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## Whole Cell Vaccines — Past Progress and Future Strategies

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### Abstract

Cancer vaccines have shown success in curing tumors in pre-clinical models. Accumulating evidence also supports their ability to induce immune responses in patients. In many cases, these responses correlate with improved clinical outcomes. However, cancer vaccines have not yet demonstrated their true potential in clinical trials. This is likely due to the difficulty in mounting a significant antitumor response in patients with advanced disease because of preexisting tolerance mechanisms that are actively turning off immune recognition in cancer patients. This review will examine the recent progress being made in the design and implementation of whole cell cancer vaccines, one vaccine approach that simultaneously targets multiple tumor antigens to activate the immune response. These vaccines have been shown to induce antigen specific T cell responses. Pre-clinical studies evaluating these vaccines given in sequence with other agents and cancer treatment modalities support the use of immunomodulating doses of chemotherapy and radiation, as well as immune modulating pathway targeted monoclonal antibodies, to enhance the efficacy of cancer vaccines. Based on emerging pre-clinical data, clinical trials are currently exploring the use of combinatorial immune based therapies for the treatment of cancer.

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Despite progress made in the field of cancer therapeutics, cancer remains a leading cause of death of both men and women. Furthermore, many cancer treatment modalities such as chemotherapy, radiation, and surgery entail side effects which decrease the quality of life for patients with advanced cancer in exchange for modest survival benefits. Immunotherapy holds the promise of a treatment which is specific and long lasting, protecting patients from cancer recurrence due to memory immune responses, and with limited adverse effects to healthy tissue. Although animal models have demonstrated the potential for effective treatment of cancer, there has so far been limited success seen in the clinic with immunotherapy. This may be due to multiple factors, including poorly designed trials that compare immunotherapy as solo therapy versus the standard of care treatment as well as limiting the inclusion of patients to those with advanced cancer who have already failed or are not eligible for conventional treatments. Rarely are single agents of any class effective in the treatment of cancer. Pre-clinical studies suggest that this holds true for immunotherapy as well due to underlying tolerance and immunosuppression. Thus, in order to successfully

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prolong survival and avoid tumor recurrence, immunotherapy will need to be used in combination with multiple immune-based or traditional modalities. Specifically, combination therapy with chemotherapy, radiation, monoclonal antibodies, and immune checkpoint inhibitors may be useful in addition to whole cell vaccine vectors to stimulate the protective immune response and inhibit the suppressive immune response.

## Whole cell cancer vaccines

Modified whole cell cancer vaccines represent one form of immunotherapy currently in development and clinical trials. The advantage of whole tumor cells used as a vaccine rather than a specific protein or peptide tumor antigen is that the cells provide a source of all potential antigens, eliminating the need to identify the most optimal antigen to target in a particular type of cancer (Figure 1). Importantly, multiple tumor antigens can be targeted at once, generating immune responses to more than one tumor antigen, thereby bypassing issues of tumor antigen loss. Furthermore, immunized lymphocyte and serologic responses can be exploited to identify novel tumor antigens or categorize the importance of a response to a particular tumor antigen through the comparison of immune responses pre- and post-vaccination and by correlating responses with prognosis.

Using autologous tumor cells in the generation of a whole cell vaccine ensures that patients are vaccinated with cells containing the same tumor antigens that their tumor expresses. However, this approach is technically limited because harvesting tumor cells and generating a vaccine line which expresses a standardized amount of cytokine is not always feasible and can be financially costly and time consuming.<sup>1</sup> Vaccines made from allogeneic cells circumvent the issue of individualizing each patient's therapy and by using several cell lines derived from different tumors in the vaccine, there is an increased likelihood that the patient's tumor will share antigens expressed by the vaccine cells, including important tumor antigens overexpressed or mutated in a high percentage of that particular cancer. A concern of using allogeneic cells is that HLA mismatch between vaccinating cell lines and the patient will result in a response directed against foreign HLA molecules rather than tumor antigens. While anti-HLA responses do develop, they have not been shown to inhibit the tumor antigen response and have actually been associated with clinical response to whole cell vaccination.<sup>2,3</sup> From an immunologic point of view, antigen presenting cells (APCs) from the patient are responsible for priming the CD8<sup>+</sup> T cell response to tumor antigens contained in the irradiated vaccine cells and the presence of foreign MHC molecules may enhance this cross-priming.<sup>2,4</sup>

Whole cell vaccines have been genetically modified to express cytokines, chemokines or costimulatory molecules to stimulate the immune response to the injected irradiated tumor cells.<sup>1</sup> Many phase I and II studies have shown this approach to be safe in patients with different types of cancer. Historically, vaccine induced immune responses have been assessed by measuring delayed-type hypersensitivity responses (DTH) to autologous tumor cells. While tumor cells alone do not elicit a DTH, DTH responses have been observed to correlate with a survival benefit in patients given gene modified vaccine cells.<sup>1</sup> One cytokine, granulocyte macrophage colony-stimulating factor (GM-CSF) was found to be superior to other cytokines tested including IL-2, IL-4, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  when transduced into irradiated melanoma cells in pre-clinical studies.<sup>5</sup> The addition of GM-CSF to a whole tumor cell vaccine resulted in a massive influx of dendritic cells (DCs), macrophages, eosinophils, and T cells at the vaccination site.<sup>1</sup> The resection of lesions post-vaccination revealed necrotic tumors with many T cells and plasma cells whereas infiltrates were not seen in non-vaccinated patients' tumors.<sup>6</sup> Many published studies now explain why GM-CSF stands out among cytokines for its ability to activate an effective tumor targeted T cell response.<sup>7</sup> GM-CSF can recruit DCs to the site of the irradiated cells and stimulate

antigen uptake, processing, and presentation.<sup>5</sup> DCs facilitate the T cell response by cross-priming CD8<sup>+</sup> T cells after uptake of GM-CSF-secreting whole tumor cells.<sup>4</sup>

## Moving forward with GM-CSF-secreting whole cell tumor vaccines

The GM-CSF-secreting whole cell cancer vaccine approach has been studied in a number of different cancer types in animal models and in patients. In pancreatic cancer, both phase I and II trials have been completed with an irradiated, allogeneic GM-CSF-transduced vaccine derived from two pancreatic tumor lines. A phase I trial in 14 patients with stage 2/3 pancreatic adenocarcinoma to assess safety and the induction of systemic antitumor immune responses found no dose limiting toxicities and a DTH correlating with disease-free survival in 3 patients surviving greater than 25 months.<sup>8</sup> All responders received the two highest dose levels and multiple vaccinations at the same dose.<sup>8</sup> A phase II follow up study in which 60 patients received one vaccine injection 8 weeks following pancreaticoduodenectomy and prior to standard chemoradiation with up to 5 additional vaccines following chemoradiation, demonstrated that immunotherapy and chemoradiation can be safely combined as adjuvant therapy.<sup>9</sup> Treatment with the vaccine resulted in survival consistent with that seen in the adjuvant setting with chemoradiation, with a significant improvement in survival over chemoradiation alone in the first two years following surgery.<sup>9</sup> CD8<sup>+</sup> T cell responses to mesothelin, a protein involved in pancreatic cancer metastasis, were enhanced following vaccination in patients with increased survival.<sup>9</sup> Interestingly, patients with greater than three years disease-free survival also exhibited an expansion of the CD8<sup>+</sup> T cell response to an increased number of mesothelin epitopes whereas the repertoire of anti-mesothelin T cells was limited for patients who survived less than 3 years, suggesting whole cell vaccination has the advantage inducing an immune response to multiple epitopes within an antigenic protein.<sup>9</sup> These findings also suggest that patients receiving this vaccine might benefit clinically from repeated boosting beyond 2 years. Studies are on-going to test this question. Phase I trials have also been completed in other advanced cancers including renal cell carcinoma, metastatic melanoma, prostate cancer, lymphoma, leukemia, breast cancer, and non-small-cell lung cancer (NSCLC) and these have provided additional evidence for the safety and ability of GM-CSF-secreting whole cell vaccines to induce immune responses.<sup>7,10</sup>

## Modification of GM-CSF-secreting whole cell vaccine vectors

Whole cell vaccines provide a source of tumor antigens, but a stimulus is required for antigens to be taken up by antigen-presenting cells in an immune stimulatory context and to recruit cells of both the innate and adaptive immune system. The genetic modification and irradiation of tumor cells generates vaccine cells that secrete cytokine but do not further proliferate in the host. Several new cancer vaccine developments are designed to stimulate the immune response by further building on the whole cell gene modified vaccine approach.

CpG oligodeoxynucleotides (ODN), a toll like receptor (TLR) 9 agonist, is one example of a molecule that stimulates TLRs expressed by antigen-presenting cells such as monocytes, macrophages and DCs to enhance the immune response to tumor antigens.<sup>11</sup> When administered with a cancer vaccine, TLR ligands function to upregulate co-stimulatory molecules and cytokine expression by APCs, driving the adaptive response to antigens delivered in the vaccine. In a mouse model using transplantable neuroblastoma tumors, co-administration of CpG ODN served to enhance the effects of a GM-CSF-secreting whole cell vaccine.<sup>12</sup> In a different vaccine approach involving dendritic cells fused with tumor cells, the addition of CpG ODN enhanced maturation of the fused cells, increasing the magnitude of the T cell response and resulting in tumor rejection after re-challenge.<sup>13</sup> CpG ODN physically cross-linked to a tumor cell vaccine proved superior against tumor

development or growth of established tumors in comparison to free CpG ODN given simultaneously with a whole cell vaccine, suggesting the co-localization of TLR agonist and tumor antigen is crucial for activation of DCs.<sup>11</sup> Clinically, TLR agonists have been used to enhance the immunogenicity of peptide vaccines but have yet to be combined with whole cell vaccines in cancer patients.<sup>14</sup>

While GM-CSF can activate antigen-processing and presentation by DCs (Fig 1), the effects of immunosuppressive molecules found in the cytokine milieu of the tumor microenvironment can inhibit this maturation and activation of APCs and T cells induced by vaccination. TGF $\beta$  secreted by suppressive cell types inhibits CD8<sup>+</sup> T cells directly through the transcriptional repression of proteins required for cytolytic effector function such as granzyme B and perforin, and indirectly through the suppression of DCs.<sup>15,16</sup> TGF $\beta$  is also responsible for the induction of T regulatory cells (Tregs) which are inhibitory to the anti-tumor response.<sup>17</sup> Therefore new efforts have been made to block TGF $\beta$  expression, starting with the vaccinating cell lines. A phase I trial of an autologous whole cell vaccine expressing both GM-CSF and a TGF $\beta$  antisense molecule represents the first combination of a whole cell vaccine with immunostimulatory and anti-immunosuppressive molecules, resulting in one complete response in metastatic melanoma and 17 out of 21 patients with stable disease for at least 2 months post-treatment.<sup>18</sup> Another phase I trial of this approach in six grade IV astrocytoma patients showed a benefit in overall survival compared to historically treated patients.<sup>19</sup> A phase II trial of belagenpumatucel-L (an allogeneic NSCLC whole cell vaccine containing antisense TGF $\beta$ ) tested 3 different doses of cells, with the medium and high dose cohorts demonstrating increased median survival compared to the low dose cohort and historical rates of median survival in NSCLC.<sup>3</sup>

## Combining other treatment modalities with whole cell vaccines

In the setting of developing cancers that have already escaped the protective immune response, it is unlikely that vaccination alone will be effective. A combination of chemotherapy, radiation and surgery, along with novel small molecule inhibitors and monoclonal antibodies, are currently administered to patients with the goal of eradicating cancer and eliminating recurrence due to resistant or mutated cancer cells. Phase I and II trials have shown promising results with whole cell vaccines through the induction of DTH and antigen-specific antibody and T cell responses. However, in phase III trials, it is difficult to show benefit beyond what is seen with conventional chemotherapy when vaccines are given as a single agent.

## Costimulatory molecules

Agonist molecules have been studied as single agents and in combination with vaccines to further stimulate the immune response. A number of monoclonal antibodies have been shown to engage co-stimulatory receptors on T cells, including OX40, 41BB, and CD40, mimicking the signals exchanged between activated antigen-presenting cells and T cells. As one example, OX40, a tumor necrosis factor receptor (TNFR) family molecule, enhanced proliferation and effector function of CD4<sup>+</sup> T cells and natural killer (NK) cells via engagement by OX40L on DCs, resulting in increased anti-tumor immunity.<sup>20–22</sup> In addition, treatment with this agonist antibody together with a GM-CSF-secreting whole cell vaccine augmented the immunodominant HER-2/neu-directed CD8<sup>+</sup> T cell response in mice expressing the HER-2/*neu* tumor antigen.<sup>23</sup> Whereas with vaccination alone, the anti-*neu* response became undetectable by day seven post-vaccine, an activated CD8<sup>+</sup> T cell response persisted for weeks in 15–20% of mice treated with OX40 co-stimulation and vaccine.<sup>23</sup> Furthermore, depletion of CD4<sup>+</sup> T cells in these mice confirmed that co-stimulation acted directly on CD8<sup>+</sup> T cells.<sup>23</sup> Emerging data also suggest that combining two co-stimulatory

agonist antibodies may be more effective than either alone. As an example, anti-OX40 and anti-41BB agonist antibodies administered with DCs pulsed with apoptotic tumor cells also enhanced the immune response and tumor rejection in HER-2/*neu* transgenic mice.<sup>24</sup> Several clinical trials are underway testing OX40 agonist antibodies in cancer patients.<sup>20</sup>

CD40 is another co-stimulatory molecule involved in bidirectional activation of APCs and T cells through signaling with its ligand, CD40L.<sup>25</sup> Using transplantable melanomas in mice, CD40 agonist antibody worked in combination with CTLA-4 blockade to enhance the CD8<sup>+</sup> T cell response to a tumor antigen delivered by an adenoviral vaccine vector.<sup>26</sup> However, one clinical trial testing the human agonist CD40 antibody failed to show benefit.<sup>27</sup> In addition, immunologic correlates demonstrated chronic B cell activation but depletion of T cells.<sup>27</sup> Another clinical trial testing GM-CSF- and CD40L-transfected bystander cells plus irradiated autologous tumor cells in stage IV cancers demonstrated a correlation between stable disease in several melanoma patients and the induction of MART-1-specific T cells.<sup>28</sup> More recently, CD40 agonist antibody was used in inoperable pancreatic cancer along with gemcitabine which induced a tumor regression in one patient while several other patients had stable disease.<sup>29</sup> Further evaluation of this treatment regimen in a mouse model of pancreatic cancer demonstrated that tumor regression was the result of CD40-mediated macrophage activation rather than T cell activation.<sup>29</sup> This was a surprising result that requires further follow up. It will also be interesting to evaluate agonist CD40 antibody activity with vaccination strategies in patients.

## Monoclonal antibodies to tumor antigens and angiogenic molecules

The importance of the humoral component of the anti-tumor response has been demonstrated in both pre-clinical and clinical studies. In one study using a DNA vaccine with constructs for HER-2/*neu* and the extracellular domain of Flt-3 ligand, the induction of *neu*-specific antibodies was required along with the T cell-mediated response for complete protection to tumor challenge.<sup>30</sup> As another example, non-transgenic mice develop an antibody and T cell response following GM-CSF-secreting *neu*-expressing whole cell vaccination and again, both components are required for elimination of *neu*-expressing tumors.<sup>31</sup> Tolerant *neu*-N mice fail to mount a protective humoral response after vaccination, but administration of an anti-*neu* monoclonal antibody with a *neu*-targeted vaccine increases survival and tumor clearance compared to vaccination alone in these mice.<sup>32</sup> Antibody therapy collaborates with vaccination to enhance the tumor antigen-specific T cell response through Fc $\gamma$  receptor-mediated uptake of the antibody-coated vaccine cells in vaccine-draining lymph nodes and subsequent DC cross-priming of *neu*-specific CD8 T cells.<sup>32,33</sup> Importantly, the timing and location of vaccine and antibody administration is such that vaccine cells continue to secrete GM-CSF for two days prior to antibody binding, allowing for optimal maturation of DCs that take up antibody-coated cells.<sup>33</sup>

The humoral response is also an important component of the antitumor response in patients. Both endogenous and administered antibodies have been shown to act via several mechanisms against tumors. As an example, endogenous antibodies to MICA (MHC class I chain-related molecule A, a ligand for the NKG2D receptors) were induced following treatment of metastatic melanoma patients with GM-CSF-secreting whole cell vaccination and the anti-CTLA-4 monoclonal antibody, ipilimumab.<sup>34</sup> Mechanistically, anti-MICA antibodies inhibited soluble MICA from binding and downregulating NKG2D, a cytotoxicity-activating receptor on the surface of CD8<sup>+</sup> T cells and NK cells.<sup>34</sup> Anti-MICA antibodies also bound the antigen on the surface of tumor cells, enhancing cross-presentation to CD8<sup>+</sup> T cells.<sup>34</sup>

A number of clinically approved monoclonal antibodies that target different tumor associated antigens including CD20 expressed by lymphomas, HER-2 expressed by breast cancers, and epidermal growth factor receptor (EGFR) expressed by head and neck and colorectal carcinomas, likely work in part through multiple immunologic mechanisms.<sup>35</sup> These antibodies have been shown to have limited efficacy when given as monotherapy.<sup>35</sup> However, when given in combination with chemotherapy, monoclonal antibody therapy can reduce the risk of recurrence by up to 30%.<sup>35</sup> While these antibodies can bind oncogenic proteins to block downstream signaling through these molecules or to clear necessary signaling molecules from the tumor cell surface, accumulating evidence suggests that therapeutically administered antibodies mediate antitumor activity through immune mechanisms as well.<sup>35</sup> Antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are two immune mechanisms that work through innate effector cells such as neutrophils,  $\gamma\delta$  T cells and NK cells that bind the antibody/tumor complex and lyse the tumor.<sup>35,36</sup> Furthermore, certain Fc $\gamma$  receptor Fc $\gamma$ R polymorphisms have been linked to outcomes in cancer patients treated with monoclonal antibodies and predict a protective or adverse outcome depending on the polymorphism.<sup>35</sup> However, the link between Fc $\gamma$ R polymorphisms and prognosis may not be entirely due to innate immune mechanisms such as ADCC and CDC. The enhanced humoral and T cell response to tumor antigens often observed after antibody therapy may represent an alternate mechanism by which these therapies add to traditional cancer treatment regimens. The kinetics of the response to monoclonal antibodies, which begin around one week post-treatment, are more consistent with DC cross-priming and development of T cell-mediated immune responses.<sup>33</sup> Monoclonal antibodies bound to the surface of tumor cells can enhance Fc-mediated antigen uptake, processing and presentation by DCs, stimulating the CD8<sup>+</sup> T cell response.<sup>33</sup> Cytokines secreted by DCs and NK cells activated through their Fc receptors can contribute to the milieu that decreases Treg mediated suppression and activates T helper 1 type and cytotoxic T lymphocytes.<sup>36</sup> Thus, the combination of a whole cell vaccine vector with monoclonal antibodies directed against a tumor antigen serves to stimulate the innate response to the vaccine cells, augmenting antigen presentation and the adaptive response to tumor antigens.

Antibodies targeting angiogenic molecules can inhibit immune-suppressive, pro-oncogenic pathways required for tumor cell survival, regardless of whether these antibodies are induced post-vaccination or administered therapeutically. GM-CSF-secreting whole cell vaccination and anti-CTLA-4 treatment was found to elicit tumor necrosis, infiltration of lymphocytes and granulocytes, destruction of tumor vessels, and induction of multiple antibodies including anti-vascular endothelial growth factor (VEGF).<sup>37</sup> Several melanoma patients treated with the GM-CSF-secreting vaccine also had elevated angiopoietin-1 and angiopoietin-2 antibodies post-vaccination.<sup>37</sup> These antibodies were capable of inhibiting Tie-2-dependent monocyte chemotaxis and angiopoietin-1/2 signaling *in vitro*, suggesting a potential role for the endogenous humoral response in controlling angiogenesis and oncogenic tumor-associated macrophage signaling.<sup>37</sup>

The survival benefit associated with treatment of patients with angiogenesis inhibitors such as the monoclonal antibody to VEGF, bevacizumab, is additive to regimens of chemotherapy.<sup>38</sup> In addition, the overall benefit is modestly significant.<sup>38</sup> Pre-clinical studies demonstrate that anti-angiogenic monoclonal antibodies may be useful in combination with immunotherapy. An anti-VEGF-R2 antibody (DC101) augments the tumor antigen-specific CD8<sup>+</sup> T cell response in wild type mice, resulting in tumor cell death and inhibition of angiogenesis.<sup>39</sup> DC101 antibody inhibited tumor growth when given with a neu-targeted vaccine in non-tolerant mice with neu-expressing tumors.<sup>39</sup> However, in tolerant *neu*-N mice, immunomodulating doses of cyclophosphamide to decrease Treg activity were required in addition to antibody and vaccine for the induction of anti-neu

CD8<sup>+</sup> T cells and tumor regression.<sup>39</sup> Despite concerns that the inhibition of neo-vascularization would impede trafficking of T cells to the site of the tumor, this was not observed and VEGF-R inhibition may have facilitated tumor infiltration by lymphocytes.<sup>39</sup> VEGF signaling pathways promote immature myeloid cells in the tumor microenvironment; thus, anti-angiogenic monoclonal antibodies can decrease immunosuppressive signaling and improve the quality and function of APCs.<sup>40</sup> These studies emphasize that angiogenic molecules are an important target of immunotherapy both when targeted directly with the use of monoclonal antibodies, and also following induction of vaccine induced anti-angiogenic responses that indirectly result in destruction of tumor vasculature by vaccine-induced effector cells.

## Radiation and chemotherapy to stimulate innate and adaptive immunity

Accumulating evidence from pre-clinical and clinical studies supports the concept that radiation and chemotherapy can enhance the anticancer immunity induced by cancer vaccines. Although radiation and chemotherapy are commonly implemented and well established cancer treatments with which immune based therapies have been co-administered, the collaborative effects of these different treatment modalities can be missed, often with loss of evidence of immune activity, if the optimal timing and dose of these approaches are not considered when integrating them. Three general approaches for effectively integrating these therapeutic modalities have emerged from prior studies. First, high doses of some chemotherapeutic drugs and radiation eliminate rapidly proliferating cells such as those of the immune system. While this is seen as an adverse effect on an existing immune response, in some circumstances, wiping out the existing response and “rebooting” the immune system can reduce immunosuppressive cell populations, promoting the expansion of effector T cells, and increasing the generation and function of effector lymphocyte activating DCs post-lymphodepletion.<sup>41</sup> As an example, mice receiving sublethal total body irradiation followed by reconstitution with syngeneic splenocytes and GM-CSF-secreting cellular vaccination experienced an enhanced anti-tumor CD8<sup>+</sup> T cell response and greater protection against a tumor challenge when compared to mice that were not lymphodepleted and reconstituted.<sup>42</sup> Chemotherapy and radiation-associated lymphodepleting regimens have been the most successful in terms of response rates in patients receiving adoptive T cell therapy in clinical trials, suggesting that more intense lymphodepletion might also enhance responses to vaccination if given along with T cell transfer.<sup>43</sup>

Second, therapeutic doses of chemotherapy and radiation therapy can enhance the immune response to tumor antigens by inducing immunogenic death of tumor cells and release of tumor antigens. As an example, dying cells treated with oxaliplatin or radiation release the TLR4 ligand HMGB1 which activates DCs.<sup>44</sup> Furthermore, several chemotherapy agents including cisplatin, doxorubicin, mitomycin C, fluorouracil, and camptothecin are capable of upregulating Fas expression on the surface of tumor cells and sensitizing cells to Fas-FasL mediated killing by lymphocytes.<sup>45</sup> In addition, the use of irradiated tumor cells as a source of tumor antigens in cancer vaccines has been shown to enhance the immunogenicity of whole cell vaccines presumably by inducing apoptotic bodies of the tumor cells that are more easily taken up by APCs for antigen processing and presentation.<sup>46</sup> Thus, this immunogenic cell death generated by radiation or chemotherapy provides additional rationale for pairing chemotherapy and immunotherapy.

Third, low doses of chemotherapy and radiation have been shown to alter sub-populations of immune suppressive cells allowing for the induction of a more effective immune response while circumventing the majority of immune cells. This is in contrast to high dose chemotherapy and radiation that eliminate most immune cells, providing a space for the

expansion of endogenous or transferred effector cells to expand preferentially. It is possible that lower dose chemotherapy and radiation may fulfill the same purpose as high dose but with fewer side effects by specifically depleting immunosuppressive populations that impair the protective antitumor response. Tregs are increased in the peripheral blood, lymph nodes and tumors of cancer patients and have been associated with poor prognosis and suppression of tumor antigen immune responses.<sup>47</sup> Low-dose total body irradiation has been shown to have immunomodulating effects, enhancing antigen-specific responses to vaccination through a decrease in Treg numbers and an increase in effector memory T cells.<sup>48</sup> Cyclophosphamide (Cy) has been shown to deplete Tregs and when administered one day prior to vaccination, allows for the activation and tumor trafficking of a previously tolerized high avidity CD8<sup>+</sup> T cell population in *neu-N* mice.<sup>49</sup> The benefits of Cy may lie in its ability to promote protective Th1 and Th17 responses in addition to depleting Tregs which suppress the tumor antigen-specific CD8<sup>+</sup> effector cells.<sup>50,51</sup> Clinically, a phase I trial of GM-CSF-secreting vaccine with or without Cy has found that the combination of both agents is safe and the addition of Cy improves median survival from 2.3 months to 4.3 months in advanced pancreatic cancer patients.<sup>52</sup> This combination of Cy and GM-CSF-secreting irradiated pancreatic tumor cells also resulted in the induction of higher avidity mesothelin-specific CD8<sup>+</sup> T cell responses which correlated with improved survival.<sup>52</sup> Other studies have also demonstrated Cy-mediated enhanced immune responses when given with a vaccine to mice and patients.<sup>53</sup>

Myeloid derived suppressor cells (MDSC) constitute another population of cells capable of suppressing antigen-specific responses induced by cancer vaccines.<sup>54</sup> Current therapies such as the previously discussed TGF $\beta$  antisense GM-CSF-secreting whole cell vaccine approach could be one method of indirectly targeting signaling molecules from suppressive cell populations. However, several chemotherapeutics have been reported to successfully decrease MDSC numbers in cancer patients. The FOLFIRI regimen, irinotecan plus infusional 5-fluorouracil and leucovorin, followed by DC vaccination is effective in promoting Th1 CD4<sup>+</sup> cells and CD8<sup>+</sup> cytotoxic T cells while decreasing suppressive Treg and MDSC populations which rebound with chemotherapy alone in a mouse model of colorectal cancer.<sup>55</sup> Docetaxel, which has been shown to have beneficial effects on the activation of APCs, selectively reduced the suppressive myeloid population while skewing myeloid cells towards a M1-like antitumor phenotype.<sup>56</sup> 5-fluorouracil and gemcitabine also selectively deplete MDSC, allowing a CD8<sup>+</sup> effector population to emerge and thus, may be useful in combination with vaccination.<sup>54</sup>

Studies have also shown that additional immune stimulatory effects result when chemotherapeutic drugs are used at subclinical doses. Paclitaxel, given at low dose (20 mg/kg in mice), stimulates DCs through TLR signaling, resulting in accelerated DC antigen processing and presentation, upregulation of costimulatory markers, and IL-12 production.<sup>57</sup> The combination of paclitaxel with a GM-CSF-secreting irradiated tumor cell vaccine and another immunomodulatory drug, Cy, resulted in the highest level of antigen-specific CD8<sup>+</sup> T cell response in *neu-N* mice.<sup>57</sup> Docetaxel, a paclitaxel analog, has been shown to enhance the effects of a GM-CSF-secreting melanoma vaccine in mice; combining the two agents at a dose where neither had impact on tumor growth or survival alone resulted in significant prolongation of survival.<sup>58</sup>

A number of clinical trials have incorporated immune modulating doses of these chemotherapeutic agents with vaccine approaches. However, extrapolating mouse doses to human doses presents a challenge. One reported clinical trial used an innovative combinatorial study design to identify the optimal doses of the most active combination of two drugs, Cy and doxorubicin (Dox) when combined with the GM-CSF-secreting whole cell vaccine in patients with metastatic breast cancer.<sup>59</sup> The trial was based on pre-clinical



studies that demonstrated that Cy given one day before and Dox given 7 days after vaccination was the optimal sequence for inducing the most effective anti-tumor immune response when combined with a HER-2/neu-targeted GM-CSF-secreting tumor vaccine in *neu-N* mice.<sup>50</sup> In the clinical trial, doses of Cy greater than 200 mg/m<sup>2</sup> negatively affected the DTH response to HER-2/neu peptides when compared to vaccine alone whereas vaccine given with 200 mg/m<sup>2</sup> Cy augmented both DTH and antibody responses to HER-2/neu.<sup>59</sup> The optimal dose of Dox was determined to be 35 mg/m<sup>2</sup> for development of HER-2/neu-specific antibodies, whereas all doses of Dox allowed for DTH responses to HER-2/neu peptides.<sup>59</sup> Interestingly, patients receiving the GM-CSF-secreting whole cell vaccine alone were observed to have progressively diminishing serum GM-CSF levels with each administration.<sup>59</sup> However, dual chemotherapy and vaccine treatment sustained peak GM-CSF levels with each treatment, perhaps by inhibiting the anti-allogeneic response while maintaining the antitumor immune response.<sup>59</sup> These studies represent alternative ways to administer chemotherapy at doses that result in fewer systemic side effects but enhanced anti-tumor immune responses.

## Immune checkpoint inhibitors

Studies in patients have long demonstrated the existence of cancer antigen-targeted CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Despite their existence, these cells are often dysfunctional with low cytokine production and increased expression of molecules associated with T cell exhaustion.<sup>60,61</sup> *In vitro* experiments have demonstrated that blockade of these “immune checkpoint” molecules can remove barriers to T cell activation and reverse exhaustion of tumor-antigen specific T cells.<sup>61</sup> CTLA-4, an inhibitory molecule controlling the expansion and activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, has become an important therapeutic target with the recent approval of a monoclonal antibody for use in metastatic melanoma.<sup>60</sup> Pre-clinical studies demonstrate that CTLA-4 blockade may be useful in enhancing the immune response generated by cancer vaccines. Blockade of CTLA-4 paired with a GM-CSF-secreting whole cell vaccine in a B16 melanoma mouse model resulted in tumor clearance in the majority of mice whereas either treatment given as monotherapy had little effect on established tumors.<sup>62</sup> Anti-CTLA-4 antibody plus GM-CSF-secreting whole cell vaccination enhanced tumor rejection in a effector T cell intrinsic manner in mice and did not seem to work through Treg-mediated effects.<sup>63</sup>

Clinically, combination therapy with a cancer vaccine and blockade of CTLA-4 appears superior to vaccination alone as demonstrated in several trials. Ipilimumab with or without a gp100 peptide vaccine improved survival in metastatic melanoma (10.0 months) compared to a gp100 vaccine alone (6.4 months), with some treatment related grade 3 and 4 autoimmune events.<sup>64</sup> Based on one study administering anti-CTLA-4 to previously vaccinated melanoma patients, the combination of GM-CSF-secreting whole cell-based vaccines with immune checkpoint blockade may be superior to the use of other vaccine vectors. In this study, patients who were treated with anti-CTLA-4 after previously receiving GM-CSF-secreting autologous tumor cells, demonstrated tumor necrosis and lymphocyte infiltration in their tumors.<sup>65</sup> In contrast, similar results have not been observed in patients receiving vaccines that target a limited set of melanoma antigens.<sup>65</sup> Additional clinical trials testing this monoclonal antibody in combination with other whole cell vaccines are ongoing.

Monoclonal antibodies to PD-1, an inhibitory co-receptor also found to be upregulated on exhausted T cells in cancer patients, are undergoing clinical testing in advanced cancers including melanoma, non-small cell lung cancer, and renal cell carcinoma.<sup>61</sup> A phase I trial testing anti-PD-1 at different doses found it to be safe at all doses for patients with advanced cancers. In addition, treatment with this antibody resulted in one complete response and a number of partial responses.<sup>66</sup> In contrast to anti-CTLA-4, which results in significant

autoimmune effects, the adverse events in this study included only one grade 3 event and a few less serious autoimmune issues.<sup>66</sup> To date, combination regimens with this antibody have only been tested experimentally in mouse models. However, PD-1 blockade given in combination with a GM-CSF-secreting tumor vaccine has been shown to enhance antitumor T cell responses and prolonged survival in mice when compared to either treatment alone.<sup>67</sup>

The possibilities for blockade of multiple immune checkpoints administered in combination with vaccines, chemotherapy, and radiation therapy to optimize anti-cancer immune responses are expanding as efforts to define the function of other molecules involved in the T cell response to tumor antigens continue. Antibody blockade of TIM3 signaling, another checkpoint molecule upregulated on tumor-infiltrating lymphocytes, demonstrated moderate results in both a tumor treatment model and in a model of suppression of carcinogen-induced tumorigenesis.<sup>68</sup> This effect was even more significant in combination with antibodies to CTLA-4 and PD-1.<sup>68</sup> LAG-3 is another inhibitory co-receptor found on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, among other immune cell types.<sup>69</sup> Deletion or blockade of LAG-3 has proven to be effective in enhancing CD8<sup>+</sup> T cell function, increasing cytokine secretion, proliferation, and tumor infiltration in a CD8<sup>+</sup> intrinsic manner, in response to a model tumor antigen in mice.<sup>69</sup> Importantly, these pre-clinical findings are being rapidly translated into the clinical setting.

## Conclusion and Future Directions

The important concepts learned from pre-clinical cancer vaccine studies have not yet translated into clinical successes in patients with cancer. However, a number of cancer vaccines have demonstrated immune activity in association with improved clinical outcomes in clinical trials. In addition, the first cancer vaccine was approved for the treatment of advanced prostate cancer. Like other cancer modalities, it is unlikely that vaccines as single agents will effectively treat existing cancers. Whole cell vaccine approaches have the advantage of targeting multiple tumor antigens at once, which should avoid antigen escape mechanisms and allow for effective targeting of the majority of tumor cells within a growing tumor. However, it has become clear that multiple inhibitory signals on T cells and the APCs responsible for activating the T cell response prevent effective immune activation and recognition of growing tumors in patients. This alone is enough to explain the lack of clinical activity of current vaccine approaches. Fortunately, these inhibitory signals are being rapidly defined and agents that modulate these signals are already demonstrating clinical responses. The future of cancer vaccines is directly linked to the success of these agents. These agents act by allowing effective induction of T cells. Most cancer patients do not have naturally existing endogenous tumor antigen-specific T cells. Thus, these agents will require cancer vaccines to generate antigen-targeted T cells. Together, these antagonist antibodies and cancer vaccines have the potential to induce optimally activated T cells in patients that will result in significant clinical outcomes.

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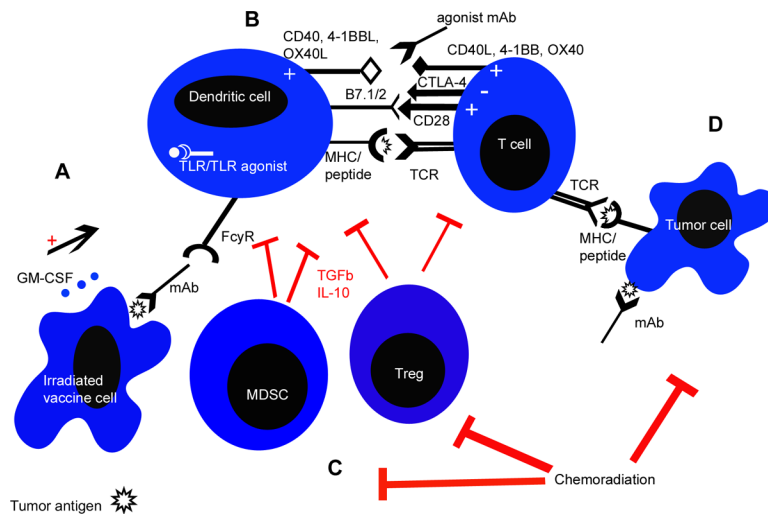
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**Figure 1.**

Interactions of the immune system with a whole cell vaccine approach (GM-CSF-secreting tumor cell vaccine as one example) and other immune modulating therapies for the treatment of cancer. **A**) GM-CSF is secreted by irradiated vaccine cells, which attract dendritic cells (DCs) to the site of antigen. The antigen is then taken up by the DCs for processing and presentation. DCs can also be stimulated by monoclonal antibodies (mAb) that bind to specific tumor antigens on the vaccine cell surface via their Fc receptor recognizing the Fc portion of the mAb. Vaccine cell lines can also be modified to secrete other cytokines, express co-stimulatory molecules that further activate DCs (such as CD40L) or express molecules that block inhibitory signals such as TGF $\beta$  (not shown). Toll-like receptor (TLR) agonists or immunomodulatory chemotherapeutic agents such as paclitaxel can also stimulate DCs through TLRs to upregulate co-stimulatory molecules, increase cytokine production, and enhance antigen processing and presentation. **B**) DCs process and present tumor antigen derived from the vaccinating cells to CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Tumor antigen is presented in the form of peptide/MHC complexes on APCs; T cells bind this complex with their T cell receptor (TCR). Additional signals are required for stimulation of T cells which can be provided by activated DCs or agonist antibodies to co-stimulatory molecule receptors such as anti-CD40, anti-4-1BB, and anti-OX40. The activation and proliferation of tumor antigen-specific T cells can also be increased with the use of blocking antibodies to immune checkpoint molecules such as CTLA-4 and PD-1. **C**) APCs and T cells can be suppressed by inhibitory cytokines and molecules such as TGF $\beta$  and IL-10 secreted by suppressive immune cell populations like MDSC and Tregs. Chemotherapy (such as cyclophosphamide and gemcitabine) and radiation, when used at immunomodulatory doses, can be used to inhibit these populations. **D**) Tumor cell killing occurs when the TCR expressed on effector CD8<sup>+</sup> T cells recognizes tumor antigens presented by MHC molecules on the surface of tumor cells. If activated efficiently, CD8<sup>+</sup> T cells can work synergistically with traditional treatments such as chemotherapy, radiation therapy, or monoclonal antibodies to kill or inhibit tumor cells.