

# The Interplay of Immunotherapy and Chemotherapy: Harnessing Potential Synergies

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## Abstract

Although cancer chemotherapy has historically been considered immune suppressive, it is now accepted that certain chemotherapies can augment tumor immunity. The recent success of immune checkpoint inhibitors has renewed interest in immunotherapies, and in combining them with chemotherapy to achieve additive or synergistic clinical activity. Two major ways that chemotherapy promotes tumor immunity are by inducing immunogenic cell death as part of its intended therapeutic effect and by disrupting strategies that

tumors use to evade immune recognition. This second strategy, in particular, is dependent on the drug, its dose, and the schedule of chemotherapy administration in relation to antigen exposure or release. In this Cancer Immunology at the Crossroads article, we focus on cancer vaccines and immune checkpoint blockade as a forum for reviewing preclinical and clinical data demonstrating the interplay between immunotherapy and chemotherapy. *Cancer Immunol Res*; 3(5); 436–43. ©2015 AACR.

## Introduction

Cancer treatment strategies are based on the rational integration of multiple distinct treatment modalities that together achieve the highest rates of disease control. Surgery and radiotherapies are used to debulk tumors and achieve locoregional disease control. In contrast, systemic therapies are used in early cancers to eradicate micrometastatic disease and increase cure rates, or in widespread incurable cancers to achieve the greatest disease control with the fewest side effects. Standard systemic therapies may include chemotherapies, pathway-specific molecular therapies, and/or tumor-specific monoclonal antibodies. Rational combinations of these systemic therapies are typically designed to impinge on distinct elements of tumor biology to achieve additive or synergistic antitumor effects. Immunotherapy—including vaccines and immune checkpoint blockade—is the newest class of systemic cancer therapies. The ultimate goal of immunotherapy is to establish a durable population of highly active, tumor-specific T cells that can lyse tumor cells and eradicate cancers. Strategically combining immunotherapies with other systemic therapies to harness potential synergies is critical for maximizing their clinical activity and realizing the greatest ben-

efits for patients with cancer. Preclinical and clinical work has evaluated mechanisms of immunomodulation by standard chemotherapy agents, revealing drug- and dose-dependent effects on various aspects of the immune system (1, 2). The schedule and sequence of chemotherapy and immunotherapy also affects tumor immunity in combination regimens (2). These critical variables differentially engage the potential additive and synergistic clinical activities of chemotherapy and immunotherapy, and are important to consider when translating chemoimmunotherapy regimens to the clinic. This Cancer Immunology at the Crossroads article summarizes the current understanding of these issues and highlights future directions for research.

## Immunotherapies: Vaccines and Immune Checkpoint Antagonists

### Cancer vaccines

The ultimate goal of cancer immunotherapies is to establish a durable pool of T cells that have potent antitumor activity. Cancer vaccines are designed to prime and expand tumor-specific T cells by delivering tumor-associated antigens in an immunologic milieu that drives effective T-cell activation (3). Various cancer vaccine platforms with a range of potencies have been tested (4). Short peptides derived from tumor antigens that contain CD8<sup>+</sup> T-cell epitopes generally activate a weak, short-lived T-cell response. In contrast, mixtures of short peptides that deliver CD8<sup>+</sup> T-cell epitopes with peptides that deliver T-helper epitopes, or long peptides that include both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell epitopes within the same peptide, activate a stronger, more durable T-cell response. Recombinant bacterial or viral vectors engineered to deliver tumor antigens both activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells and provide additional inflammatory signals that bolster vaccine-activated immunity. Peptide-pulsed dendritic cells (DC) provide an optimal means of effectively cross-priming the tumor-specific immune response. Furthermore, DC-based vaccines derived from whole tumor cells can activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells

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specific for a range of tumor antigens, decreasing the likelihood of selecting for antigen loss variants as a means of immune escape.

### Immune checkpoint antagonists

Whereas vaccines prime the tumor-specific immune response by driving T-cell activation and expansion, immune checkpoint antagonists abrogate negative signals that diminish T-cell activation during the priming process, or inhibit effector T-cell activity at the tumor site (5). Immune checkpoints, represented by the interaction of the cell-surface proteins cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) with their respective ligands, transmit a negative signal to T cells. Immune checkpoint signaling thus decreases T-cell function, including proliferation, cytokine release, and cytotoxic granule secretion. CTLA-4 binds to its coreceptors B7-1 (CD80) or B7-2 (CD86), providing a negative feedback signal at the T cell–antigen-presenting cell (APC) interface. PD-1 binds to its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC), which are present on APCs, host stromal cells at the tumor site, and tumor cells themselves. Tumors co-opt these immunoregulatory pathways to circumvent immune surveillance and promote their growth and progression. Blocking immune checkpoints with antagonist monoclonal antibodies "takes the brakes off," restoring immune surveillance and unleashing T-cell function. Ipilimumab (Yervoy; Bristol-Myers Squibb) is a humanized IgG<sub>1</sub> monoclonal antibody that blocks CTLA-4 signaling and is FDA approved for advanced melanoma (6). Pembrolizumab (Keytruda; Merck) and nivolumab (Opdivo; Bristol-Myers Squibb) are humanized IgG<sub>4</sub> monoclonal antibodies that are FDA approved for metastatic melanoma (7); nivolumab received FDA approval in March of 2015 for squamous non-small cell lung cancer (NSCLC). Agents that target the PD-1 pathway also have clinical activity in a range of other cancer types, including some (urothelial bladder cancer and triple-negative breast cancer) that have been traditionally considered immunologically inert (7).

### Evasion of Immune Attack by Cancers

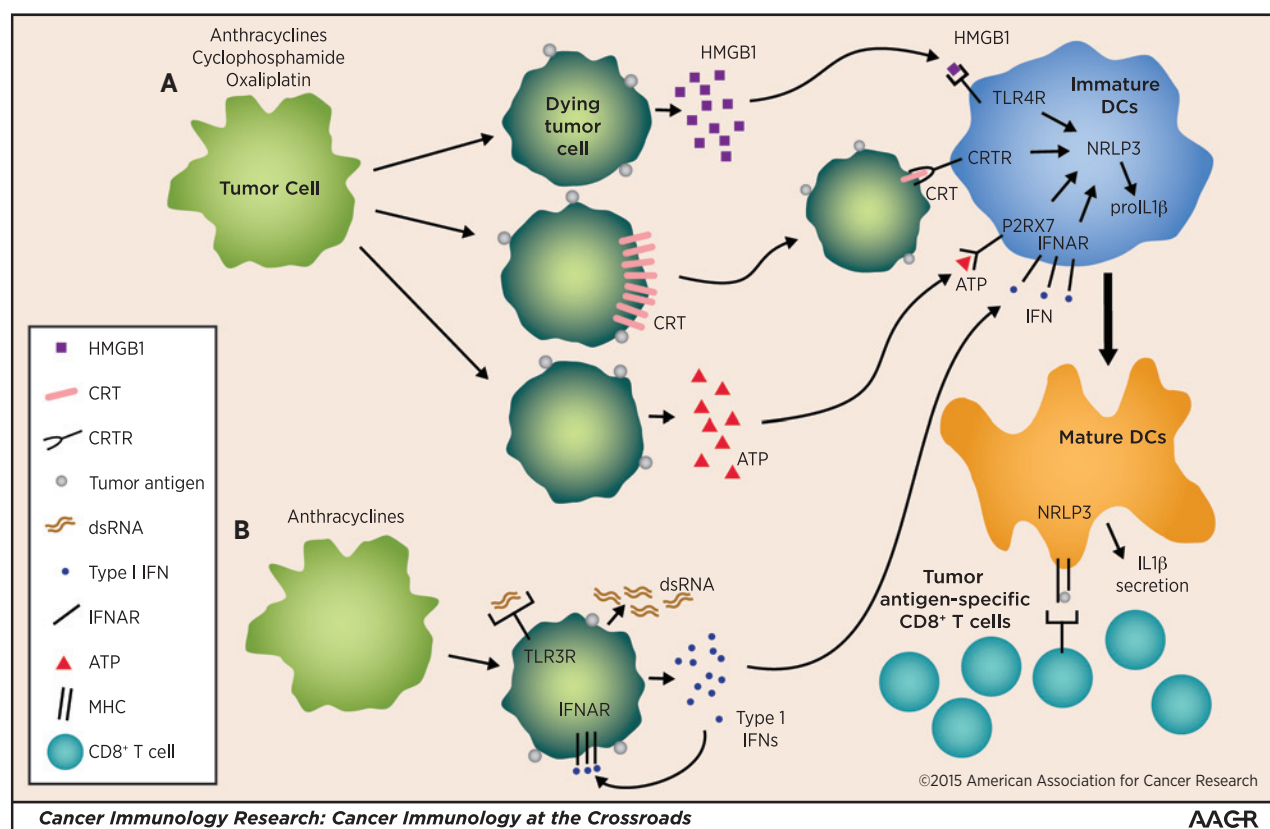
As with other systemic cancer therapies, resistance to immunotherapy may lead to therapeutic failure. Defining these mechanisms, and developing strategies for overcoming immune resistance, is critical for the optimal efficacy of immunotherapy. Tumor cells are known to evade immune surveillance by a variety of mechanisms (8). They downregulate tumor antigens, MHC class I and II proteins, and other molecules involved in antigen processing and presentation. Cross-talk between cancer cells and the host immune system results in the intratumoral accumulation of immune suppressive cells, including regulatory T cells (Treg), interleukin (IL)-17–secreting T cells, myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages (TAM). Tumor cells may express immune checkpoint ligands, such as PD-L1, either through constitutive oncogene-driven expression or through upregulation in response to interferon (IFN)- $\gamma$  released by T cells at the tumor site (5). High levels of immune-suppressive cytokines within the tumor microenvironment, including transforming growth factor- $\beta$  (TGF $\beta$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and IL10, further shut tumor-specific immune responses down (8).

### Mechanisms of Immunomodulation by Chemotherapy—Standard Doses

Standard cancer chemotherapy can promote tumor immunity in two major ways: (i) inducing immunogenic cell death as part

of its intended therapeutic effect; and (ii) disrupting strategies that tumors use to evade the immune response. A large body of data demonstrates that some chemotherapy drugs at their standard dose and schedule mediate their antitumor effect, at least in part, by inducing immunogenic cell death (Fig. 1; ref. 9). This process involves the concomitant release of tumor antigens and the emission of danger-associated molecular patterns (DAMP) in the tumor microenvironment during cell death. Anthracyclines activate expression of the pattern recognition receptor (PRR) Toll-like receptor-3 (TLR3), the rapid secretion of type I IFNs, and the release of the chemokine CXCL10; a type I IFN gene signature predicted response to anthracycline therapy in breast cancer patients (10). Phylogenetically conserved chemokine signaling by CXCL8 increases the exposure of calreticulin on the tumor cell surface, which is critical for the recognition and engulfment of dying tumor cells by DCs (11). High mobility box binding protein-1 (HMGB-1) or ATP released into the tumor microenvironment bind to their respective PRRs, TLR4, and the purinergic receptor P2RX7. This activates the NLRP3 inflammasome, with resulting IL1 $\beta$  secretion and activation of IFN $\gamma$ -secreting CD8<sup>+</sup> T cells (8, 12). Underscoring the possible clinical relevance of these pathways, TLR4 and P2RX7 loss-of-function polymorphisms are associated with a higher risk of breast cancer relapse after adjuvant anthracycline-based chemotherapy (13, 14), and TLR4 loss-of-function polymorphisms with shorter progression-free survival (PFS) and overall survival (OS) in patients with advanced colorectal cancer (15) and squamous cell head and neck cancer (16). Loss-of-function polymorphisms in TLR4 or P2RX7 fail to affect clinical outcome in patients with NSCLC (17), suggesting that tumor biology, chemotherapeutic agent, or both may influence whether tumor cell death is immunogenic, and which cell death pathway is activated. Other forms of immunogenic chemotherapy-induced cell death include autophagy (18) and necroptosis (19).

Alternatively, chemotherapy can modulate distinct features of tumor immunobiology (Fig. 2) in a drug-, dose-, and schedule-dependent manner (reviewed in ref. 2). The optimal integration of immunotherapies with standard cancer therapies to minimize antagonistic interactions and engage potential synergies is therefore of great importance. One obvious strategy is to give immunotherapy in the setting of minimal residual disease, after the tumor mass has been optimally reduced with surgery and systemic chemotherapy. This sequencing strategy minimizes the negative impact of tumor bulk on the potency of the antitumor immune response. It also allows chemotherapy to modulate the immune phenotype of any residual tumor cells. In addition to inducing immunogenic cell death and type I IFN secretion, anthracyclines promote the CCL2/CCR2-dependent recruitment of functional APCs into the tumor site, but not into tumor-draining lymph nodes (20). Distinct chemotherapy drugs may modulate the intrinsic immunogenicity of tumor cells through a variety of mechanisms (reviewed in ref. 2). Chemotherapy can enhance tumor antigen presentation by upregulating the expression of tumor antigens themselves, or of the MHC class I molecules to which the antigens bind. Alternatively, chemotherapy may upregulate costimulatory molecules (B7-1) or downregulate coinhibitory molecules (PD-L1/B7-H1 or B7-H4) expressed on the tumor cell surface, enhancing the strength of effector T-cell activity. Chemotherapy may also render tumor cells more sensitive to T cell–mediated lysis through fas-, perforin-, and Granzyme B–dependent mechanisms.

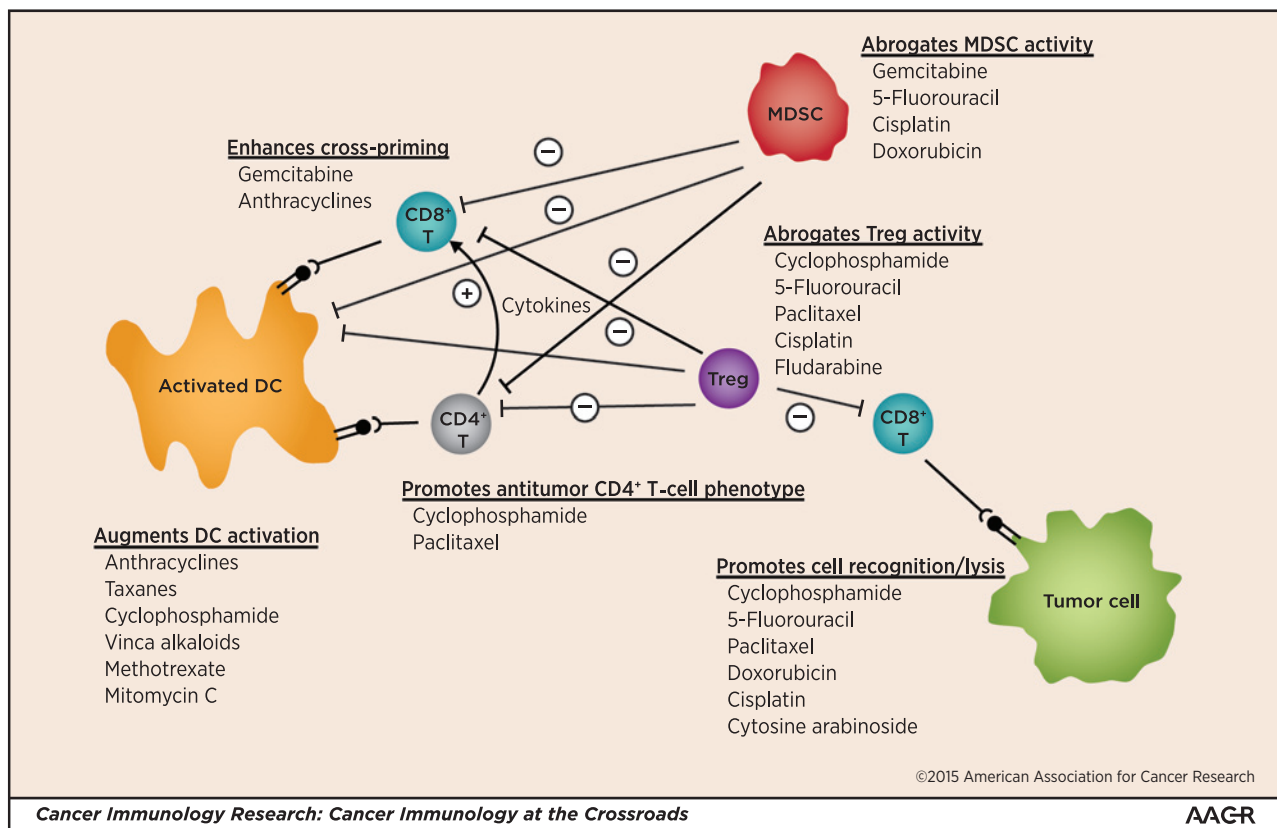


**Figure 1.**

Mechanisms of immunogenic tumor cell death induced by chemotherapy. Certain chemotherapy agents can cause immunogenic cell death through diverse pathways. **A**, anthracyclines, cyclophosphamide, and oxaliplatin induce immunogenic cell death through the release of tumor antigens, the translocation of CRT (an eat-me signal for phagocytosis by DCs) to the cell surface, and the secretion of the danger-associated molecules HMGB1 and ATP (a find-me signal for phagocytosis by DCs). These cell death-associated molecules bind their respective receptors, the calreticulin receptor (CRTR), the TLR4 receptor (TLR4R), and the P2RX7 receptor. This results in activation of the NLRP3 inflammasome, the production of pro-IL1 $\beta$ , DC maturation, and the secretion of IL1 $\beta$  to support the evolution of tumor-specific CD8<sup>+</sup> T cells. **B**, tumor cell death induced by anthracyclines results in the release of dsRNA, which binds to TLR3 and results in the tumor cell autonomous secretion of type I IFNs. This pathway is analogous to the response to viral infection and supports the evolution of tumor immunity. CRT, calreticulin; HMGB1, high mobility binding box 1; NLRP3, inflammasome.

One example of an immunomodulatory standard-dose chemotherapy is gemcitabine, which has pleiotropic immune effects. It induces tumor cell apoptosis and enhances the cross-priming of CD8<sup>+</sup> T cells in animal models (21). It also reverses defective cross-presentation of tumor antigens by tumor-infiltrating DCs (22). Giving gemcitabine "before" vaccination or a CD40 agonist augmented the survival of mice treated with chemoimmunotherapy (21, 23). Conversely, gemcitabine + cisplatin given "after" immunotherapy with an adenoviral vector expressing IFN $\alpha$  (AdIFN $\alpha$ ) also had greater antitumor activity than either chemotherapy or AdIFN $\alpha$  alone, by increasing antigen-specific tumor-infiltrating lymphocytes (TIL) numbers, activation, and trafficking (24). Another study showed that, although levels of antigen-specific peripheral T cells were decreased, concomitant gemcitabine increased the efficacy of a DC-based vaccine by both increasing T-cell trafficking and sensitizing tumor cells to T cell-mediated lysis (25). Decreased peripheral immunity was avoided by giving gemcitabine after two cycles of vaccination. In addition, gemcitabine significantly reduced MDSCs in preclinical animal models (24, 26, 27). These principles were explored in the

TeloVac study, a phase III clinical study designed to strategically harness the impact of standard-dose gemcitabine on immunity and clinical responses to the promiscuous MHC class II telomerase vaccine GV1001 given with GM-CSF adjuvant (28). This study randomized 1,062 patients with advanced or metastatic pancreatic cancer 1:1:1 to standard gemcitabine/capecitabine chemotherapy (GemCap arm 1), two cycles of GemCap followed by vaccination days 1, 3, and 5, then weekly  $\times$  3, and at week 6, then monthly thereafter until disease progression at which time patients returned to GemCap chemotherapy (sequential arm 2), or concurrent GemCap for six cycles with GV1001 + GM-CSF given as in arm 2 (concurrent arm 3). The primary endpoint of this study was OS. OS in the concurrent arm was virtually identical to the control GemCap arm, with a trend toward inferior OS in the sequential arm. Objective response rates and PFS were significantly worse in the sequential arm relative to those of the other two arms. Importantly, the sequential arm of this study was designed based in part on the data summarized above, in which a short course of chemotherapy prior to vaccination could enhance antigen cross-presentation, and a return to

**Figure 2.**

Chemotherapy modulates tumor immunity by mechanisms distinct from immunogenic cell death. Various chemotherapy drugs can modulate the activity of distinct immune cell subsets or the immune phenotype of tumor cells through enhancing antigen presentation, enhancing expression of costimulatory molecules including B7.1 (CD80) and B7.2 (CD86), downregulating checkpoint molecules such as programmed death-ligand 1 (PD-L1), or promoting tumor cell death through the fas, perforin, or Granzyme B pathways. Reviewed in greater detail in ref. 2.

chemotherapy after vaccination could boost vaccine-primed immunity by releasing tumor antigens and DAMPs. Synergy between chemotherapy and immunotherapy in the TeloVac study may have been prevented by at least three factors. First, many patients on the sequential arm never returned to chemotherapy due to rapid disease progression after beginning the vaccination phase of the sequence. This problem supports an argument for testing such a strategy in patients with a slower pace of disease, giving vaccination time to establish a deep and robust antitumor immune response (29). Second, the induction of tumor cell apoptosis is necessary for the enhancement of antigen cross-presentation by gemcitabine (21); analysis of apoptosis induction by GemCap in the TeloVac study revealed that apoptosis was induced in <25% of patients. Even in patients with evidence of apoptosis induction, there was no evidence of an enhanced peripheral immune response after vaccination (G. Middleton and colleagues; unpublished data). In addition, the outcome of patients returning to chemotherapy after vaccination was no better than the outcome of patients treated with chemotherapy alone (28). Finally, modulation of the tumor microenvironment by chemotherapy may also have been limited by the stromal characteristics of pancreatic cancer, as therapeutic synergy between vaccine and gemcitabine in preclinical pancreas tumor models was seen with subcutaneous pancreas tumors but not when the same

pancreatic cancer cells were implanted orthotopically (25). Also of relevance, analysis of MDSCs in 19 patients receiving standard GemCap failed to demonstrate a reduction in Lin<sup>-</sup>DR<sup>-</sup>CD11b<sup>+</sup> MDSC numbers, but MDSC numbers decreased in 19 of 21 patients receiving GemCap concurrently with GV1001 + GM-CSF (30). Nine of these 21 patients developed T cells specific for GV1001, and MDSCs declined in 8 of 9 of these patients.

Other clinical trials have shown that standard chemotherapy may inhibit immunotherapy. A phase II study integrated pancreas GVAX with standard adjuvant therapy in 60 patients with stage II and III pancreatic cancer (31). In this clinical trial, participants developed mesothelin-specific T-cell responses after one vaccination prior to surgery, then went on to adjuvant 5-fluorouracil (5-FU)-based chemotherapy before receiving three additional boost vaccinations. The vaccine-induced mesothelin-specific T-cell response was reduced during adjuvant chemotherapy and was restored by boost vaccinations (E. Lutz and colleagues; unpublished data). A phase III study tested prostate GVAX combined with standard-dose docetaxel chemotherapy, randomizing patients to GVAX every 3 weeks with docetaxel but no prednisone, or docetaxel with prednisone, 10 mg daily (32, 33). The study was closed after 408 patients were randomized because of an imbalance of deaths on the vaccine arm relative to the control arm. At least



two major factors may have contributed to the failure of this study. Although docetaxel has been reported to suppress MDSC and enhance DC function (34), and also to induce translocation of calreticulin (35), this vaccine had never been tested with full-dose docetaxel in the clinic. The chemotherapy may thus have impaired vaccine-induced immunity. In addition, prednisone is a critical component of standard docetaxel therapy for these patients, and it was omitted from the vaccine arm due to concern that it could inhibit T-cell responses. In another trial, the activity of the canary pox vaccine ALVAC-CEA-B7.1 was tested in combination with 5-FU + leucovorin + irinotecan in 118 patients with metastatic colorectal cancer, where three cycles of vaccine alone followed by vaccination combined with chemotherapy was given to two groups, and four cycles of chemotherapy followed by vaccination in patients without disease progression on chemotherapy was given to the last group (36). No significant differences between the groups emerged, and chemotherapy did not inhibit CEA-specific T cells. Finally, a phase II trial of a pox virus-based vaccine encoding mucin-1 (MUC-1) and IL2 (TG4010) enrolled 148 patients with MUC-1<sup>+</sup> tumors who received six cycles of gemcitabine + cisplatin or vaccination with TG4010 and concurrent chemotherapy with six cycles of gemcitabine + cisplatin, where vaccination continued until disease progression (37). CD16<sup>+</sup>CD56<sup>+</sup>CD69<sup>+</sup> natural killer cells were present at normal levels in 73.2% of patients, and were associated with a more favorable safety profile, improved time to progression (HR, 0.5), and OS (HR, 0.6) in patients receiving TG4010. Patients with high levels of activated natural killer cells who received TG4010 had worse outcomes. On the basis of these data, a phase III trial is in the planning stages. Multiple small trials enrolling 10 to 28 patients have shown that concurrent or phased standard chemotherapy does not inhibit, and may enhance vaccine-induced immunity (2).

Immune checkpoint antagonists have also been combined with standard-dose chemotherapy, either concurrently or in sequence. A seminal phase III trial tested the CTLA-4 antagonist ipilimumab or placebo combined with standard-dose dacarbazine (850 mg/m<sup>2</sup>) in 502 patients with stage IV melanoma (38). This study demonstrated improved survival with ipilimumab combined with dacarbazine relative to dacarbazine alone. Two clinical trials examined concurrent versus phased chemotherapy with ipilimumab in 204 patients with NSCLC (39) and 130 patients with extensive-stage small-cell lung cancer (SCLC; ref. 40). In these trials, standard paclitaxel (175 mg/m<sup>2</sup>) and carboplatin (AUC 6) were used. Patients received four cycles of chemotherapy + ipilimumab, followed by two cycles of chemotherapy, or two cycles of chemotherapy followed by four cycles of chemotherapy + ipilimumab. For NSCLC, improved immune-related and standard PFS were observed if patients received phased treatment with chemotherapy first, followed by chemotherapy + ipilimumab, whereas for SCLC improved immune-related but not standard PFS was observed for this same phased therapy. Finally, a small clinical trial tested recombinant soluble LAG-3 immunoglobulin (Ig) fusion protein, IMP321, as one component of first-line paclitaxel therapy in 30 patients with metastatic breast cancer (41). IMP321 binds with high avidity to MHC class II, resulting in activation of APCs and subsequent activation of memory T cells. The 6-month PFS rate was 90%, and biomarker analyses revealed a sustained increase in activated APCs and a greater percentage of natural killer and CD8<sup>+</sup> effector memory T cells.

## Mechanisms of Immunomodulation by Chemotherapy—Immune-Modulating Doses

Various chemotherapy drugs, including cyclophosphamide, paclitaxel, cisplatin, and temozolomide, can be used at low doses in a schedule-dependent manner to modulate tumor immunity (reviewed in ref. 2). Of these, the adjuvant activity of cyclophosphamide has been most studied. A single low dose of cyclophosphamide given 1 to 3 days before antigen exposure overcomes systemic immune tolerance to enhance both antibody and T-cell responses, whereas the same treatment given after or at the same time as antigen exposure induces antigen-specific tolerance (2). In preclinical models, low-dose cyclophosphamide depletes Tregs, promotes DC maturation, shifts the CD4<sup>+</sup> T-helper phenotype from type 2 to type 1, induces the differentiation of T-helper type 17 cells, and promotes the evolution of a durable CD44<sup>hi</sup> T-cell memory response through IFN $\alpha$  secretion (2). Clinically, cyclophosphamide doses of 200 to 300 mg/m<sup>2</sup> given 1 day prior to vaccination or 600 mg/m<sup>2</sup> given 7 days prior to vaccination can decrease Tregs; metronomic cyclophosphamide is similarly effective (42, 43). The taxanes also have pleiotropic immune-modulating effects. Low-dose paclitaxel promotes the TLR4-dependent maturation of DC in mice (44) and shifts the CD4<sup>+</sup> T-helper phenotype from type 2 to type 1, thereby promoting proinflammatory cytokine secretion and enhancing the priming and lytic activity of CD8<sup>+</sup> T cells (45). Doxorubicin also has immunomodulatory properties, although the precise mechanisms remain unclear (1). In preclinical models of tumor antigen-specific immune tolerance, a low dose of cyclophosphamide or paclitaxel given 1 day prior to cell-based vaccination depletes Tregs, augments vaccine-induced T-cell responses, and promotes tumor-free survival in tumor-bearing mice (45). Moreover, a low dose of doxorubicin given 7 days after vaccination enhances vaccine activity and delays tumor outgrowth. Combining cyclophosphamide and doxorubicin at this low dose and schedule results in the greatest effect, curing some of the mice. In this model, cyclophosphamide selectively depletes Tregs, allowing the recruitment of high-avidity tumor-specific T cells specifically in animals cured of their tumor (not in mice whose tumors grew; ref. 46).

Cyclophosphamide has historically been used in the clinic to inhibit the influence of suppressor T cells based on how they were defined in the 1970s and 1980s. Several clinical trials showed that patients who received 300 mg/m<sup>2</sup> of cyclophosphamide given 3 days before vaccination with a sialyl-Tn-keyhole limpet hemocyanin (KLH) vaccine developed higher antibody titers and lived longer than patients who received the vaccine alone (47). On the basis of these data, a phase III clinical study randomized patients with metastatic breast cancer, with 523 patients to receive 300 mg/m<sup>2</sup> cyclophosphamide followed by vaccination with sialyl-Tn-KLH 3 days later, and 505 patients to receive 300 mg/m<sup>2</sup> of cyclophosphamide followed by vaccination with KLH alone (48). PFS and OS were no different between the two groups, though an unplanned subset analysis showed a trend toward improved survival in those patients on concurrent endocrine therapy. Thus, like the TeloVac study and the phase III study of prostate GVAX, this study failed. In this case, it is possible that the trial design was flawed, as there may have been some immunomodulatory activity associated with the control intervention. In

addition, concurrent endocrine therapy could have confounded the results. A third limitation is that the vaccine only delivered one antigen, setting the stage for immune escape.

More recently, a phase II clinical trial enrolled 68 patients with advanced renal cell carcinoma to receive 17 vaccinations with the multi-peptide vaccine IMA901 (49). In this trial, 33 patients received 300 mg/m<sup>2</sup> of cyclophosphamide 3 days before vaccination with IMA901 + GM-CSF adjuvant, and 35 patients received vaccination with IMA901 + GM-CSF adjuvant alone. This single-dose of cyclophosphamide reduced Tregs by 20% within 3 days relative to baseline, with a decrease in the percentage of proliferating Tregs relative to all Tregs noted; these effects were not seen in the group of patients who received IMA901 alone. There was no change in absolute lymphocyte count on either arm. Interestingly, the immune response rates between the two groups were not different, suggesting that cyclophosphamide did not alter the induction of tumor antigen-specific T cells. Among immune responders, patients who received cyclophosphamide and IMA901 had longer OS than patients who received IMA901 alone (HR, 0.38; *P* = 0.01); there was no difference related to cyclophosphamide in immune nonresponders (HR, 0.92; *P* = 0.87). Overall, there was no difference in PFS between the two groups, but there was a trend toward longer OS in patients who received cyclophosphamide with IMA901 compared with those who received IMA901 alone (median OS, 23.5 vs. 14.8 months). Patients who developed multi-peptide immune responses survived longer than those who did not, suggesting that clinical benefit may track with a diverse tumor-specific immune response. Finally, among six predefined MDSC populations, two were prognostic for OS, and among over 300 serum biomarkers, APOA1 and CCL17 were predictive for immune response to IMA901 and OS.

These two studies used a dosage of 300 mg/m<sup>2</sup> of cyclophosphamide for immune modulation based on its ability to deplete suppressor T cells as they were historically defined, not based on its ability to augment antigen-specific immune response induced by vaccination. A distinct study tested an HER2<sup>+</sup>, GM-CSF-secreting breast tumor vaccine alone, or with a range of low, immune-modulating doses of cyclophosphamide and doxorubicin given in a specifically timed sequence defined by preclinical modeling (45, 50). This study used an innovative response surface design to detect interactions among the vaccine, cyclophosphamide, and doxorubicin, where the vaccine was held constant. The study was designed to test cyclophosphamide at doses of 0, 200, 250, or 350 mg/m<sup>2</sup> 1 day prior to vaccination, and doxorubicin at doses of 0, 15, 25, and 35 mg/m<sup>2</sup> 7 days after vaccination in order to identify doses of chemotherapy that optimized HER-2-specific immunity. Vaccination alone induced new HER-2-specific delayed-type hypersensitivity (DTH), with low levels of HER-2-specific antibody also induced. Cyclophosphamide given at 200 mg/m<sup>2</sup> maintained the DTH response, and enhanced the HER-2-specific antibody response; higher doses of cyclophosphamide abrogated HER-2-specific DTH and did not augment HER-2-specific antibody responses. The optimal chemotherapy dose combination tested as measured by the magnitude of HER-2-specific antibody responses was cyclophosphamide at 200 mg/m<sup>2</sup> and doxorubicin at 35 mg/m<sup>2</sup>, which is close to the doses predicted by response surface analysis at 193 and 25 mg/m<sup>2</sup>, respectively. Detailed analyses showed that the lowest doses of cyclophosphamide tested induced the selective apoptosis of CD4<sup>+</sup> Tregs relative to effector T cells, creating a window for the

effective activation of effector T cells by the vaccine (51). These data highlight the importance of innovative trial designs and appropriate biomarker analyses in order to identify meaningful interactions between chemotherapy and immunotherapy in patients.

Few studies have examined low-dose chemotherapy in combination with immune checkpoint blockade. One study in a model of cervical cancer showed that anti-PD-1 antibody combined with low-dose cyclophosphamide synergistically induces antigen-specific immunity, the infiltration of CD8<sup>+</sup> and CD4<sup>+</sup> FoxP3<sup>-</sup> T cells into the tumor, and tumor-free survival (52). The addition of anti-PD-1 to cyclophosphamide augments and prolongs the effect of cyclophosphamide in decreasing both systemic and tumor-infiltrating Tregs. To date, there are no clinical trials exploring low-dose immune-modulating chemotherapy with immune checkpoint blockade in patients.

## Conclusions and Future Directions

The success of immune checkpoint antagonists heralds the dawn of a new age in cancer therapy, in which harnessing the power of the immune system to treat cancer is becoming a key strategy for clinical management. Combination therapies that integrate distinct immunotherapies, including immune checkpoint antagonists and cancer vaccines, with chemotherapy, radiotherapy, and targeted molecular therapy are under active investigation. Understanding the cellular and molecular mechanisms underlying the interplay of traditional cancer therapies, and their dose- and schedule-dependent activities, will be essential for effective translation to the clinic. Novel trial designs that arise from and build upon clinically relevant preclinical data to investigate dose and schedule will be essential. Careful assessment of patterns of clinical response and appropriate clinical endpoints will be key for success. Biologic correlates that not only evaluate the most relevant immunologic endpoints (tumor antigen-specific CD8<sup>+</sup> T cells, for example) but also dissect mechanisms of interaction between chemotherapy and immunotherapy are also important to build into trials testing chemotherapy in combination with vaccines and immune checkpoint modulators. The first generation of clinical trials described here has yielded important insights that can inform the way forward as harnessing the antitumor immune response becomes the backbone of cancer therapy.

## Disclosure of Potential Conflicts of Interest

Under a licensing agreement between Aduro, Inc., and the Johns Hopkins University, the University and L.A. Emens are entitled to milestone payments and royalties on sales of the GM-CSF-secreting breast cancer vaccine. The terms of these arrangements are being managed by the Johns Hopkins University in accordance with its conflict-of-interest policies. L.A. Emens receives research funding from Genentech, Inc., Merck, Inc., EMD Serono, Maxcyte, Inc., and Amplimmune, Inc. and is a consultant/member of the advisory board for Vaccinex, Celgene, Aveo and Bristol-Myers Squibb. G. Middleton has received research funding from KAE-L-GemVax.

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