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Combination strategies to enhance the potency of monocyte-derived dendritic cell-based cancer vaccines

Dendritic cells (DCs) are potent inducers of adaptive immunity and their clinical use in cancer vaccine formulations remains an area of active translational and clinical investigation. Although cancer vaccines applied as monotherapies have had a modest history of clinical success, there is great enthusiasm for novel therapeutic strategies combining DC-based cancer vaccines with agents that 'normalize' immune function in the tumor microenvironment (TME). Broadly, these combination vaccines are designed to antagonize/remove immunosuppressive networks within the TME that serve to limit the antitumor action of vaccine-induced T cells and/or to condition the TME to facilitate the recruitment and optimal function and durability of vaccine-induced T cells. Such combination regimens are expected to dramatically enhance the clinical potency of DC-based cancer vaccine platforms.

First draft submitted: 26 May 2016; Accepted for publication: 24 June 2016; Published online: 8 September 2016

Keywords: cancer • combination immunotherapy • dendritic cell • immunoconditioning • immunoregulation • targeted therapy • vaccine

Dendritic cells (DCs) are prototypic professional antigen-presenting cells (APCs) that bridge the innate and adaptive arms of the immune system. These bone marrow-derived leukocytes specialize in the induction and shaping of antigen-specific immune responses by influencing conditions under which cognate T cells undergo activation and subsequent polarized functional differentiation to become T effector (Te) and T memory cells. Based on prior environmental conditioning (i.e., the tissue milieu in which antigen is acquired), the phenotype and functional properties of antigen-loaded DC dictate whether subsequent interaction with cognate naive T cells results in no response, the induction of anergy (hypo-responsiveness) or activation. If activation occurs, the balance of DC-provided signals (including cytokine profiles and the balance of intercellular interactions involving co-stimulatory vs co-inhibitory/checkpoint molecules) determines the degree of T-cell proliferation

versus apoptosis, and the functional polarity of the resulting Te (i.e., characterized by T-cell expression of canonical transcription factors and cytokine production; T helper [Th]1 [Tbet, IFN- γ], Th2 [GATA3, IL-4/IL-5], Th17 [ROR γ t; IL-17], Treg [Foxp3, IL-10/TGF- β], among others).

In peripheral tissues under noninflammatory conditions, DCs are commonly found in an immature state which is specialized for exogenous antigen sampling/uptake (i.e., via pinocytosis, endocytosis, phagocytosis, etc.) and antigen processing/presentation. Tissue DCs undergo 'spontaneous' maturation, or they can be induced to mature under (pro) inflammatory conditions, during which they modulate their cytokine profile and co-stimulatory/inhibitory molecule expression and acquire CCR7 expression, facilitating their trafficking to tissue-draining lymph nodes and interaction with cognate T cells. As a consequence of their conditioning

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tissue environment, DCs become specialized to drive functionally polarized T-cell responses presumably of greatest benefit to the host in reacting to the ‘insult’ impacting the tissue of most recent DC origin. Type I polarizing DCs, often referred to as DC1, produce high levels of IL-12p70 that promote biased differentiation of naive CD4⁺ Th cells into IFN- γ -secreting Th1 effector cells and naive CD8⁺ T cells into IFN- γ producing Tc1/cytotoxic T-lymphocytes (CTLs). Conversely, Type II-polarized DCs, often referred to as DC2, produce very little IL-12p70 but high levels of IL-4 (aka B cell growth factor) that polarize naive CD4⁺ Th cells into Th2 cells (IL-4, IL-5, IL-6, IL-13-secreting) in support of B-cell expansion and differentiation, and the evolution of antigen-specific antibody production. Regulatory DCs express high levels of co-inhibitory molecules and preferentially produce suppressive factors, such as IL-10, TGF- β and indoleamine 2,3-dioxygenase (IDO), that sponsor T_H apoptosis or the development of regulatory T cells (Treg). The practical ability to manipulate/imprint DC functional polarity (*ex vivo*) in order to predictably drive T-cell responses of a given functional polarity allows one to theoretically tailor DC-based vaccine formulations for greatest clinical benefit based on the nature of the disease being treated. In the cancer setting, DC1 would be preferred based on their capacity to elicit inflammatory/cytotoxic T-cell-mediated immunity, while in the transplant/autoimmune settings, regulatory DC would be preferred to foster antigen-specific T-cell anergy/peripheral tolerance.

Enhancing the bioactivity of DC-based vaccines

The safety/tolerability and efficacy of DC-based vaccines to promote type 1 antigen-specific immune responses has fueled the continued use of this immunotherapeutic approach in patients harboring a variety of cancer types. The use of DC-based vaccines to induce anticancer immunity has been actively studied since the mid-1990s, with the DC-based therapeutic vaccine, Sipuleucel-T, being US FDA-approved in 2010 for the treatment of metastatic castration-resistant prostate cancer [1]. Overall, the performance of DC-based vaccines as a monotherapy has been relatively disappointing in the clinical setting, with only rare reports of durable, objective clinical responses based on RECIST criteria [2]. However, it is broadly believed that the use of DC-based cancer vaccines in combination with alternate cancer immunotherapies or radio/chemotherapies may define treatment regimens with enhanced potency in support of protective or therapeutic antitumor immunity. This review will highlight novel combination vaccine strategies currently being developed for the treatment of cancer.

Enhancing the IL-12-producing, type-1 phenotype of vaccine DCs

A major goal of DC-based cancer vaccines is to promote specific Type-1 antitumor immune responses, making it vital to immunize patients with autologous DC that are imprinted to mediate Type-1 polarizing potential. A most advantageous APC type for this end point is DC1 producing the Th1-promoting cytokine, IL-12p70. The Type 1-polarizing cytokine cocktail (TNF- α , IL-1 β , poly-I:C, IFN- α and IFN- γ) preferentially conditions Type 1-polarized, high IL-12p70 producing DC (α DC1) *in vitro*, that elicit high frequencies of melanoma-specific Tc1/CTL effector cells *in vitro/in vivo*. Notably, vaccine DC production of IL-12p70 was observed to positively correlate with time-to-progression amongst patients with recurrent malignant glioma [3,4]. Tumor peptide-loaded α DC1 vaccines are currently being evaluated in a number of combination clinical trials for the treatment of melanoma (NCT01876212), as well as carcinomas of the breast (NCT02479230), colon (NCT02615574), peritoneum (NCT02151448) and prostate (NCT00970203). It is important to note that Type 1-polarized DCs are very responsive to CD40-ligand (CD40L) signaling, based on heightened production of IL-12p70. CD4⁺ Th cells express CD40L and this interaction with CD40 on the Type 1-polarized DCs appears important for amplifying and sustaining Th1-biased immunity *in vivo*. Additional means by which to enhance CD40-CD40L signaling via molecular engineering in the context of Type 1-polarized DC-based cancer vaccines are currently being evaluated [5,6].

DC antigen source

Tumor-specific immunity resulting from DC-based vaccination in cancer patients depends in large part on the type and format of antigen used in the vaccine formulation. The most common antigens used are shared tumor-associated antigens (TAAs), which are highly expressed in tumor cells but expressed at lower levels in normal tissue, theoretically providing a degree of safety in the vaccine-induced (auto)immune response. TAAs can be overexpressed, nonmutated ‘self’ proteins (i.e., pigment-associated gene products in melanoma cells; tyrosinase, TRP-1, TRP-2) or mutated proteins that function as oncogenic drivers common to one or more tumor types, such as the BRAF^{V600E} mutation which is harbored by more than 50% of metastatic melanomas [7]. A drawback for use of such shared, nonmutated TAA in vaccines is that the induction of specific Th1/Tc1 responses requires the breaking of operational tolerance, leading to the development of typically low-avidity T-cell responses. Despite this limitation, TAA usage in cancer vaccines has resulted

in treatment-associated clinical responses in Phase I/II trials [8]. Methods to help overcome ‘self-tolerance’ in cancer therapy are being employed and will be further discussed in the combination strategies section below. New methods to utilize tumor-specific ‘neoantigens’ as a source antigen for DC vaccines are also being developed. Neoantigens are newly-expressed protein antigens in a given patient, which have not been subjected to central tolerance mechanisms in the host, making it possible for personalized DC-based vaccines to activate high-avidity effector T cells that might mediate superior antitumor benefit(s). This technical strength is also a logistic weakness, since DCs targeting neoantigens in a vaccine must be customized to a given patient. However, advancements in high-throughput gene/protein sequencing technologies should facilitate the rapid identification of neoantigens and their application in patient-specific vaccine formulations in the near future [9,10].

In addition to the induction of tumor-specific Tc1/CD8⁺ CTL responses, it has become increasingly clear that the induction of CD4⁺ Th1 helper cell responses is important for CD8⁺ T-cell support and for mediating direct antitumor effects in their own right (i.e., promote tumor inflammation, direct killing via Fas/Fas-L, TNF/TNFR or TRAIL/TRAILR interactions, etc.). Th1-associated cytokines were identified in peripheral blood mononuclear cells and sentinel lymph nodes analyzed in the majority of melanoma patients that received a helper peptide vaccine [11]. In a clinical trial treating advanced-stage melanoma patients, patient response to vaccination with a pool of six melanoma helper peptides was found to be directly correlated with clinical performance and extended overall survival [12].

Combination strategies to enhance DC vaccine potency

Over the past decade great advances have been made in the development and application of targeted therapies for cancer treatment. Most recently, small molecule inhibitors of cell proliferation pathways, such as the mitogen activated protein kinase (MAPK) pathway, as well as immune checkpoint inhibiting antibodies, such as anticytotoxic T-lymphocyte antigen-4 (CTLA-4), antiprogrammed cell death protein-1 (PD-1) and anti-PD-1 ligand (PD-L1) have demonstrated clinical success in cancer patients. Unfortunately, as single modalities these therapies do not result in high rates of durable clinical responses. As a consequence, these and emerging therapies are being explored as ‘adjuvants’ in combination with DC-based vaccinations in translational and clinical studies. The effects these adjuvant therapies have on the immune system are complex and for simplicity sake, we have clustered these strategies

into two broad categories; in other words, immunoregulatory versus immunoconditioning strategies (Figure 1).

Immunoregulatory strategies

An important aspect of tumor biology that contributes to cancer progression and to immune responsiveness to therapy is the evolving nature of the tumor microenvironment (TME). In addition to the tumor cells, stromal and infiltrating immune cells, as well as the endogenous secretome (i.e., cytokines, chemokines, soluble danger signals and suppressive factors such as gangliosides) and cellular stressors (hypoxia, acidosis, high interstitial pressure, nutrient deprivation due to poor tissue perfusion) all contribute to the pathologic TME. The TME in progressive disease recruits and fosters the development of immunoregulatory populations of cells such as Tregs and myeloid derived suppressor cells (MDSC) that act to prevent/retard the function and viability of protective antitumor cell-mediated immunity. Effector cells, both innate (NK) and adaptive (Th1/Tc1 T cells), are in fact capable of targeting tumor cells; however, these effector cells are commonly inhibited/dysregulated in the TME, thereby limiting the efficacy of endogenous and therapeutically-induced host antitumor immunity. However, a range of new treatment strategies designed to antagonize regulatory circuits in the TME hold great promise as stand-alone or co-immunotherapeutic treatment options for cancer patients (Figure 1B).

Targeting checkpoint pathways

Activated tumor-specific T effector cells are frequently found in progressively growing tumors. As an immune defense, tumor cells hijack the immune system’s inherent braking (i.e., checkpoint) pathways designed to limit the exuberance of inflammation under normal physiologic conditions. Hence tumor and TME stromal cell expression of co-inhibitory molecules (CTLA-4, PD-L1 among others) renders tumor-specific T cells inert or promotes their apoptosis. These inhibitory circuits are initiated when the inhibitory checkpoint protein receptors bind to their cognate ligands. Checkpoint proteins are expressed by activated CD4⁺ and CD8⁺ T cells; however, they are constitutively expressed by Tregs which are often recruited to the TME in high numbers. Receptor-ligand interaction on activated T cells dampens the effector response, whereas receptor-ligand interaction on Tregs enhances the inhibitory activity of regulatory cells in support of the immunosuppressive TME. Silencing of these inhibitory pathways by targeting of checkpoint receptors with antagonistic monoclonal antibodies represents a major ongoing area of translational research

Figure 1. Therapeutic modulation of immunity in the tumor microenvironment. (A) In the progressive disease setting, the tumor microenvironment (TME) is typically characterized by an abundance of recruited immunoregulatory cell populations, including Tregs and MDSCs, that restrict/silence the protective action of CD4⁺ and CD8⁺ anti-tumor tumor-infiltrating lymphocyte (TIL). Regulatory cells and tumor cells inhibit or promote the death of protective TIL effector cells via a wide array of mechanisms, including negative signals contributed through immune checkpoint molecules, catabolism of key amino acids (e.g. IDO catabolism of TRP), elaboration of immunosuppressive cytokines (TGF- β , IL-10; not depicted), among others. (B) Immunoregulatory therapy: targeting immune checkpoint proteins (CTLA-4-, PD-1/2-, PD-L1/L2-, TIM-3-, LAG-3-antagonists) or mechanisms of immune suppression (IDO-inhibitor, GITR agonist) with novel antibody- or small molecule-based treatments antagonize regulatory circuits in the TME. Such agents may resurrect existing immune function in the TME or reinforce the anti-tumor action of recruited T cells elicited by DC-based vaccines. (C) Immunoconditioning therapy: small-molecule inhibitors of MAPK, tyrosine kinases, HSP90 and HDACs, as well as conventional chemotherapy and radiotherapy regimens, not only mediate direct tumoricidal activity, but have immunoconditioning effects on the TME (leading to increased CD4⁺ and CD8⁺ T effector cell infiltration, improved APC function, and reduced recruitment/persistence of immune suppressor cell populations such as Treg and MDSCs) that may bolster the efficacy of anti-tumor T cells invoked by DC-based vaccines. Ab: Antibody; APC: Antigen-presenting cell; DC: Dendritic cell; IDO: Indoleamine 2,3-dioxygenase; MDSC: Myeloid-derived suppressor cell.

that is expected to dominate the landscape of immuno-therapeutic clinical trial design for many years to come.

In 2011, the FDA approved ipilimumab, an anti-human monoclonal antibody against CTLA-4, for metastatic melanoma followed by the approval of two different antihuman antibodies targeting PD-1, pembrolizumab and nivolumab, in 2014. Motivated by the preclinical and clinical efficacy for these checkpoint inhibitors when applied as monotherapies or together in combined immune checkpoint blockade (ICB) protocols, including those integrating DC-based vaccines are ongoing or currently under development for the treatment of patients afflicted with a broad range of cancer types. In a successful clinical trial (NCT00090896), MART-1 peptide-pulsed DCs

administered in combination with anti-CTLA-4 yielded increased rates of tumor regression and durable objective clinical response (RECIST) when compared with patients treated with either single modality in the setting of advanced-stage melanoma [13]. Furthermore, results from a Phase II study (NCT01302496) of vaccination with autologous monocyte-derived DCs electroporated to express melanoma-associated antigens (TirMixDC-Mel) [6,14] in combination with anti-CTLA-4 therapy show highly durable antitumor responses in the peripheral blood of advanced-stage melanoma patients, with 38% of vaccinated patients achieving objective clinical response, including 20% with complete responses [15]. In murine models, increased tumor-infiltrating Te activity and improved

overall survival rates were observed in tumor-bearing animals treated with immature DC and radiation plus anti-CTLA-4 antibody [16] or those treated with anti-CTLA-4 and anti-CD134 (OX40) in combination with anti-DEC205/HER2 monoclonal antibody that specifically targets the HER2 TAA into cross-presenting DCs [17].

Additionally, combination anti-PD-1 antibody plus DC-based vaccines are being actively explored by a number of groups, with early-phase clinical trials underway. As a treatment for follicular lymphoma, the enhancement of antitumor CD8⁺ T-cell responses is the goal of a Phase II trial (NCT02677155) combining anti-PD-1 antibody administration with sequential intranodal immunotherapy using rituximab (anti-CD20 antibody) and DCs plus radiotherapy. An autologous DC/myeloma fusion vaccine stimulates more robust antitumor T-cell responses when combined with anti-PD-1 antibody *in vitro* [18], with a clinical trial (NCT01067287) integrating PD-1 blockade following DC/myeloma vaccination promoting increased frequencies of tumor-reactive CD8⁺ T cells and reduced frequencies of Treg in patient peripheral blood, with complete responses noted in a subset of treated patients [19]. Phase II trials examining blockade of PD-1 in conjunction with DC-tumor cell fusion-based vaccines are ongoing for the treatment of acute myelogenous leukemia and kidney cancer (NCT01096602, NCT01441765).

PD-1 expression is upregulated on activated T cells as an immunoregulatory mechanism. In addition to the overexpression of PD-1 ligands by tumor cells, PD-1 ligands (PD-L1/PD-L2) are also commonly upregulated by tumor cells by (immune cell elaborated) inflammatory cytokines, such as IFN- γ . In this regard, DC vaccine-induction of Type-1 T-cell responses are likely to directly contribute to upregulated expression of PD-L1 in the TME, and to the subsequent muffling of protection afforded by therapy-associated PD-1⁺ tumor-infiltrating lymphocytes (TILs). It stands to reason that the introduction of anti-PD-1-based therapy into combination DC-based vaccines will help in alleviating this self-limiting clinical paradigm.

Emerging checkpoint targets

Novel checkpoint molecules continue to be identified as potential targets for the development of interventional therapeutic agents. T-cell immunoglobulin and mucin containing protein-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) are just a few new emerging targets that serve as negative regulators of T-cell activation, function and survival. LAG-3 is expressed by activated T cells, natural killer (NK) cells and B cells, whereas TIM-3 is largely restricted in expression to

activated CD4⁺ and CD8⁺ T cells. TIM-3+ NY-ESO-1-specific CD8⁺ T cells have been demonstrated to be dysfunctional in melanoma patients, with TIM-3 blockade capable of restoring Te activity [20]. Among CD8⁺ TIL, LAG-3 is commonly co-expressed with PD-1 [21], prompting the performance of a Phase I trial examining the clinical/immunologic benefits of treating cancer patients with antagonist LAG-3 monoclonal antibody alone or in combination with anti-PD-1-based immunotherapy (NCT01968109). A LAG-3-Ig fusion protein that targets the natural ligand of LAG-3 (i.e., MHC-II), is also currently being evaluated as an adjuvant within the context of a Phase I MART-1 peptide-based vaccine trial (NCT00324623), where the combination vaccine has shown the ability to induce higher levels of more durable MART-1-specific CD8⁺ T cells, while coordinately decreasing Treg frequencies in the peripheral blood of treated patients [22]. The implementation of these new checkpoint inhibitors as single modalities and in combination with alternate forms of immunotherapy, including DC-based vaccines holds great current/future promise in the clinical management of cancer patients.

Suppressing immunosuppression

One mechanism supporting the development of immunosuppressive Treg cells involves the catabolic conversion of tryptophan into kynurenine by the enzymes, indoleamine 2, 3-dioxygenase 1 (IDO1) and tryptophan 2, 3-dioxygenase (TDO). Tumor cells express high levels of IDO1 and TDO [23,24]; consequently, these enzymes represent two rational new targets for antagonism in the context of combination immunotherapies for cancer. Although there are no FDA-approved IDO1-inhibitors, the IDO1-inhibitor, 1-methyl tryptophan (1-MT), is being clinically developed for treatment of cancer (Indoximod) (NCT00567931). Like immune checkpoint inhibitors, IDO-inhibition is being explored clinically in combination with conventional chemo/radiotherapies and/or immunotherapies. IDO1-inhibition is being investigated in combination with DC-based vaccination in a Phase I/II trial for metastatic breast cancer patients (NCT01042535), in addition to a Phase II trial where it is being combined with Sipuleucal-T for the treatment of patients with prostate carcinoma (NCT01560923). TDO-inhibitors are still under active development [25].

An alternate target for suppressing immunosuppression is the costimulatory molecule glucocorticoid-induced TNF receptor related protein (GITR). GITR is constitutively expressed by naive Tregs but not naive CD4⁺ and CD8⁺ T cells. Activated Tregs and conventional T cells express very high versus high

expression of GITR, respectively. A murine anti-GITR agonist antibody (DTA-1) has been demonstrated to reverse Treg-mediated immunosuppression *in vitro* [26]. Additionally, effector T cells activated through GITR/GITR-L interactions are resistant to subsequent suppression by Tregs [27,28]. Cancer therapies employing GITR-agonists in conjunction with DC-based vaccines are currently open for patient accrual [29]. Finally, NK cells also express high levels of GITR and mediate enhanced cytolytic activity after engagement with GITR-L. Hence, NK-centric immunotherapies combined with GITR agonists and DC-based vaccination also represent potential avenues of study as future treatment options for cancer patients.

Immunoconditioning strategies

The etiology of a variety of cancer types has been associated with driver mutations and overexpressed oncogene products, such as BRAF^{V600E} found in the tumors of more than 50% of patients with malignant cutaneous melanoma [7], BCR-ABL protein tyrosine kinase in chronic myelogenous leukemia patients [30], overexpressed human EGF receptor 2 (HER2) in human breast cancers [31] and anaplastic lymphoma kinase in non-small-cell lung carcinoma [32], among others. The identification of these genetic alterations has led to the research and development of rationally designed small molecule inhibitors that not only mediate direct tumoricidal activity, but also exhibit immunoconditioning effects *in vivo*. In addition to directly killing tumor cells, many conventional chemotherapy and radiotherapy regimens are currently being re-examined for their immunoconditioning qualities. Preferred immunoconditioning agents modify tumor cells or the TME to respond more favorably to immunotherapy approaches, including DC-based vaccines (Figure 1C).

MAPK inhibitors

The BRAF-inhibitors (BRAFi), dabrafenib and vemurafenib, specifically inhibit oncogenic signaling mediated by (constitutively activated) mutated BRAF^{V600E} in relevant forms of cancer (i.e., melanoma), consequently preventing tumor cell proliferation via the MAPK signaling pathway. These inhibitors are highly-efficacious and provide improved progression-free survival (PFS) in treated patients. Unfortunately, the improved PFS is short-lived, with disease relapse occurring in less than 12 months for the vast majority of patients. However, these inhibitors could potentially be combined with specific DC-based vaccines to extend patient survival. Interestingly, BRAFi have been shown to improve antigen presentation by DC and to support enhanced T cell recognition of, and functional activity against,

tumor cell targets [33–36]. Melanoma cells harboring the BRAF^{V600E} mutation suppress DC function when these cells are co-cultured *in vitro*, however, the BRAFi vemurafenib prevents/reverses tumor-associated DC dysfunction in the absence of overt (off-target) deleterious effects on DCs themselves [37]. Additionally, BRAFi have been shown to promote T-cell infiltration into human metastatic melanomas, suggesting their potential use in combination with immunotherapies such as DC-based vaccines or adoptive cell therapy (ACT; [38]). Interestingly, in a melanoma case report, a patient that relapsed following initial treatment with BRAFi subsequently exhibited objective regression of metastatic disease after receiving immunotherapy (ipilimumab + DC-based vaccination) prior to reinitiation of BRAFi treatment [39].

MEK is another protein kinase in the MAPK pathway that lies downstream of RAF and upstream of MAPK. The MEK-inhibitor (MEKi), trametinib, has been FDA-approved for treatment of BRAF^{V600E} unresectable or metastatic melanoma. Objective clinical responses have been reported when administering MEKi as a monotherapy, but when combined with BRAFi, treated patients displayed statistically-significant extension in overall survival [40]. This has prompted the FDA to recently approve the combined use of trametinib with dabrafenib in BRAF^{V600E} unresectable or metastatic melanoma. Similar to BRAFi, MEKi promote increased expression of melanocyte differentiation antigens by melanoma cells, leading to enhanced recognition by antigen-specific T cells [33]. This suggests that MEKi might be best used in conjunction with DC-based vaccines targeting melanocyte differentiation antigens. Furthermore, the combination of dabrafenib and trametinib plus adoptive T-cell therapy promotes complete tumor regression in concert with the presence of enhanced Tc1 TIL in a murine melanoma model [41]. Unlike BRAFi, however, MEKi have been shown to inhibit T-cell function(s) when applied at high doses [33], which would have to be carefully monitored/managed in the context of any combination immunotherapy approach.

Tyrosine kinase inhibitors

Additional targeted therapies using small molecule inhibitors are being explored for use in conjunction with DC cancer vaccines. Tyrosine kinase inhibitors (TKIs) are orally-active, small molecules that compete with the ATP-binding site of oncogenic tyrosine kinases and have demonstrated specificity to tumor cells with fewer off-target toxicities than other conventional chemotherapies [42]. TKIs are being used as monotherapies or in combination with other chemotherapeutic

agents; however, their use in combination with immunotherapies is increasingly being explored in preclinical models and in the clinical setting. In addition to demonstrating direct tumoricidal effects, TKIs are now known to also modulate immune function. Dasatinib (SPRYCEL®), a BCR/Abl, Src, c-Kit (and others) kinase inhibitor that has demonstrated clinical efficacy as a single agent in multiple cancer types, increases the number of tumor infiltrating CD8⁺ T cells and promotes tumor regression in murine melanoma models [43]. When applied in combination with a DC/peptide-based vaccine *in vivo*, the therapeutic efficacy of dasatinib was enhanced with increased tumor infiltrating CD8⁺ T cells and decreased numbers of Tregs and MDSC when compared with either single modality [43]. Combination therapy utilizing dasatinib and DC-based vaccination against tumor blood vessel-associated antigens is currently being explored in the clinic, generating promising preliminary results in a pilot Phase II trial for HLA-A2+ patients with advanced-stage melanoma (NCT01876212). Combinational therapies employing other TKI, such as axitinib (Inlyta®) and sunitinib (SUTENT®), with dendritic cell vaccinations are also being studied in preclinical models of melanoma [44,45], as well as in a Phase II clinical trial of metastatic renal cell carcinoma (NCT02432846). In a Phase III clinical trial (NCT01582672), sunitinib enhanced the therapeutic efficacy of a novel autologous DC/peptide-based vaccine for patients with metastatic renal cell carcinoma [46]. Treatment with the combined regimen similarly led to decreased numbers of Treg and a coordinate expansion in CD28⁺ effector-memory Tc1 cells, with an associated improvement in patient median overall survival [47]. Furthermore, TKIs are being used in combination with non-DC cancer vaccines to enhance their antitumor efficacy. In this regard, sunitinib has been shown to enhance the induction of tumor-specific immune responses as monitored in peripheral blood, to increase tumor infiltration by specific CD8⁺ T cells, and to decrease MDSC content when combined with a viral-based cancer vaccine [48].

HSP90 inhibitors

The antitumor efficacy of DC-based vaccines might also be improved by combining them with heat shock protein 90 (HSP90)-inhibitors (HSP90i). HSP90 is a highly-abundant molecular chaperone that guides and maintains the proper folding of thousands of different client proteins, many of which can support tumor growth, survival and invasiveness [49]. Tumor overexpression of HSP90 leads to an accumulation of tumor promoting wild-type and mutated proteins, making HSP90 an attractive target for disruption in order to

limit tumor-intrinsic pro-oncogenic processes. A range of HSP90i therapies have been developed over the past several decades for use as anticancer agents, with many now known to also modulate immune function. In a murine sarcoma model, a first-generation HSP90i, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), improved the antitumor efficacy of a peptide-based DC vaccine by reconditioning the TME and leading to increased recruitment of therapeutic T cells (effector CD4⁺ and CD8⁺) into the TME, with downmodulation of tumor-associated Treg and MDSC populations also coordinately observed [50]. In addition, accumulated HSP90 client proteins in tumor cells have been reported to undergo conditional proteasome-dependent degradation after treatment with the HSP90i 17-DMAG, resulting in the enhanced loading of MHC-I complexes with client protein-derived peptides, and in the facilitated recognition of HSP90i-treated tumor cells by client protein-specific T cells. Several second-generation, rationally designed small molecule HSP90i are currently being evaluated as single agents for their safety and therapeutic efficacy in ongoing Phase II/III clinical trials in the cancer setting. Further exploration of HSP90i in conjunction with DC-based cancer vaccines or other forms of immunotherapy is strongly anticipated in the near future.

HDAC inhibitors

Histone deacetylases (HDACs) are enzymes that epigenetically alter oncogenic gene transcription. Their expression is commonly dysregulated in tumor cells, engendering profound interest for the development of HDACi for use as anticancer agents (reviewed in [51]). Vorinostat and romidepsin are two FDA-approved HDACi currently being investigated as single modality therapeutic agents in the setting of cutaneous T-cell lymphoma. More recently, HDACi have been studied for their ability to enhance the efficacy of cancer immunotherapies. Somewhat counter-intuitively, HDACi were initially reported to exert anti-inflammatory effects [52]; however, there is emerging evidence supporting the immune stimulating effects of HDACi and their potential utility when applied in combination with existing immunotherapy platforms (reviewed in [53]). HDACi can directly affect tumor cell recognition by T cells by increasing tumor cell expression of MHC (I and II) and costimulatory molecules, as well as by enhancing the function of the antigen processing/presentation machinery in tumor cells [53]. Selective HDAC6 inhibition results in hyper-acetylation of HSP90 and to an inhibition in its chaperone activity [54,55], suggesting HDAC6i may mimic HSP90i in enhancing the antitumor efficacy of DC-based vaccines

(see above). Panobinostat, a pan-HDACi, enhanced the antimelanoma efficacy of ACT T cell-based immunotherapy in mice [56], with selective inhibition of HDAC6 by rocilinostat resulting in the accumulation of central-memory CD4⁺ and CD8⁺ T cells in both peripheral blood mononuclear cells and TIL in melanoma patients. Interestingly, ACT using rocilinostat-treated T cells also improved the antitumor efficacy of this treatment approach against established B16 melanomas [57,58]. When co-administered with an oncolytic vesicular stomatitis virus vaccine during the boosting phase, entinostat (an HDAC1/3 inhibitor), enhanced secondary immune responses against the tumor, but suppressed the primary response against the vaccine vector, thereby focusing the immune response against TAAs [59]. Overall, the utility of HDACi to enhance antitumor immunity, particularly in conjunction with DC-based cancer vaccination holds great promise for future translation into the clinic. However, there remain details to be resolved with regard to selection of the type, dose and schedule of HDACi to be administered for optimal combination immunotherapy benefit in the absence of off-target (immune) deleterious events.

Chemo/radiotherapy

In addition to their direct cytotoxic effects, conventional chemotherapies also have immune conditioning/enhancing qualities that disrupt immune suppression and are being further explored for their effectiveness when applied in combination chemoimmunotherapy strategies [60]. Alkylating antineoplasms drugs, such as temozolomide (TMZ) and cyclophosphamide, promote genotoxic stress in tumor cells with eventual cell death; however, they have also been shown to remove regulatory cells from the TME, notably when administered metronomically or at low doses, which improves their safety profile [60]. When TMZ was given in combination with CpG nanoparticle-based vaccination, enhanced tumor-specific T cell-mediated cytotoxicity was seen in a glioblastoma model [61]. In a Phase II clinical trial for patients with metastatic prostate cancer, a personalized peptide vaccination plus cyclophosphamide induced peptide-specific CTL with a decrease in Treg (but increase in MDSC) compared with personalized peptide vaccination alone [62]. Also, cisplatin has been reported to enhance tumor-specific CD8⁺ T-cell responses and to decrease MDSC content in a model of cervical cancer, when applied prior to vaccination [63]. Furthermore, enhanced effector CD4⁺ and CD8⁺ TILs were detected in mice treated with low-dose paclitaxel prior to intratumoral DC vaccine in mice with Lewis lung carcinoma [64], making chemotherapy an attractive partner for implementation with

DC-based vaccines. In a Phase I trial for the treatment of recurrent glioblastoma, there was strong correlation between improved clinical outcome and vaccine-induced immunity among a subset of patients that received TMZ prior to vaccination with DC preloaded with autologous tumor cell antigens [65].

In addition to its cyto-reduction capacity, cancer radiotherapy similarly appears to modulate systemic immune responses in treated patients, with a new appreciation for its adjuvant-like characteristics. The global immune-stimulating consequences of radiotherapy are referred to as the abscopal effect, in which immune-mediated delay/regression of tumor growth in a nonirradiated tumor occurs distal to a 'primary' lesion that was initially treated with localized irradiation [66]. An adjuvant effect for radiotherapy has been demonstrated when combined with immunotherapy (anti-CTLA-4 and anti-PD-1) in patients with metastatic melanoma [67]. Radiotherapy, along with chemotherapy, has also been reported to promote so-called tumor 'immunogenic cell death,' in which tumor cells die via a mechanism that enhances their immunogenicity (via membrane expression of calreticulin and HSPs), leading to tumor cell uptake by local DC that become competent to cross-prime antitumor immune responses (*in situ* priming). The rationale of using DC in conjunction with an inducer of immunogenic cell death, such as radiotherapy, is being actively explored in the clinical setting. Notably, PFS was increased in glioblastoma patients that received conventional radio/chemotherapy plus an autologous DC-based vaccine when compared with conventional treatment alone [68]. It is important to note that radiotherapy may have inhibitory effects on DC Th1-polarizing potential. In both human and mouse DC, ionizing radiation inhibits Th1-polarizing IL-12 production as well as the Th17-polarizing IL-23 production [69–71] without substantially impacting their viability and/or tumor-infiltrating capacity [69,71,72]. Conversely, ionizing radiation has little impact on DC production of IL-6 or IL-10, resulting in a net potential for these APC to enforce anti-inflammatory or tolerogenic immunity. These points highlight the need to better understand the dose and schedule of (locoregional) radiotherapy in combination with DC-based vaccinations in order to predictably elicit, direct and sustain a preferred proinflammatory antitumor T-cell repertoire in (the TME of) cancer patients.

Conclusion

Optimizing immunotherapeutic strategies is essential for achieving robust antitumor immunity capable of mediating durable objective clinical responses in cancer patients. DC are the most potent antigen presenting

cells for inducing primary immune responses and their use and development in DC-based vaccines continues to expand in the clinical arena. Although DC-based cancer vaccines as a monotherapy have resulted in only modest clinical success to date, their use in combination protocols along with immunomodulatory or TME-conditioning ‘adjuvants’ (i.e., genotoxic chemotherapies and radiotherapies, ICB and/or targeted therapies) holds great promise for improved potency. Such combined strategies are envisioned to hold the power to downmodulate immunosuppression in the TME and to recruit and sustain vaccine-induced antitumor T cells into TME where they may mediate therapeutic tumor regression. These combinational DC-based vaccine strategies along with renewed vigor in the identification of neoantigens will help to foster in the age of optimal personalized immunotherapeutic strategies.

Future perspective

Immunotherapies have reached a turning point and they currently represent standard of care for many forms of cancer (i.e., anti-CTLA-4 and anti-PD-L1/PD-L1 antagonist antibodies for the treatment of melanoma). However, treatment with ICB is most effective against tumors that are already infiltrated with T cells – ‘inflamed tumors’ [73], therefore integration of co-therapies that induce antitumor T-cell recruitment into the TME are logical starting points for the design of novel combination immunotherapies that are likely to impact clinical disease. In particular, we would speculate that optimized DC-based vaccines will be a crucial ingredient in future efficacious combination immunotherapies. Additionally, although conventional DC-based vaccines are thought to cross-prime specific T-cell responses in vaccine site-draining lymph nodes, DCs injected directly into tumor lesions under certain conditions may nucleate the formation of tertiary lymphoid structures (TLS) in the TME where tumor antigen-specific T-cell priming may occur. TLS are a lymph node-like organized network of innate and adaptive cells that act as sites of adaptive immune induction and these can be found in the TME, where they have generally been associated with improved clinical prognosis [74,75]. We have previously reported that intratumoral delivery of DC engineered to express the Type I activator T-bet (DC.Tbet) promotes the active recruit of immune cells into TLS, leading to specific T-cell induction and slowed tumor growth in the murine MCA205 sarcoma model [76]. Therapeutic expression of LIGHT, a promoter of Type I immunity (also known as TNF superfamily member 14; TNFSF14), in the TME has been similarly demonstrated to pro-

mote TLS formation, effector T-cell recruitment and tumor regression [77]. Thus, therapeutic induction of TLS in tumors by DC-based ‘vaccines’ may represent a particularly attractive option for clinical translation as a prospective immunotherapeutic strategy.

NK cells are also emerging as essential contributors to antitumor immune responses [76] and their activation may be targeted by DC-based vaccines [78]. The ‘IL-15 DC,’ human monocyte-derived DC differentiated with GM-CSF and IL-15, potentially stimulate NK cell antitumor activity in an IL-15- and contact-dependent manner [79], suggesting that DC-based vaccine strategies are also viable options as coordinate activators of antitumor NK cells and T cells. Also, an emerging model suggests that ‘licensed’ human $\gamma\delta$ T cells operate as potent professional APCs similar to DC by cross-presenting exogenous antigen to tumor-reactive $\alpha\beta$ T cells. The full licensing of $\gamma\delta$ T cells requires the interaction of $\gamma\delta$ T cell CD16 with antibody-opsonized tumor cells following $\gamma\delta$ TCR ligation at tumor site [80,81]. Therapies incorporating licensed $\gamma\delta$ T cells as antigen presenting cells in combination with monoclonal antibody therapy may represent an effective cancer treatment strategy in future.

Cancer immunotherapies integrating blood-derived primary DC subsets are also actively being pursued. Naturally-circulating DC represent rare events in peripheral blood, but improved isolation methods have helped to accelerate their use in clinical vaccine trials. Early trial results suggest vaccination with patient peripheral blood-derived (plasmacytoid or myeloid) DC is safe and well-tolerated among patients with advanced-stage melanoma or prostate carcinoma [82]. Blood-derived DC do not require lengthy *ex vivo* culturing periods and differ biologically from cultured monocyte-derived DC, with plasmacytoid DC-based vaccines suggested to promote more durable antitumor T-cell responses in patients versus homologous vaccines integrating myeloid DC [83]. Clearly, efforts to mobilize more primary DC for harvest from patient blood, to improve isolation techniques and to refine short-term *ex vivo* conditioning regimens for primary DC subsets may all lead to breakthroughs for the general use of these APC in optimized cancer vaccine formulations [83]. In this regard one must also consider the differential effects of a given conditioning regimen on the functional competency of the various subsets of DC; in other words, MAPK inhibitors blunt IL-12 production from monocyte-derived DC, but augment its production by primary CD1c⁺ DC [84]. DC subset conditioning protocols are expected to dramatically impact future vaccine design and implementation, and will be heavily investigated in the near term.

Executive summary

Enhancing the bioactivity of DC-based vaccines

- Dendritic cell (DC) vaccines are safe, well-tolerated and capable of promoting effective Type 1 anticancer immune responses.
- Aside from US FDA-approved agent Sipuleucel-T, DC-based vaccines so far exhibited only modest clinical efficacy in cancer setting.
- Use of a Type I-polarizing, high IL-12p70-producer DC (α DC1) may be particularly advantageous in the context of vaccine formulations designed to invoke Th1/Tc1 immunity.
- Shared tumor-associated antigens are the most common antigens used in DC vaccine, but tumor-associated neoantigens are being considered for potential use in DC vaccines as the field evolves toward patient-specific protocols.
- Targeting the induction and appropriate functional polarity of CD4+ T helper cells is important for optimal CD8+ T effector cell function, survival and memory.
- Use of primary plasmacytoid and/or myeloid DC subsets rather than cultured monocyte-derived DC in order to elicit more robust/durable antitumor immunity.

Combination strategies to enhance DC vaccine potency

- Combination strategies for use in enhancing the potency of DC-based vaccines may be broadly categorized into two groups: immunoregulatory and immunoconditioning.
- Immunoregulatory strategies are designed to antagonize/remove immunosuppressive networks within the tumor microenvironment.
- Immunoconditioning strategies are designed to (pre)condition the tumor microenvironment (TME) to respond more robustly to DC-based vaccines by facilitating the recruitment and optimal function and durability of vaccine-induced antitumor T cells.

Immunoregulating strategies

- Targeting immune checkpoint pathways with anti-CTLA-4 and/or anti-PD-1 in combination with DC vaccines enhances tumor-specific CD8+ T-cell responses, in concert with tumor regression (objective clinical response), and extended PFS/OS in the clinical setting.
- Emerging checkpoint targets such as TIM-3 and LAG-3 are being investigated in combination with immunotherapy approaches (including DC-based vaccine studies) for the treatment of cancer.
- IDO-1 inhibitor, 1-methyl tryptophan, suppresses the action of regulatory DC and Treg cells by inhibiting the catabolism of tryptophan, and is currently being evaluated in the clinic in combination with DC-based vaccines.
- An alternate target for suppressing immunosuppression is agonism of the costimulatory molecule GITR.
- GITR agonism of effector T cells makes them more resistant to suppression mediated by Tregs, thus improving their antitumor efficacy when applied in adoptive cell therapy-based approaches.
- Therapeutic strategies against cancer that employ GITR-agonists in conjunction with DC-based vaccinations are in progress.

Immunoconditioning strategies

- Mitogen-activated protein kinase pathway inhibitors such as the BRAFi, dabrafenib and vemurafenib, and the MEKi, trametinib, improve T-cell recognition of tumor cells by coordinately modulating tumor cell antigen-presenting cells (APCs) function and T effector cell function, supporting combination therapy approaches with DC-based vaccines.
- Tyrosine kinase inhibitors such as dasatinib and sunitinib increased CD8+ tumor-infiltrating lymphocytes frequencies and decrease numbers of Tregs and myeloid derived suppressor cells in the TME, leading to significantly improved median overall survival of cancer patients in combination DC-based vaccine trials.
- HSP90i show promise when applied in combination with DC-based vaccines given their ability to conditionally improve tumor cell MHC-I loading with antigenic peptides recognized by specific CD8+ T cells.
- HDACi promote increased tumor cell expression of MHC (I and II) and costimulatory molecules, thus improving tumor cell immunogenicity.
- Timing of HDACi use is a factor that affects the biological consequence when combining HDAC inhibition with immunotherapy.
- Conventional chemotherapies may eliminate the immunosuppressive TME by decreasing Treg populations and enhancing the recruitment and function of tumor-specific T effector cells.
- An immune-stimulating consequence of radiotherapy is the abscopal effect, which is the (systemic) immune-mediated delay/regression of a nonirradiated tumor distal to the treated tumor.
- Radiotherapy, along with certain forms of genotoxic chemotherapy, induces immunogenic tumor cell death *in vivo*, leading to enhanced immunogenicity, uptake of dying tumor cells by DC capable of cross-priming specific T-cell responses.

Conclusion

- DC are considered the most potent APCs for inducing immune responses; their use in DC-based vaccines continues to increase and evolve.
- Combination of DC-based vaccines with immunoregulatory and/or immunoconditioning therapies, such as chemotherapies, radiotherapies, alternate immunotherapies and/or targeted therapies, is very promising strategy for enhancing the potency/antitumor efficacy of DC-based vaccines.
- Combinational strategies involving the use of DC-based vaccines will be integral to effectively fostering in the age of personalized immunotherapeutic strategies.
- Future development of clinical vaccine protocols implementing γ/δ T cells or primary DC subsets from patient peripheral blood as APC holds significant promise.

(Primary or cultured) DC-based vaccines may also be readily incorporated into combination protocols that integrate ACT, including chimeric antigen receptor (CAR)-engineered T-cell therapies. In CAR-T cell therapies, autologous T cells are genetically engineered to produce tumor-targeting receptors and adoptively transferred back into the patient, and have demonstrated great clinical success, particularly for the treatment of B-cell malignancies [85]. CAR-T cells that target different cancer types are being evaluated in multiple ongoing clinical trials. DC-based vaccines would be envisioned to serve as a co-therapy option to best maintain and periodically expand the systemic population of CAR-T cells in the treated cancer patient. Indeed, the combination of DC-based vaccination plus ACT has been reported to result in significantly improved recurrence free- and overall survival among hepatocellular carcinoma patients [86]. Although logistically complex, such combination immunotherapies could

be envisioned as future standard of care treatments for many if not all forms of cancer.

Acknowledgements

The authors would like to thank K Fabian for her thoughtful review and critical comments provided during the preparation of this manuscript.

Financial & competing interests disclosure

This work was supported by NIH R01 grant CA169118 (to WJ Storkus) and a Career Enhancement Program Award from the University of Pittsburgh's Melanoma and Skin Cancer SPORC P50 CA121973 (to RJ Fecek). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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