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# Combination Therapies to Improve Delivery of Protective T Cells into the Melanoma Microenvironment

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## 1. Introduction

Incidence rates for melanoma continue to rise between 2-3% each year in the United States [1]. Although melanoma accounts for 5% of new cancer cases, the disease is responsible for most deaths resulting from skin cancer. Five-year survival rates for localized disease have historically been greater than 95% after successfully excising tumors that are less than 1 mm thick [2]. Yet, despite intense efforts in the field, the ability to improve patient survival with invasive forms of the disease has changed little over the past two decades. Current five-year prognostic rates for regional and metastatic melanoma are approximately 66% and 15%, respectively [1].

FDA-approved agents dacarbazine (DTIC), interferon- $\alpha$ , and high-dose IL-2 have long been employed as palliative therapies in advanced-stage melanoma patients (albeit with significant adverse side effects) [3]. Recent exciting data from large multicenter clinical trials has helped usher in the FDA approval of two new therapies that significantly improve upon the efficacy of existing first-line treatments such as DTIC. Ipilimumab is a humanized monoclonal antibody that functionally blocks the CTLA-4 molecule involved in suppressing T cell activation. In a randomized, double-blind phase III study, metastatic melanoma patients with unresectable stage III or IV disease were administered ipilimumab, ipilimumab plus a peptide vaccine specific to the melanosomal antigen gp100, or gp100 vaccine alone [4]. Ipilimumab therapy resulted in at least a 10 month median overall survival compared to 6.4 months for the gp100 vaccine treatment arm, but no statistical differences were observed between the ipilimumab treatment groups. In a fol-

low-up phase III trial, patients with treatment-naive stage III or IV melanoma received DTIC alone or combined ipilimumab and DTIC therapy [5]. Although there was an improvement to median overall survival (9.1 versus 11.2 months, respectively), treatment with ipilimumab/DTIC significantly improved survival rates in patients at 3 years of follow-up. Vemurafenib is a small molecule drug that inhibits the activity of mutant BRAF (BRAF V600E) molecules in melanoma cells that constitutively signal via the MAPK pathway, promoting tumor cell proliferation and preventing cancer cell apoptosis [6]. Patients with previously untreated metastatic melanoma were first screened for the BRAF V600E mutation and then randomized to receive vemurafenib or DTIC in a phase III clinical trial [7]. At 6 months post therapy, vemurafenib resulted in an improved overall survival rate of 84% relative to 64% for DTIC treatment. Objective responses were also observed in 48% of vemurafenib-treated patients compared to 5% confirmed responses in the DTIC treatment arm. Although these preliminary findings are promising, the follow-up time of the study was inadequate to address the final objective and evaluation of progression-free survival rates for these patients is currently ongoing [8]. In a similarly structured phase II trial, vemurafenib administration in previously-treated BRAF V600E-selected melanoma patients led to a median overall survival of 15.9 months, which exceeds that previously observed for standard first-line treatments in patients with metastatic melanoma [9]. Unfortunately, the current level of care for metastatic melanoma remains far below the general expectations of wide-spread durable responses since most patients relapse from the above mentioned therapeutic interventions and eventually succumb to disease.

## 2. Supposed barriers to effective treatment

Improving tumor stage classification, candidate drug/therapy selection, and prediction of a patient's outcome to treatment could result from defining molecular events involved in the transformation of normal melanocytes into melanomas [2]. The delineation of these molecular patterns has proven difficult, however, since melanoma contains high frequencies of dissimilar gene mutations, deletions, duplications, and translocations across the range of patients evaluated [10]. A number of inherited events have been illuminated (transmissible through genetic or epigenetic means) that appear directly involved in initiating a melanocyte's pathway to malignancy by first inducing the clonal selection and outgrowth of cells [11]. Examples include alterations in the kinases BRAF and KIT and the tumor suppressor protein PTEN. The activating BRAF (BRAF-V600E) point mutation occurs in approximately 50% of melanomas (more commonly in cutaneous melanomas) and constitutively drives the MAPK pathway - without upstream RAS activation - leading to cell proliferation and survival [12]. The frequency of BRAF mutations is also preserved among primary and metastatic melanoma lesions, supporting the hypothesis that genetic disruption of BRAF is an early event that does not drive metastasis alone [12, 13]. KIT alterations account for up to 25% of acral and mucosal melanoma subtypes [6, 14]. The most common genetic change in KIT is an activating point mutation that stimulates

the MAPK and PI3K-AKT signaling pathways, promoting cell growth and migration and preventing apoptosis [15]. Additional common melanoma defects are cells disrupted/deficient in the gene encoding PTEN. Under normal physiologic conditions, growth factors bind their respective cell surface receptor tyrosine kinase (RTK) and induce PI3K activity. PTEN serves to block PI3K function by preventing phosphorylation of PIP2 to PIP3, which ultimately drives signaling events through the PI3K-AKT pathway. In the absence of the phosphatase activity of PTEN, the AKT signaling cascade is unrestrained, driving the cell into a pro-survival mode. Simultaneous PTEN and BRAF alterations are two of the more widely documented correlative markers in late-stage melanoma patients and highlight the importance of the overlapping and non-overlapping functions of the AKT and MAPK pathways, respectively, in maintaining a malignant state. The common melanoma genetic aberrations (e.g., BRAF, KIT, PTEN) are not currently utilized for clinical diagnosis or prognosis, though, considering the seemingly paradoxical instances where gene markers do not correlate with independent classifiers of tumorigenesis [2]. For example, PTEN expression profiles have been reported to predict more aggressive forms of melanoma in cases of PTEN gene disruption [16] or activation [17] alongside clinico-pathological results. Drug-candidate discovery and testing has instead flourished with the improved knowledge of recurring primary genetic aberrations that appear to induce melanoma, as highlighted above for the FDA-approved BRAF inhibitor vemurafenib. Many other potential therapies have entered into clinical trials and have been well-described in a recent review [6]. One such promising drug is the RTK inhibitor dasatinib. With regard to melanoma, dasatinib targets KIT (and a limited range of alternate RTKs), leading to the disruption of the MAPK and PI3K signaling pathways. In a recently completed phase I trial, unselected patients with stage III or IV metastatic melanoma were administered dasatinib along with DTIC [18]. Combined treatment resulted in an objective response rate of 13.8% and median progression free survival of 13.4 weeks and appeared to be more active than either agent applied alone based on historical controls. Although these results are promising and support follow-up studies with this TKI, clinical evidence suggests that dasatinib preferably inhibits mutated KIT (occurring at exon 11 or 13) versus overexpressed wild-type KIT in melanoma patients [19-21]. It will, therefore, be of interest to closely monitor the differential anti-tumor efficacy of dasatinib treatment in melanoma patients harboring KIT mutations in future trials in order to select the most suitable patient population for clinical trial accrual.

Monotherapeutic use of drugs specific to the more commonly disrupted signaling pathways in melanoma has several drawbacks. At best, known drug/molecular target combinations are available for no more than 50% of melanoma patients (as in the case with vemurafenib and mutated BRAF), which severely limits treatment options for excluded patients. Drug resistance also presents a major concern in melanoma patients treated under these regimes. Tumor cells are capable of thwarting the benefits of targeted molecular approaches based on a number of innate and acquired mechanisms that include utilizing compensatory cell signaling pathways [22] and survival signals provided by the supportive TME [23]. In the instance of vemurafenib treatment, most BRAF-V600E-selected patients respond to therapy in the short-term (~80%) but fail to maintain durable re-

sponses [24]. Such clinical observations are not specific to melanoma but describe a wider phenomenon of eventually developing resistance to molecularly-targeted approaches in solid tumors [25, 26]. It has been hypothesized that therapy administration actually promotes the natural selection of a resistant tumor mass in the host [27]. These problematic corollaries will have to be overcome through the prudent use of combinational strategies that coordinately attack tumor cells and/or the tumor stroma at multiple, non-redundant levels. As one example, tyrosine kinase inhibitor (TKI) drugs (e.g., sunitinib, axitinib, dasatinib) remain attractive front-line agents to improve the efficacy of other co-applied strategies such as immunotherapy since these small molecule inhibitors may enable heightened responses to immune intervention based on the removal of suppression pathways inherent in the TME (as discussed in subsequent sections).

The initial driver mutations occurring in a melanocyte (e.g., BRAF, KIT, PTEN) are directly implicated in arresting cell cycle control points and promoting the clonal selection and expansion of cells that may disseminate systemically [11]. These primary genetic aberrations also induce an array of secondary events – all of which may contribute to molecular intra- and inter-patient heterogeneity. The pattern of tumor growth typically follows a course, whereby, melanomas transition from a benign radial phase in the epidermis (i.e., nevus) to vertical growth into the dermis and eventual systemic spread [28]. Upon reaching a size of 1-2 mm, a primary tumor nodule is growth-limited based on the need to develop a blood supply capable of providing sufficient nutrients to cells and effectively discharging metabolic waste [29]. To progress beyond this 1-2 mm limit, molecular signals in the tumor must be initiated to promote neovascularization. Hypoxia serves as one stimulus to initiate the expression of vascular endothelial growth factor (VEGF) by melanoma cells [30]. VEGF secretion by tumor cells can also result from inflammatory cytokines derived from infiltrating immunosuppressive cell populations such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). In general terms, locoregional VEGF production recruits endothelial precursor cells by binding its cognate high affinity receptor VEGFR2 [29, 30]. Endothelial cells in turn promote pericyte trafficking and coverage via elaboration of platelet-derived growth factor (PDGF). The effects of angiogenic pathways induced under conditions of tumor growth, however, do not resemble normal physiologic conditions. There is no hierarchical structure of arterioles to venules to capillaries. Instead, the tumor blood supply consists of a chaotic distribution of immature and mature endothelial cells, which are partly due to continued VEGF signaling by melanoma and endothelial cells and pericytes. Chronic VEGF expression serves to antagonize the interaction of endothelial cells and pericytes (by inhibiting PDGF/PDGFR binding) as well as to promote an ongoing cycle of endothelial cell recruitment and proliferation. The end-results are blood vessels comprised of loosely connected endothelial cells with little-to-no pericyte coverage. Consequently, blood flow is severely restricted in areas of the tumor while fluid build-up (e.g., plasma protein extravasation) occurs in the tumor interstitium, all of which contributes to heightened hypoxia, acidosis, and interstitial pressure. These TME dynamics in late-stage disease may help account for melanoma's intrinsic resistance to chemo/radiotherapies [31]. First, the delivery of anti-tumor strategies is impaired due to deficiencies in the tumor-derived blood supply and increased interstitial pressure. The hypoxic environment also directly contrib-

utes to a reduced efficacy of drug function such as in the case of radiotherapies. Lastly, conventional strategies that incorporate cytotoxic drugs have a diminished effect on tumor cells selected for growth under hypoxic and acidic conditions.

### **3. Improving treatment strategies**

#### **3.1. Vascular reconditioning hypothesis**

Correcting deficiencies in the tumor vasculature could potentially circumvent many of the problems that serve to limit the effective treatment of late-stage metastatic melanoma patients as outlined above. Historically, vasculature disruption was hypothesized to starve tumors, leading to apoptosis/necrosis and lesional regression. In reality, anti-vasculature measures appear to primarily modulate the overall tumor blood vessel architecture through actions on immature endothelial cells [32]. These effects lead to transient improvements in blood flow (thereby, diminishing hypoxia and acidosis) and reduced interstitial pressure in the tumor mass [31]. In phase II clinical trials, patients with either metastatic melanoma or colorectal cancer have experienced improved response rates when bevacizumab (an anti-VEGF monoclonal antibody therapy) was combined with a standard of care treatment such as chemotherapy [33-37]. Although bevacizumab monotherapy exhibits minimal clinical impact [38], the antibody appears to exert a helper action by improving the bioavailability/activity of co-delivered cytotoxic drugs via its disruption of the melanoma-associated vasculature. This overarching paradigm has been formally tested in a number of preclinical models showing the improved distribution and efficacy of anti-tumor agents subsequent to tumor blood vessel "normalization" [29]. One caveat to this strategy is the need to consider the optimal schedule for application of each modality to yield superior anti-tumor efficacy. Our laboratory has recently reported that delayed TKI administration in a therapeutic melanoma mouse model negated protection from a dendritic cell (DC) vaccine based on subcutaneous tumor growth kinetics [39]. These studies and others indicate a window of therapeutic opportunity where anti-vasculature measures are highly effective in enhancing co-administered anti-tumor therapies. Melanomas, however, would be expected to become refractory to the action of anti-vascular drugs based on the selection of mature blood vessels that are effectively stabilized by pericytes [32]. As noted with molecular targeting strategies, tumor cells are also likely selected based on their ability to induce angiogenesis via alternate signaling pathways that do not overlap those sensitive to the originally-administered agents. In the absence of an effective second line strategy, increased tumor growth following anti-vasculature monotherapy may instead occur [40].

#### **3.2. Immunotherapy and melanoma**

The immune system provides a promising platform for consideration of inclusion in combined anti-melanoma therapies as it holds many theoretical advantages over standard treatment options such as chemotherapy or bulk cytokine (biologic modifier) administration. Namely, immunotherapies can be tailored to specifically target and kill tumor cells

while leaving the surrounding normal tissue intact. Immune memory (recall) can also aid in sustained therapeutic action as a result of active vaccination, allowing for the maintenance of sub-clinical residual disease (in the adjuvant setting) or the prevention of recurrent tumor variants (i.e., through mechanisms of immune cross-priming and epitope spreading in the protective T cell repertoire). Several clinical studies have highlighted the proof-of-principle for immunotherapy in mediating objective clinical responses in melanoma patients. Therapies incorporating ipilimumab and bevacizumab have been discussed in preceding sections. Impressive clinical results have also been obtained using *ex vivo* expanded tumor infiltrating lymphocytes (TIL; T cells) in combination with rhIL-2 and non-lethal irradiation therapy, although this is a highly specialized process limited to a few locations worldwide [41, 42]. Durable complete responses (CR) (RECIST) have been observed in 22% of patients undergoing this form of treatment and most responses have been durable for > 3 years irrespective of prior treatments. Not all patients are suited to this approach, however, due to the technical constraints of resecting and culturing TIL (approximately 45% of patients are eligible at this stage) and severe toxicities associated with IL-2 administration and lymphodepletion.

The general failure of immunotherapeutic strategies to date likely involves a number of issues. As noted, melanoma is a vascularized cancer that maintains an aberrant blood vessel system. Immunologic strategies that rely on the anti-tumor properties of effector cells such as CD8<sup>+</sup> T cells or antibodies may be unable to penetrate areas of the tumor based on the abnormal dynamics of blood flow and high interstitial pressure. Other melanoma characteristics such as reduced oxygen content and low pH serve to further reduce the function of cytotoxic CD8<sup>+</sup> T cells if they should even be recruited into the TME. First-line strategies that recondition the melanoma-associated vasculature would be expected to overcome such obstacles and allow for the improved delivery and cytotoxic action of immunotherapeutic moieties.

Melanoma is an inherently immunogenic tumor, given the anti-tumor properties of resected TIL *in vitro* [43] and clinical observations that patients with higher frequencies of TIL have improved overall survival [43, 44]. However, the late-stage TME is also quite immunosuppressive. Due in part to the hypoxic nature of the TME, immunosuppressive cells such as regulatory T cells (Tregs), TAMs, and MDSCs become enriched within the tumor and reinforce their own survival/function while coordinately opposing the survival/function of protective T effector cells and Type-1 polarized DCs via soluble mediators and direct cell-to-cell contact [45, 46]. Elaboration of cytokines such as IL-10 and TGF- $\beta$  sustain Tregs and inhibit T cell Type-1 polarization and DC maturation [47-49]. T cells are further suppressed by MDSC secretion of reactive oxygen and nitrogen species, TGF- $\beta$ , VEGF, and arginase (i.e., through L-arginine depletion) [50]. Additionally, melanoma cells can express inhibitory molecules such as PD-L1 on their cell surface that interacts with T cell-expressed PD1, leading to T cell dysfunction and death [51]. Melanoma cells might also prevent DC processing/presentation or T cell targeting through defects in the antigen presenting machinery and/or antigen loss. Therefore, combined immunotherapies must counteract the suppressive TME at some level (e.g., ipilimumab's anti-CTLA-4 mode of action). By reversing the balance of

immunosuppression toward inflammatory Type-1 immunity, one can envision improved clinical benefits for coordinately-applied cancer vaccines. Yet, the optimization of the vaccine sub-component of such regimens remains an area of intense study [52].

### 3.3. Focus on dendritic cell vaccination

DCs provide a theoretical advantage over other vaccine types since they potently stimulate antigen-specific *de novo* and memory recall T cell responses [47]. Under steady-state conditions, a mature DC first migrates out of the periphery and into the TME where antigen is sampled and processed/presented in the form of MHC class I/II-peptide complexes [53]. After upregulating CCR7 expression, antigen-loaded DC become competent to migrate to tissue-draining lymph nodes, where it may provide an antigenic target, costimulation (e.g., DC CD80/86 binding the T cell receptor CD28), and cytokines to allow for the activation of antigen-specific CD4+ and/or CD8+ T cells. These educated effector T cells then return to the blood circulation where a portion of these cells may enter the tumor and perform anti-tumor activities.

Effective vaccination against melanoma antigens (many of which are non-mutated and expressed by normal melanocytes) presents a formidable challenge. Indeed, most tumor-associated antigens are on the whole less immunogenic than tumor specific antigens that arise as a result of viral infection (e.g., HPV induced cervical cancer). Assuming that host central and peripheral tolerance mechanisms have not deleted the appropriate T cell repertoire, the maturation status of the DC may be key to whether specific anti-melanoma T cell responses can be invoked at all. For example, improperly matured DCs may engage responder T cells and induce either anergy or death rather than T cell activation, expansion, and differentiation into effector cells. In addition, the immunosuppressive TME can adversely condition both endogenous DC and T cell survival/function. Immuno-oncologists have attempted to tackle these confounding issues by adoptive transfer of *ex vivo* manipulated DCs (and T cells) that exhibit preferred (normal) bioactivity. In the case of DCs, these cells may be harvested as blood precursors from cancer patients and subsequently polarized to a Type-1 phenotype through genetic manipulation or exposure to a cocktail of inflammatory-prone soluble mediators in culture. After further loading with target antigens associated with tumor cell growth and progression, this cellular vaccine may be reinfused back into the patient. Fully-mature DCs generated in this fashion are able to efficiently home to draining lymph nodes and activate/instruct resident effector-prone T cells while remaining functionally-resistant to TME inhibitory factors such as IL-10, TGF- $\beta$ , VEGF, IL-6, and PGE<sub>2</sub> [54]. The framework for the autologous DC delivery strategy in cancer patients has been validated to some degree with the FDA-approved cellular immunotherapy designated sipuleucel-T. In this protocol, peripheral blood mononuclear cells (PBMCs) are harvested from men with castration-resistant prostate cancer and incubated with a fusion protein containing prostatic acid phosphatase and GM-CSF, a cytokine important for DC maturation [55, 56]. The stimulated PBMCs are then delivered back into patients every two weeks for a total of three injections. In a phase III double-blind multicenter trial, sipuleucel-T resulted in a median survival advantage of 4.1 months in 22% of individuals versus the placebo group [55]. Sipuleucel-T promoted



heightened Type-I T cell and antibody responses against the vaccine fusion protein in a majority of patients presumably due to the enhanced maturation state of infused activated DCs [56]. Overall survival correlated with improved specific immunity in responding patients suggesting that sipuleucel-T's mechanism of action includes immune targeting of prostate carcinoma cells by vaccine-induced T cells.

Many clinical studies have highlighted the ability of DC-based adoptive therapy to boost resident anti-tumor T cell responses and to mediate corollary clinical activity in patients with melanoma [57-65]. In one of the first reported DC-based therapy trials in the melanoma setting, DCs were harvested from patients (regardless of their HLA type), cultured in the presence of rhGM-CSF and rhIL-4 for one week, and pulsed with melanoma-associated peptides (e.g., HLA-A2 restricted gp100, tyrosinase, and Melan-A/MART1 peptides) or autologous tumor lysates [59]. The cellular vaccines were delivered into tumor uninvolved inguinal lymph nodes at least 4 times at weekly intervals. Eleven out of 16 (69%) enrolled patients developed DTH reactions to intradermal injections of DCs loaded with either vaccine-derived peptides or tumor lysates following DC vaccine therapy. Subsequent analysis of infiltrating T cells in representative biopsied DTH sites revealed peptide-specific reactivity to antigenic components of the vaccine. Overall, 2 CR and 3 PR were observed with these same patients also exhibiting vaccine-specific reactivity as evidenced in DTH testing. In a separate phase I clinical trial reported by Ribas and colleagues, GM-CSF/IL-4 *ex vivo* cultured DCs were loaded with a Melan-A/MART1 peptide and delivered intradermally into metastatic melanoma patients a total of 3 times every 2 weeks alongside tremelimumab (anti-CTLA-4) treatment [66]. Tetramer and ELISPOT analysis revealed increases in the frequency of peripheral Melan-A/MART1-reactive CD8+ T cells as a consequence of specific vaccination in 9 of 15 (60%) individuals, although tremelimumab therapy did not appear to enhance Melan-A/MART1 T cell frequency and function. Four vaccinated patients experienced objective clinical responses (2 CR, 2 PR) with 3 individuals also displaying an improved MART-1 T cell response post-DC vaccination. Although such studies provide proof-of-principle, major improvements are still needed in order to achieve durable clinical responses and prolonged survival rates in a majority of patients undergoing autologous DC therapy. A potential improvement to DC activity *in vivo* may reside with how DCs are manipulated *ex vivo* following leukopheresis. In cases where DCs are stimulated to an underwhelmed (use of GM-CSF/IL-4) or exhausted (use of PGE<sub>2</sub>) Type-1 state, effector T cells suffer from an inability to effectively mediate anti-tumor responses [49]. One promising DC polarizing method incorporates IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\alpha$ , IFN- $\gamma$ , and poly-I:C in the *ex vivo* culturing phase to effectively mature DCs (designated  $\alpha$ -DC1). Twenty-two patients with recurrent malignant glioma were administered up to 4 vaccinations intranodally of  $\alpha$ -DC1 loaded with glioma associated antigens at 2 week intervals [67]. At the conclusion of the immunization cycle, 58% of evaluable patients demonstrated a response to at least one antigenic component of the vaccine based on PBMC specific activity through IFN- $\gamma$  ELISPOT or tetramer analysis. Upregulated gene expression profiles of Type-1 cytokines (e.g., IFN- $\alpha$ , IFN- $\gamma$ ) and chemokines (e.g., CXCL10) were also observed in PBMCs from  $\alpha$ -DC1 treated patients, suggesting that the vaccine therapy enhanced the cytolytic activity and trafficking ability of immune cells. Progression free survival was extended to 12 months in 9 of 22 pa-

tients receiving the  $\alpha$ -DC1 vaccine. The ability of  $\alpha$ -DC1 to produce IL-12 (and, hence, stimulate CD4<sup>+</sup> and CD8<sup>+</sup> T cell function) correlated to prolonged progression free survival. Based on the safety profile and relative success of this trial, the  $\alpha$ -DC1 generation protocol is currently being evaluated in a phase I trial in patients with metastatic melanoma (NCT00390338).

Another way to improve the immunogenicity of autologous DC-based therapy involves the choice of antigenic target for presentation to T cells (i.e., therapeutic selection of the responding anti-tumor T cell repertoire for expansion). Most DC-based vaccine trials have incorporated melanoma-associated antigens such as gp100, tyrosinase, Melan-A/MART1 and MAGE in the vaccine formulation. Despite the surprisingly high immunogenic nature of these “self” antigens in vaccinated patients, tumor cells can continue to grow progressively by evading the effector T cell system via various well-described mechanisms [46, 48, 53]. For instance, the tumor mass is composed of a heterogeneous mixture of cancer cells that exhibit a range of defects/deficiencies in the antigen presentation machinery that limits effective presentation of tumor antigen-derived peptides in MHC complexes and leads to poor recognition by the immune system. As such, a fraction of tumor cells may become “invisible” to the adaptive immune system, resulting in the negative selection of treatment-resistant tumor cells in progressor lesions [68]. This scenario can be avoided in part by the use of vaccines incorporating antigens that represent proteins required for maintenance of the transformed state, progressive growth, or metastasis. Alternatively, one may consider the inclusion of antigens expressed not by tumor cells themselves but by the supportive stromal cells (whose phenotype is uniquely modified by the TME) that enable the formation of large bulk tumors. We hypothesize that peptides associated with tumor angiogenesis (summarized in Table 1) may provide an ideal source of targets for DC/peptide vaccine design. In effect, targeting the underlying tumor stroma (e.g., vascular cells, pericytes) would disrupt melanoma growth and promote tumor-specific immunity and protection. Our laboratory has previously demonstrated the ability to successfully treat HLA-A2<sup>+</sup> transgenic mice bearing established colon carcinoma or melanomas using DC-based vaccines containing antigens differentially associated with the tumor vasculature [69]. Animals administered peptide-loaded DC vaccines displayed enhanced protection from established tumor growth and ability, in instances of complete regression, to provide durable protection from dormant disease. Interestingly, active vaccination against tumor stromal antigens led to the corollary cross-priming of T cell responses directed against alternate vascular-associated antigens that were not originally comprised in the vaccine therapy as well as *bona fide* tumor cell-associated antigens. Normal donors and melanoma patients also exhibited immune reactivity to many of the stromal antigens upon *in vitro* sensitization, indicating that operational tolerance to such “self” antigens may be broken using a DC/peptide-based vaccination approach [70]. Importantly, this vaccine strategy appears safe in treated mice since we have not observed deleterious immunologic responses against the normal tissue vasculature, disruptions to the normal cutaneous wound healing process, or aberrations in the fertility/litter size of pre-vaccinated female animals [69, 70].

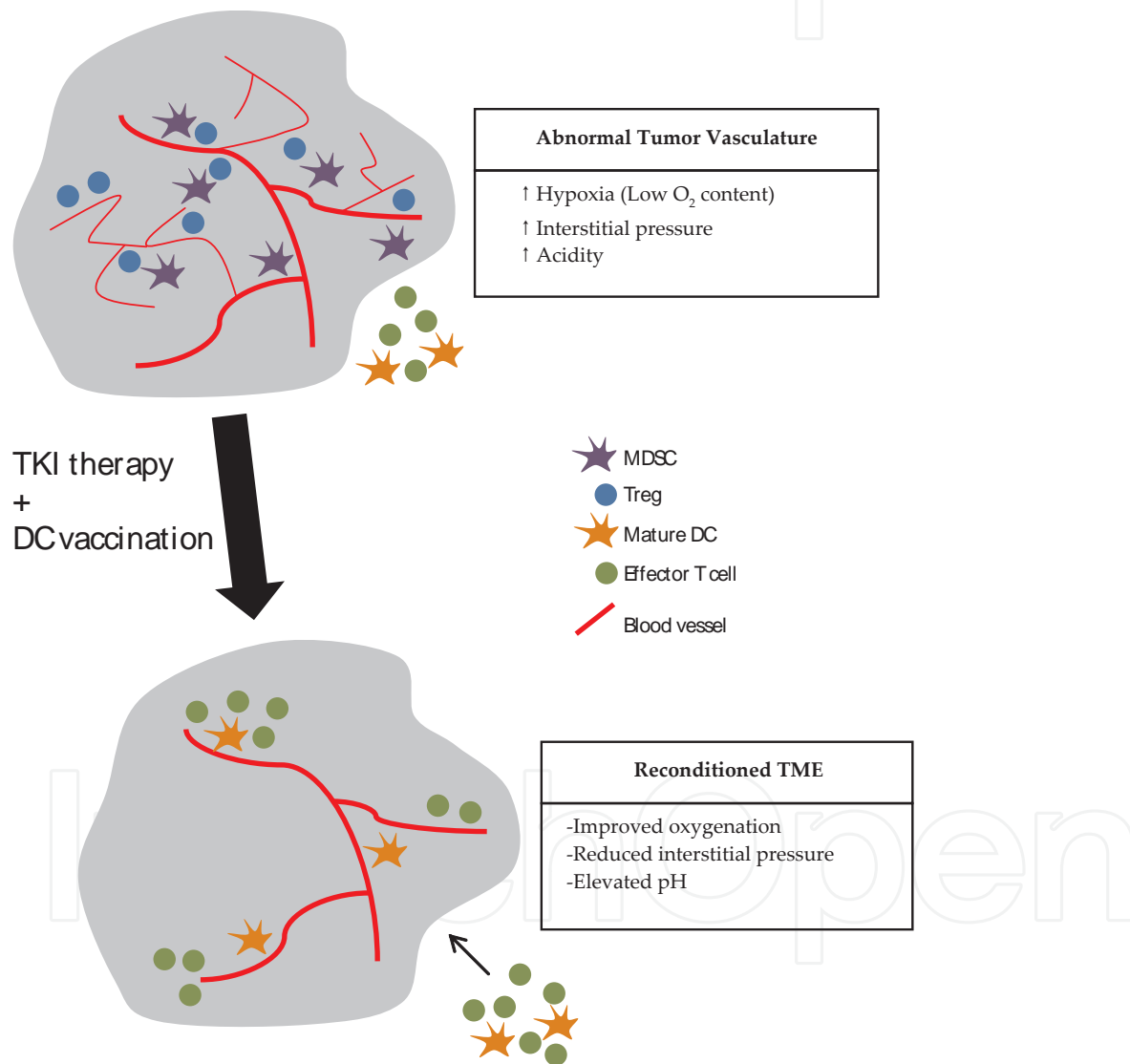
Stromal antigen	Cell expression	AA positions	Peptide sequence	CD8+ T cell response		
				HLA-A2+ transgenic mice	HLA-A2+ normal donors	HLA-A2+ melanoma patients
DLK1	P	269-277	RLTPGVHEL	++	+	++
		310-318	ILGVLTSLV	++	+	++
		328-336	FLNKCETWV	+++	+	++
EphA2	VEC	883-891	TLADFDPRV	+++	+	++
HBB	P	31-39	RLLVVYPWT	+	+	++
		105-114	RLLGNVLVVCV	+	+	+
NG2	P	770-778	TLSNLSFPV	-	-	++
		2238-2246	LILPLL FYL	+	-	++
NP1	P	331-339	GLLRFVTAV	+	+	+++
		433-441	GMLGMVSGL	++	+	+++
		869-877	VLLGAVCGV	+++	+	+
NP2	P	214-222	DIWDGIPHV	-	-	++
		328-336	YLQVDRFL	-	-	++
PDGFR $\beta$	P	890-898	ILLWEIFTL	+++	+	+
PSMA	VEC	441-450	LLQERGVAYI	+	-	+
RGS5	P	5-13	LAALPHSCL	+	-	++
TEM1	VEC/P	691-700	LLVPTCVFLV	+	++	++
VEGFR1	VEC/P	770-778	TLFWLLLTL	++	+	+

**Table 1.** Candidate melanoma-associated vascular peptides for DC vaccine design. CD8+ T cell response summaries are provided from previous work by our laboratory [69, 70]. Naive HLA-A2+ transgenic mice were vaccinated bi-weekly with DCs pre-pulsed with the appropriate antigen-derived peptide. One week following the second DC vaccine, splenic CD8+ T cells were harvested and co-cultured 48 hours with the HLA-A2+ T2 cell line pulsed with the relevant peptide. CD8+ T cell elaboration of IFN- $\gamma$  (as a read-out for Type-1 activity) was then determined through ELISA. Human CD8+ T cell responses to stromal peptides were determined by first isolating PBMCs and stimulating cells in the presence of antigen-loaded autologous DCs for 1 week. Normal donor samples underwent 2 rounds of IVS while PBMCs obtained from melanoma patients were subjected to 1 round of IVS. CD8+ T cell IFN- $\gamma$  expression was assessed as similarly described for HLA-A2+ transgenic mice. Abbreviations used: AA, amino acid; P, pericyte; VEC, vascular endothelial cell; -, No observed activity; +, low activity; ++, medium activity; +++, high activity; IVS, *in vitro* sensitization

### 3.4. Combining small molecule drugs with DC vaccination

In addition to empirically improving DC vaccine design (e.g., via the *ex vivo* conditioning of the APC and a rationale selection of the included antigenic targets), the effectiveness of such treatments would be expected to improve by mitigating the functional constraints on vaccine-induced T effector cells imposed by the generally suppressive TME. As previously

mentioned, the aberrant dynamics of the tumor vascular architecture and enrichment of regulatory cell populations (e.g., MDSC, Treg) in the TME consort to diminish the recruitment, vitality, and tumoricidal activity of immune cells *in situ*. Therefore, the conditional abrogation of the negative attributes of the TME would be predicted to improve infiltration and function of vaccine-expanded T cell populations, leading to more durable objective clinical responses in melanoma patients as diagrammed in Figure 1. What follows are examples of three FDA-approved TKI drugs that could be utilized in DC-based vaccine combination immunotherapies to achieve this goal.



**Figure 1.** Paradigm for effective combination treatment of melanoma. Established vascularized cancers such as melanoma are entrenched with a chaotic blood vessel network and immunosuppressive cell populations. These TME properties serve to prevent the intratumoral delivery and function of single-agent cytotoxic therapies, including specific active vaccination. In cases of combined therapeutic strategies where the melanoma-associated vasculature is first modulated through TKI drug sensitization, for example, immature blood vessels (i.e., endothelial cells loosely decorated by or absent in pericyte coverage) may be disrupted, resulting in a normoxic TME with reduced interstitial pressure and acidity. Frequencies of MDSC and Treg cells are also minimized through mechanisms that are not entirely clear.

Consequently, vaccine-initiated effector T cells can better traffick into tumors and exert their anti-tumor functions. Mature DCs are also able to infiltrate the tumor lesion and sample material from dying cells or necrotic tissue for cross-presentation purposes to unknown/untargeted tumor associated antigens, leading to activation of a broad T cell repertoire that is competent to promote durable anti-tumor immunity. Abbreviations used: TKI, tyrosine kinase inhibitor; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; DC, dendritic cell; TME, tumor microenvironment

Sunitinib binds to and inhibits a range of tyrosine kinases including the vascular associated molecules VEGFR and PDGFR. The drug is approved for use in patients with metastatic renal cell carcinoma (mRCC) or gastrointestinal stromal tumors, where most patients respond favorably to treatment in the short-term [71, 72]. In one recently reported phase I trial, metastatic melanoma patients harboring KIT mutations were administered sunitinib using the FDA-approved regimen of 50 mg/day for 4 weeks followed by 2 weeks off drug [73]. Out of 10 evaluable patients, 1 individual had a CR that lasted 15 months while 2 PR endured between 1-7 months. A separate clinical study, reported on the ability of sunitinib to work in concert with docetaxel therapy in patients with solid tumors including melanoma [74]. Two PR were confirmed in a total of 12 metastatic melanoma patients treated with the combination regimen, supporting a potential tumor vascular "reconditioning" role of sunitinib in improving the delivery and function of cytotoxic therapies within the TME. Our own animal studies support a similar paradigm for combination immunotherapies [39]. Protection from established melanoma progression (based on tumor growth kinetics and survival) were enhanced in mice receiving both sunitinib and DC/peptide-based vaccination versus either agent administered as a monotherapy. Sunitinib co-treatment facilitated the recruitment of DC-"primed" Type-1 CD8<sup>+</sup> T cells into melanoma lesions based in part on the upregulated expression of VCAM-1 (on vascular endothelial cells) and CXCR3 ligand chemokines (e.g., CXCL9, CXCL10, CXCL11) within the TME. This TKI also reduced frequencies of immunosuppressive cell populations such as MDSC and Tregs in the tumor and tumor draining lymph node (TDLN), which was associated with increased cytotoxic potential mediated by vaccine-induced CD8<sup>+</sup> T cells. Sunitinib therapy has similarly been reported to prevent the peripheral accumulations of MDSCs and Tregs in mRCC patients [75-77]. Although the molecular mechanism underlying these alterations remains an open question, sunitinib inhibits STAT3 activation (via inhibition of upstream tyrosine kinases) which may prove core to its perceived anti-tumor actions [39, 75].

Axitinib is a potent TKI targeting VEGFRs (VEGFR1, 2, and 3) that support tumor angiogenesis [30, 78]. Following the completion of a recent phase III trial [79], axitinib was granted approval by the FDA as a second-line therapy for mRCC patients refractory to first-line treatment options including sunitinib. Axitinib has also been used to treat patients with melanoma. Pre-clinical studies have supported a role for axitinib monotherapy to disrupt angiogenesis and tumor formation in xenograft melanoma models [80]. A multicenter phase II trial also justified the continued use of axitinib-based treatment in metastatic melanoma patients [81]. Individuals receiving this TKI experienced reductions of VEGFR2 and VEGFR3 and increased levels of soluble VEGF in their plasma. Treatment with axitinib was associated with an overall objective response rate of 18.8%, which is comparable to historical response rates for chemotherapy and IL-2-based therapies. Given the relative clinical success for axitinib monotherapy, we assessed the impact of axiti-

nib on DC/peptide-based vaccination on established melanoma growth in murine models [82]. Melanoma-bearing mice administered axitinib and specific vaccines were protected from tumor growth and displayed enhanced survival for up to 80 days following melanoma implantation. Axitinib-sensitization improved the trafficking and retention of vaccine-induced CD8<sup>+</sup> T cells in the TME, with the Type-1 functionality (as assessed by IFN $\gamma$  expression) of CD8<sup>+</sup> T cells elevated in both the tumor site and the TDLN. Similar to our observations with sunitinib [39], axitinib reduced systemic frequencies of MDSCs and Tregs and promoted a Type-1 TME, as evidenced by the upregulated expression of Tbet, IFN- $\gamma$ , CXCR3, and CXCL10 gene transcripts.

Dasatinib has already been reported to selectively abrogate mutated KIT activity in human melanomas [19, 83]. This TKI also inhibits other tyrosine kinases such as the Src family of kinases (impacting PI3K-AKT signaling) involved in melanoma adhesion, motility, and invasion [84, 85]. As a monotherapy, dasatinib was well-tolerated in melanoma patients, yielding an objective response rate comparable to alternate current first-line treatment options [18]. Dasatinib diminishes tumor angiogenesis by inhibiting the tyrosine kinases EphA2 and PDGFR that play significant roles in endothelial and pericyte biology, respectively [84]. In unpublished results from our laboratory, dasatinib mediates anti-TME effects that are similar to sunitinib and axitinib in melanoma-bearing mice [39, 82]. Animals treated with dasatinib undergo a restructuring of the tumor vasculature in association with reduced hypoxia and MDSC/Treg frequencies and increased accumulation of T effector cells in the TME, particularly when combined with a DC/peptide-based vaccine. The combined therapy also yielded greatest objective clinical benefit when compared with either monotherapeutic approach. Overall, these studies have supported the design of a pilot phase II trial (dasatinib + DC/tumor stromal antigen-based vaccine) at the University of Pittsburgh planned to begin enrolling patients in Q4 2012. In this trial, HLA-A2<sup>+</sup> patients with advanced-stage melanoma will be administered dasatinib and an autologous  $\alpha$ DC1/peptide vaccine, with frequencies of antigen-specific T cells monitored in patient blood and tumor biopsies over time along with objective clinical responses.

#### 4. Conclusions

The emergence of ipilimumab and vemurafenib as treatment alternatives to the long-standing DTIC-, IL-2-, and IFN- $\alpha$ -based therapies attests to progress made in treating patients with metastatic melanoma. Although the genetic heterogeneity of melanoma cells has confounded high-throughput sequencing technologies, patterns of molecular aberrations are becoming clearer and help support the clinical application of FDA-approved small molecule drugs (such as TKIs) as therapeutic options in eligible patients. Select TKIs (e.g., sunitinib, axitinib, dasatinib) not only directly inhibit melanoma growth and progression by specifically disrupting cell intrinsic signaling pathways, but these drugs indirectly perturb tumorigenesis based on their “normalizing” effects on the TME. Central to this therapeutic paradigm is the ability of the drugs to recondition the chaotic architecture and fluid dynamics of the blood vasculature in the TME. The short-term consequences of TKI sensitization

are impressive and include a reversal of hypoxia, acidosis, and interstitial pressure in the TME, which allows for a corollary improvement in the accumulation and action of co-applied cytotoxic therapies (including immunotherapies).

Combinational immunotherapies hold great promise in minimizing/preventing the emergence and progression of (same) therapy-resistant melanoma populations, as has typically been observed in cases of single-agent treatment strategies. These approaches also have potential to result in a state of perpetual disease dormancy which may extend patient overall survival [69]. The current challenge to the field is to determine the best combination (dosing and scheduling) of agents to best affect a state of durable clinical benefit in the advanced-stage disease setting. From our work, and that of many others, immunotherapy represents one promising component of such combined treatment strategies, particularly when integrated with agents that act as immune adjuvants, inhibitors of immune regulatory cells, and “normalizers” of the TME. Preclinical studies have clearly justified the combined strategy of TKI drug therapy alongside specific DC/peptide-based vaccination. In particular, TKI administration essentially serves as an “immune adjuvant” by reversing the inherent immunosuppression of the TME upon diminishing frequencies of suppressive cell populations and physically manipulating the tumor vasculature architecture. Vaccine-initiated effector T cells are then able to more effectively infiltrate a tumor lesion in order to perform their clinically-beneficial cytolytic functions. Prospective clinical trials will test the validity of this operational biologic paradigm on patient outcome and define a series of safe and effective combination treatment options for melanoma patients.

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