009). In this model, chemotherapy plays the central effector role, while immunotherapy is used to sensitize the tumor and its microenvironment to the cytotoxic effect of chemotherapy. This strategy employs the use of nonspecifically activated CD4+ T lymphocytes (aCD4) as a chemosensitizer of tumor cells followed by treatment with chemotherapeutic drugs. The rationale for this strategy is based on the known ability of activated T cells to secrete multiple cytokines that can regulate proliferation and/or apoptosis of tumor cells and the ability of activated T cells to exert direct activity on tumor cells through apoptotic pathways such as the Fas/Fas ligand pathway; for instance, tumor cell apoptosis is initiated when the Fas ligand, present on activated T cells, binds the Fas receptor, present on tumor cells. This model does not depend on Ag-specific activation of T cells and is, therefore, potentially applicable to a wide variety of tumor types and patients regardless of their HLA status. Indeed, preclinical studies examining the role of chemocentric chemoimmunotherapy in melanoma, breast, colon and prostate cancer cell lines have shown a dramatic enhancement of induced tumor-cell apoptosis. With the added advantage of ease of use and cost effectiveness in the clinic, chemocentric chemoimmunotherapy is a promising future methodology that is widely applicable, in terms of patient character schema, cost and basic technical utility, in the clinical setting.

4.1 Chemosensitization with nonspecifically activated CD4+ T cells

According to conventional wisdom, it is counterintuitive to administer chemotherapy immediately after cell therapy because the transferred immune cells will be eliminated by chemotherapy. Although this may be true in the setting of conventional immunocentric chemoimmunotherapy, where immunotherapy plays the major effector role and chemotherapy is used to prepare the host, this approach is rational in the context of chemocentric chemoimmunotherapy, where chemotherapy exerts the principal effector function and immunotherapy is used transiently for the purpose of presensitization.

We have recently demonstrated that presensitization of tumor cells with nonspecifically activated CD4+ T cells (aCD4) greatly enhanced the cytotoxic effect of chemotherapy in both *in vitro* and *in vivo* models (Radfar et al., 2009). This activity was observed for all seven tumor cell lines as well as all four chemotherapeutic agents tested. Soluble factors secreted from the activated CD4+ T cells were found to be responsible for the observed effect, with IFN-γ playing a major role in the chemosensitization of tumor cells. IFN-γ by itself, however, was consistently inferior to activated CD4+ T cells in the chemosensitization of tumor cells.

For three human metastatic melanoma, two human breast, one prostate and one colon cancer cell line, treated with 5-FU, temozolomide (TMZ), carboplatin (Carbo), or paclitaxel, chemosensitization with aCD4 had a dramatic impact on tumor cell viability and *in vivo* tumor formation. TMZ, the oral imidazotetrazine derivative of dacarbazine that is also capable of crossing the blood-brain barrier, ultimately produces the same dacarbazine compound after intake and has been shown in clinical trial to be similarly effective in treating metastatic melanoma as dacarbazine (Middleton et al., 2000). Treatment of the A375 melanoma cell line with TMZ after presensitization with aCD4 resulted in near complete cell death, with residual cell viability as low as 5%, compared to 67% viability for cells treated with TMZ that were not presensitized with aCD4. Similar study of two more melanoma cell lines provided comparable results. Further tests using Carbo, a drug with minimal activity against melanoma, resulted in a decrease of melanoma cell viability of ~83% at the optimal aCD4 concentration, compared with no change in viability after Carbo treatment alone. Non-melanoma cell lines treated with paclitaxel, Carbo or 5-FU all gave similar results.

Presensitization was found to be mediated in large part via cytokines, principally IFN-γ, and not through cell-cell contact. aCD4 supernatants were screened for 13 common Th1 and Th2 pro- and anti-inflammatory cytokines. IL-1b, IL-5, and IL-12p70 levels were negative or minimally expressed. The remaining 10 were tested for in combination against aCD4 alone for their chemosensitizing ability, assessed based on viability after TMZ treatment. There was no increase in chemosensitizing ability of combination IL-2/-4 over cells without cytokine addition, and combinations IL-6/-8 and IL-10/-12p40/-13 produced enhanced activity of 7% and 8%, respectively. Combination IFN-γ/TNF-α/GM-CSF significantly enhanced the cytotoxic activity of TMZ by 17%. While most individual cytokines provided little to no enhancement of TMZ cytotoxicity, IFN-γ in combination with other cytokines consistently provided the greatest impact on chemosensitization, and IFN-γ combinations showed no improvement over IFN-γ itself; however, the effects of IFN-γ by itself or in combination lagged the single combination of all 10 tested cytokines. Administration of aCD4 alone prior to TMZ treatment as a positive control yielded the best results, showing significant enhancement of TMZ cytotoxic cell killing over the 10-cytokine combination.

Lysates from A375 cells presensitized with aCD4 followed by treatment with TMZ were analyzed via a transwell system for cytoplasmic histone-associated DNA fragments as a measure of apoptosis. Melanoma cells treated with TMZ alone or aCD4 alone resulted in slightly higher levels of DNA fragments, ~49 arbitrary units (AU) and 54 AU, respectively, as compared 23 AU for nontreated cells. However, when A375 cells were presensitized with aCD4 and treated with TMZ, the level of DNA fragments detected, 1041 AU, was more than 21-fold over that of the TMZ alone group, 19-fold over the aCD4 alone group, and 45-fold over the nontreatment control. In addition, a significantly higher level of caspase-8 activity, ~2300 RFU, was detected in cells in the experimental group compared with the control

groups, i.e., no treatment (524.4 ± 3.5 RFU), TMZ treatment only (508.6 ± 28.3 RFU), or aCD4 treatment only (392.7 ± 4.0 RFU). A reduction in the relative expression of Bcl-2 protein was also detected in A375 cells treated with aCD4 alone or the combination of aCD4 followed by TMZ, but not in cells treated with TMZ alone. These data demonstrated that presensitization of tumor cells with aCD4 led to a decrease in Bcl-2, and that treatment with aCD4 and TMZ together resulted in enhanced caspase-8 activity, resulting in significant reduction in cell viability through an increase in apoptosis. The combination of aCD4 and chemotherapy yielded dramatic results at 24 hours in viability experiments. To evaluate true pro-apoptotic potential of aCD4 presensitization, apoptosis assays were performed at earlier time points, 12 hours instead of 24 hours after treatment with chemotherapy. For caspase-8 activity, chemotherapy treatment was stopped after 5 to 6 hours. Even at these early time points when aCD4 alone or chemotherapy alone had no discernable effects, the combination of aCD4 and chemotherapy resulted in a dramatic increase in apoptosis and caspase-8 activity.

Two human tumor xenograft models provided evidence of significant tumor growth delay for melanoma and breast cancer after presensitization with aCD4 and subsequent treatment with TMZ or paclitaxel, respectively. The melanoma xenograft also displayed significant growth inhibition. In the first model, injections of aCD4 were made intratumorally, followed 48 hours later with intraperitoneal administration of TMZ, to athymic nude mice bearing human melanoma A375 xenograft. There was a significantly pronounced delay in tumor growth compared with mice receiving no treatment or either treatment alone. Similarly, mice bearing aggressive MDA-MB-231 human breast tumors treated with aCD4 followed by paclitaxel had significant delay in tumor growth compared with each of the control groups. The difference between aCD4 plus paclitaxel versus paclitaxel alone was statistically significant. Interestingly, aCD4 alone had a significant growth inhibition effect on the melanoma xenograft but not on the breast cancer xenograft. This was consistent with the known immuno sensitivity of melanoma compared with other solid tumors. To assess toxicity resulting from the experimental treatment, animal weights were measured and recorded during the course of the study. No statistically significant difference in weight was found between the experimental group and control groups up to day 30 after initiation of treatment.

Some recent studies may help shed light onto the possible mechanisms that underlie the dramatic chemosensitizing effect of aCD4. Inflammation and inflammatory cytokines, as represented by a mixture of IL-1β, TNF-α, and IFN-γ, were shown to generate nitric oxide through the induction of nitric oxide synthase, resulting in global inhibition of DNA repair activity in cholangiocarcinoma cells (Jaiswal et al., 2000). Perrotta et al. (Perrotta et al., 2007) further described chemosensitization through dendritic-cell-mediated intratumoral administration of nitric oxide in a B16 mouse melanoma model. Other studies demonstrated that IFN-β or IL-24 could overcome TMZ resistance in neuroblastoma and melanoma, respectively, through down-regulation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase expression and activity (Rosati et al., 2008; Zheng et al., 2008). Additionally, several recent clinical studies have reported improved response rates and survival with salvage chemotherapy in patients who previously received cancer vaccination and developed an immune response (Antonia et al., 2006; Arlen et al., 2006; Gribben et al., 2005; Schlom et al., 2007; Wheeler et al., 2004). Our model may provide a plausible mechanism to explain these observations as well as to facilitate further understanding, design, and development of improved methods for chemoimmunotherapy in cancer.

5. Conclusion

Despite intense research efforts, scientists are still struggling with the simplest principles of tumor cell proliferation, cell cycle kinetics and the genetic basis of malignant transformation and tumor progression. In particular, treatment options for melanoma are few, and those with metastatic disease have been afforded only meager therapeutic options in the past, maintaining a disappointing 15% five-year survival rate. In somewhat of a contradiction, melanoma is now at the forefront of targeted and immunological therapeutic study, evident by the surging number of relevant publications and the recent addition of ipilimumab to a short list of FDA-approved therapies for metastatic melanoma patients. One of the most popular and theoretically promising of these directions currently being studied is combination chemoimmunotherapy.

While many chemoimmunotherapeutic regimens have resulted in lackluster responses, our novel adaptation of this methodology for melanoma and other cancers, utilizing non-tumor-specific activation of CD4+ T cells as a chemosensitizer, has provided exciting results. We have shown dramatic increases in the efficacies of four functionally unique chemotherapeutic drugs in seven cell lines comprising four different cancer types with our 'chemocentric chemoimmunotherapeutic' strategy. After presensitization with aCD4, cytotoxicity was greatly enhanced for all chemo drugs, both *in vitro* and *in vivo*, against all cell types tested. In addition, we recently described the presensitization of tumor cells with aCD4 prior to gamma-irradiation to significantly enhance cancer cell growth inhibition (Wang et al., 2010). Soluble factors released by aCD4, particularly IFN-γ, were primarily responsible for the observed activity, and TNF-α, though inactive by itself, significantly augmented the radiosensitizing activity of IFN-γ.

Not only are the time and monetary costs of producing non-tumor-specific activated CD4+ T cells far and away less than the current standard of activating against a specific set of patient tumor cell antigens, but, as our data shows, aCD4 may also be applied in patients with a variety of cancer types in synergistic conjunction with the most currently effective and applicable chemotherapy for the individual patient. Regardless of the cell line used or its sensitivity or resistance to a particular chemotherapeutic drug, in all our tested cases, presensitization with aCD4 greatly enhanced the cytotoxicity of the drugs, resulting in near complete cell death. Therefore, patients with a typically chemoresistant cancer, such as metastatic melanoma, may be able to benefit from enhanced chemotherapeutic efficacy with aCD4 presensitization. Additionally, utilization of a much broader range of chemotherapeutic agents may be possible for patients who might normally have only a small number of available options, specific for their tumor type and physical condition. Patients may thus be able to receive treatment sooner, accessing the readily available wealth of well-studied chemotherapeutic drugs without the need to await new drug development and testing. To this end, drug selection may ultimately be better tailored to patient tolerance, creating a therapeutic strategy with greater anti-tumor activity, therefore, less time for development of chemoresistance, and better tolerance which will allow more patients to remain on their particular regimen for its appropriate duration. Indeed, the utilization of this novel technique may be able to provide a more effective and efficient means to combat melanoma and other cancers.

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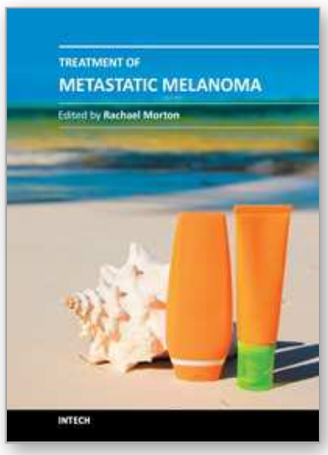

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Surgery continues to be the mainstay treatment for melanoma localized to the primary tumor and/or lymph nodes. Results from randomized controlled trials indicate that sentinel node biopsy for the treatment of cutaneous melanoma of intermediate thickness has a beneficial effect on recurrence rates, and adjuvant radiotherapy to regional lymph node fields following surgical resection reduces loco-regional recurrence in patients at high risk of relapse. Isolated limb perfusion, electrochemotherapy, and photodynamic therapy continue to be evaluated for treatment of stage IV disease. However, the greatest excitement in new treatment has been with targeted therapies for genetic mutations. In particular, the promising results of partial and complete tumor response in stage IV disease from early phase trials of the B-RAF kinase inhibitors. This book provides a contemporary insight into the therapeutic treatment options for patients with metastatic melanoma and is relevant to clinicians and researchers worldwide. In addition, an update on current clinical trials for melanoma treatment has been included, and two chapters have been reserved to discuss the treatment of oral and uveal melanoma.

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Phone: +385 (51) 770 447
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Phone: +86-21-62489820
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