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Re-purposing cancer therapeutics for breast cancer immunotherapy

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Abstract

After decades of work to develop immune-based therapies for cancer, the first drugs designed specifically to engage the host anti-tumor immune response for therapeutic benefit were recently approved for clinical use. Sipuleucel-T, a vaccine for advanced prostate cancer, and ipilimumab, a monoclonal antibody that mitigates the negative impact of cytotoxic T lymphocyte antigen-4 signaling on tumor immunity, provide a modest clinical benefit in some patients. The arrival of these drugs in the clinic is a significant advance that we can capitalize on for even better clinical outcomes. The strategic and scientifically rational integration of vaccines and other direct immunomodulators with standard cancer therapeutics should lead to therapeutic synergy and high rates of tumor rejection. This review focuses on the use of cyclophosphamide, doxorubicin, and HER-2-specific monoclonal antibodies to dissect mechanisms of immune tolerance relevant to breast cancer patients and illustrates how appropriate preclinical models can powerfully inform clinical translation. The immune-modulating activity of targeted, pathway-specific, small molecule therapeutics is also discussed. Fully understanding how cancer drugs impact the immune system should lead to the ultimate personalized cancer medicine: effective combinatorial immunotherapy strategies that simultaneously target signaling pathways essential for tumor growth and progression, and systematically break multiple, distinct immune tolerance pathways to maximize tumor rejection and effect cure.

Keywords

PIVAC 11; Chemotherapy; Cyclophosphamide; Monoclonal antibody; Immunotherapy; Breast cancer

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Introduction

Manipulating the immune system for therapeutic benefit in cancer patients has been studied for well over 100 years. Despite intensive investigation, the first cancer therapies designed to directly manipulate the antitumor immune response have taken their place in the cancer treatment arsenal only recently. One of these, sipuleucel-T (Provenge^{R}), is a patient-specific, dendritic cell-based vaccine loaded with a recombinant prostate acid phosphatase (PAP)–granulocyte–macrophage colony-stimulating factor (GM-CSF) fusion protein. This vaccine was approved for use by the Food and Drug Administration of the United States (US FDA) based on a survival advantage of 4 months in late-stage prostate cancer patients [1]. The other, ipilimumab (Yervoy^{R}), is a monoclonal antibody that blocks the negative activity of the immune checkpoint molecule cytotoxic T lymphocyte antigen-4 (CTLA-4). This drug was approved for use by the US FDA based on a survival benefit for both untreated and treatment-refractory metastatic melanoma patients [2, 3]. Although only a small subset of patients derive a limited but distinct clinical benefit from treatment with either of these agents, both drugs demonstrate an overall survival benefit in patients with few other treatment options. These new developments highlight the potential of immune-based therapy for cancer treatment.

Concomitant with the development of these approved drugs, a large body of data has been established that supports a significant role for the antitumor immune response in the efficacy of standard cancer therapeutics [4]. Chemotherapy can have a number of positive effects on the immune system, with potential for eliciting immunogenic tumor cell death, enhancing other aspects of tumor cell immunogenicity, inducing homeostatic T cell proliferation, modulating the suppressive influence of $CD4^{+}CD25^{+}$ $FoxP3^{+}$ regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs), and conditioning dendritic cell function to support tumor rejection [5]. Whether the effect of chemotherapy is positive, negative, or neutral depends on the chemotherapy drug, its dose, and its schedule of administration [6]. These variables highlight the importance of thoughtful trial design when testing combined chemoimmunotherapy strategies.

Similarly, therapeutic monoclonal antibodies function not only in a target-specific fashion to antagonize oncogenic pathways, but also by modulating intrinsic tumor cell immunogenicity and supporting the cross-priming of the adaptive tumor-specific immune response [4, 7]. In addition, in some cases (depending on the target), monoclonal antibody therapy can be viewed as a passive reconstitution of the humoral immune response against tumors. The ability of therapeutic monoclonal antibodies to enhance the clinical efficacy of standard chemotherapy and radiotherapy effectively illustrates the potential of combinatorial immune-based approaches. Taken together, these data suggest that combining both immune-modulating chemotherapy and tumor-specific monoclonal antibodies with a tumor vaccine has high potential for success. This review summarizes a body of preclinical and clinical work systematically investigating the optimal integration of chemotherapy, HER-2-specific monoclonal antibodies, and a cell-based cancer vaccine that secretes GM-CSF.

The preclinical model

The genetically engineered *neu*-N transgenic mouse was derived from the parental FVB/N strain by placing the gene for the rat HER-2 protein under the regulatory control of the promoter for the mouse mammary tumor virus (MMTV) [8]. As a result, the rat HER-2 protein is expressed specifically in mammary tissue, and *neu*-N mice spontaneously develop mammary tumors beginning at about 4–6 months of age. These breast cancers progress through similar stages that human breast cancers do, including hyperplasia, atypical hyperplasia, ductal carcinoma in situ, and invasive carcinoma. Moreover, because the

immune system develops in the context of rat HER-2 protein expression during development, the rat HER-2 protein is viewed by the immune system as self, with multiple mechanisms of immune tolerance working in concert to keep immune responses to rat HER-2 shut off [9]. Together, the parental FVB/N mouse and the *neu*-N mouse represent a powerful model system for testing the efficacy of immunotherapy strategies in the presence (*neu*-N mouse) and absence (FVB/N mouse) of marked tumor antigen-specific immune tolerance utilizing rat HER-2 as a model tumor antigen.

To interrogate antigen-specific tumor immunity in this model system, a vaccine was developed comprised of 3T3 fibroblast cells genetically engineered to secrete high levels of GM-CSF alone (the control vaccine 3T3GM), or to secrete high levels of GM-CSF and deliver the tumor antigen rat HER-2 (the specific vaccine 3T3neuGM) [9]. In addition, tumor cell lines that express high levels of HER-2 (NT) were derived from the spontaneous tumors that arise in the *neu*-N mice [9]. When FVB/N mice bearing orthotopic HER-2-expressing tumors of about 1 cm in size are vaccinated with the 3T3*neu*GM vaccine, the growing mammary tumors initially stabilize in size and then being to shrink, with an ultimate rejection rate of 100 % [10]. Conversely, these same tumor burdens in FVB/N mice vaccinated with the control 3T3GM vaccine continue to grow relentlessly. In striking contrast, when *neu*-N mice bearing nonpalpable orthotopic NT tumors are vaccinated with either the control 3T3GM or the antigen-specific 3T3neuGM vaccine, the tumors grow out progressively at identical rates, illustrating a complete lack of vaccine efficacy when HER-2-specific immune tolerance is present [10]. Evaluation of the tumor-specific immune responses in these mice showed that FVB/N mice develop high levels of HER-2-specific antibody and a population of HER-2-specific T cells with a predominance of high avidity T cells specific for the immunodominant epitope of rat HER-2, RNEU420-429 [10, 11]. In contrast, vaccinated *neu*-N mice develop very low levels HER-2-specific antibodies, and a low level, heterogeneous HER-2-specific T cell response with a paucity of T cells specific for RNEU420-429 [11, 12]. With this as a platform, strategies that abrogate distinct mechanisms of HER-2-specific immune tolerance in *neu*-N mice, and shift the immune response profile toward the profile observed in vaccinated FVB/N mice, should be the most promising for clinical translation. Exploring interactions with drugs in current clinical use is the most practical place to start developing combination immunotherapies.

Chemotherapy enhances HER-2-specific immunity in vaccinated *neu*-N mice

A variety of chemotherapy drugs in common use for cancer treatment were first tested for their ability to enhance vaccine activity in FVB/N mice [10]. These included ranges of doses of cyclophosphamide, doxorubicin, paclitaxel, and cisplatinum given either 1 day prior to vaccination, at the time of immune priming, or 1 week later, at the time of T cell expansion. In nontolerant FVB/N mice, low doses of cyclophosphamide or paclitaxel given 1 day prior to vaccination enhanced vaccine activity, but completely inhibited the vaccine if given 1 week later. This effect was dose dependent and disappeared as the peripheral T cell count dropped in response to higher chemotherapy doses. In FVB/N mice, doxorubicin or cisplatinum were unable to enhance vaccine activity regardless of dose or schedule. Some of these observations translated to the tolerant *neu*-N mice, where cyclophosphamide or paclitaxel were also able to render the 3T3neuGM vaccine active in *neu*-N mice, with a significant delay in tumor outgrowth in tumor-bearing *neu*-N mice compared with either the group treated with the 3T3neuGM vaccine alone or the control group treated with 3T3GM sequenced with the proper dose and schedule of either cyclophosphamide or paclitaxel [10]. One difference between the parental FVB/N mice and the *neu*-N mice was that *neu*-N mice treated with doxorubicin given 1 week after vaccination displayed significantly slower tumor outgrowth compared with *neu*-N mice treated with the 3T3neuGM vaccine alone, or

the control 3T3GM vaccine sequenced with doxorubicin [10]. This observation is similar to the reported ability of doxorubicin administered in a similar schedule to facilitate the vaccine-induced cytotoxic $CD8^+$ T cell response in the CT26 murine model of colon cancer [13]. Combining both cyclophosphamide and doxorubicin into a polychemotherapy vaccination regimen resulted in the best delay in tumor outgrowth and was associated with a durable complete tumor clearance rate of about 30 % in tolerant *neu*-N mice [10].

Analyzing the immune effector response of these regimens yielded multiple insights. The addition of either cyclophosphamide or paclitaxel to the vaccine reversed immunologic skew, promoting the evolution of a HER-2-specific T helper type 1 response [10]. In addition, cyclophosphamide was demonstrated to abrogate the suppressive influence of cycling $CD4^+CD25^+FoxP3^+$ Treg in *neu*-N mice, thereby facilitating the recruitment of high avidity $CD8^+$ T cells specific for RNEU420-429 that otherwise remain idle in the tolerant setting [14].

HER-2-specific monoclonal antibodies enhance HER-2-specific T cell responses in vaccinated *neu*-N mice

Since the vaccine-induced HER-2-specific antibody response in *neu*-N mice is negligible [10], the role of antibody in contributing to the complete tumor clearance observed in nontolerant FVB/N mice was explored by conducting adoptive transfer experiments in SCID mice [15]. These studies showed that either HER-2-specific immunoglobulin (IgG) or a $CD8^+$ T cell line specific for HER-2 [11], both derived from vaccinated FVB/N mice, could partially protect against the outgrowth of an NT tumor cell challenge. However, only the adoptive transfer of both HER-2-specific IgG and $CD8^+$ T cells specific for HER-2 fully protected the mice from an NT tumor cell challenge [15]. Building on these observations, the efficacy of adding exogenous HER-2-specific monoclonal antibodies to vaccination in order to passively restore the humoral immune response was investigated. Sequencing two distinct HER-2-specific monoclonal antibodies (7.9.5 and 7.16.2) with vaccination could markedly delay tumor outgrowth, with rates of protection of 70 and 30 % in the prevention and treatment settings, respectively [16]. This effect was T cell dependent, and resulted in greater numbers of HER-2-specific cytokine-secreting $CD8^+$ T cells, enhanced tumor antigen processing and presentation, and greater lysis of HER-2-expressing tumor cells [16]. For clinical translation, the murine monoclonal antibody 7.16.4, similar to the therapeutic HER-2-specific monoclonal antibody Trastuzumab ($Herceptin^R$) in wide use for breast cancer treatment, was similarly evaluated [17]. Adding 7.16.4 monoclonal antibody to vaccination led to a higher HER-2-specific $CD8^+$ T cell response, and the protection of about 60 % of mice from a subsequent NT tumor challenge. Notably, both of these endpoints were dependent on the Fc portion of the antibody. Further experiments demonstrated that the antibody enhanced locoregional immune priming through the Fc-mediated activation of dendritic cells, resulting in higher levels of proliferation and cytokine production by HER-2-specific $CD8^+$ T cells in vivo [17]. Furthermore, antibody-modulated vaccination promoted the evolution of the $CD44^+CD62L^+CD8^+$ HER-2-specific central memory T cell response. Finally, the combination of low dose cyclophosphamide, 7.16.4 monoclonal antibody, and vaccination generating the highest numbers of HER-2-specific $CD8^+$ T cells, and protected up to 70 % of mice from the outgrowth of established tumors (Emens, unpublished data).

The human vaccine

In order to validate these preclinical observations in breast cancer patients, we developed a human GM-CSF-secreting breast tumor vaccine [18]. We chose two cell lines based on cell growth rates in vitro, transfection efficiency, breast cancer profile (expression of estrogen

receptor (ER) and HER-2) and tumor antigen delivery. T47D and SKBR3 were selected to represent $ER_{pos}/HER\text{-}2_{neg}$ and $ER_{neg}/HER\text{-}2_{pos}$ breast cancers, respectively, and to deliver tumor antigens other than HER-2 (MUC-1, CEA, p53). They were then genetically modified by plasmid DNA transfection to generate the subclones 2T47D-V and 3SKBR3-7 that secrete high levels of GM-CSF. The two subclones are mixed together to formulate a generalizable vaccine that secretes a sufficient level of GM-CSF for immune activation and that delivers HER-2 at levels sufficient to enable immune monitoring of vaccine-induced immune responses by characterizing correlative HER-2-specific antibody and T cell responses.

Timed sequential therapy with cyclophosphamide, doxorubicin, and a GM-CSF-secreting tumor cell vaccine for metastatic breast cancer

The allogeneic, HER-2-expressing GM-CSF-secreting breast tumor vaccine was first tested as a single agent, and then in sequence with a range of low doses of cyclophosphamide (given the day prior to vaccination) and doxorubicin (given 7 days after vaccination) in 28 patients with stable metastatic breast cancer [19]. This clinical study utilized an innovative three by three factorial matrix design to efficiently determine the best predicted dose combination of cyclophosphamide and doxorubicin when given with a fixed dose of vaccine cells (5×10^8 cells). Cyclophosphamide was tested at doses of 0, 200, 250, and 350 mg/m^2, and doxorubicin was tested at doses of 0, 15, 25, and 35 mg/m^2. These doses were chosen in order to encompass the human equivalent of the chemotherapy drug doses found to be effective in the murine model, where the most effective doses were 100 mg/kg cyclophosphamide (equivalent to a human dose of 225 mg/m^2), and 5 mg/kg doxorubicin (equivalent to a human dose of 12 mg/m^2) [10]. Eligible patients received three monthly cycles of chemotherapy-modulated vaccination, followed by a fourth boost cycle of the same intervention provided disease was stable or better [19]. The study demonstrated that chemotherapy-modulated vaccination is safe, and associated with the induction of HER-2-specific $CD4^+$ T cell-dependent HER-2-specific immunity as measured by delayed type hypersensitivity (DTH) and antibody levels. Optimal chemotherapy doses (cyclophosphamide 200 mg/m^2 and doxorubicin 35 mg/m^2) significantly augmented the relatively low levels of HER-2-specific antibody induced by vaccine alone. Cyclophosphamide doses greater than 200 mg/m^2 completely inhibited both DTH and antibody responses specific for HER-2, illustrating the importance of studies defining cancer vaccine-drug interactions in patients. Interestingly, the effective dose of cyclophosphamide was similar in the murine and human settings, whereas the best doxorubicin dose was quite different for the murine model as compared to breast cancer patients.

The mechanism by which cyclophosphamide and doxorubicin augment vaccine activity is under investigation. Initial analysis of peripheral Treg levels with time after each vaccination cycle suggests that they do not change significantly (Emens, unpublished data). Importantly, the addition of chemotherapy to vaccination does appear to impact immune priming [19]. With each vaccine cycle, serum GM-CSF levels peaked at day 2, and declined thereafter. When the vaccine was given as a single agent, peak levels were highest with the first vaccine, and declined with each subsequent vaccine cycle. When cyclophosphamide and doxorubicin were sequenced with the vaccine, peak levels of GM-CSF were maintained across all 4 cycles of vaccination. Further investigation of this observation, as well as analysis of the impact of chemotherapy dose on HER-2-specific T cell responses is ongoing.

This study has at least two broader implications. Importantly, this study defined a cyclophosphamide dose of 200 mg/m^2 within the dose range tested as most optimal for enhancing vaccine-induced immunity compared with doses of 250 or 350 mg/m^2, which were ineffective [19]. Historically, cyclophosphamide doses of 300 mg/m^2 have been used

for enhancing immunotherapies in Phase II and III cancer vaccine trials. This study provides one possible explanation for the lack of vaccine efficacy observed in those trials. As a result, we are taking a cyclophosphamide dose of 200 mg/m^2 forward as the best dose of cyclophosphamide for immune modulation in current and future breast cancer vaccine trials. In addition, these results suggest that analysis of vaccine-induced DTH and/or antibody responses may be one straightforward measure of antigen-specific immunity in optimization trials designed to define the best immunotherapy drug doses and schedules. This study was initially designed to test a range of cyclophosphamide doses that included 0, 250, 350, and 450 mg/m^2. In conducting the study, we observed the induction of de novo HER-2-specific DTH with vaccine alone in the first six patients. Once chemotherapy was added to the vaccination regimen, we noticed in real-time that the induction of HER-2-specific immunity by DTH skin testing disappeared and some chemotherapy-related side effects appeared (hair loss, mild nausea). Therefore, we modified the study design to drop the highest dose of cyclophosphamide planned for testing (450 mg/m^2) and add a lower dose than originally planned (200 mg/m^2). Thus, these simple measures can be used for realtime analysis of immune responses to guide dose and schedule selection in proof of principle studies. They may also have an important role in studies that involve multiple sites where the T cell analysis might be more likely compromised by variations in sample collection, storage, and transport.

Therapy with cyclophosphamide, Trastuzumab, and a GM-CSF-secreting tumor cell vaccine for HER-2-expressing metastatic breast cancer

A clinical trial testing the combination of weekly Trastuzumab, cyclophosphamide, and the GM-CSF-secreting vaccine in women with measurable or evaluable HER-2$^+$ metastatic breast cancer (for whom Trastuzumab is standard breast cancer therapy) has also been completed [20]. This was a single arm, single institution, open label study of CY (300 mg/m^2), and the allogeneic HER-2-expressing GM-CSF-secreting breast tumor vaccine with standard weekly Trastuzumab. Doxorubicin was not used in this vaccination regimen due to its widely recognized synergistic cardiac toxicity with Trastuzumab [21], and cyclophosphamide was used at 300 mg/m^2 based on historical data since the trial described above was not complete when this study was designed. This clinical trial demonstrated the safety of the combination regimen, with clinical benefit rates of 50 % at 6 months, and 35 % at 1 year. Seven of the 20 vaccinated patients developed new or increased immunity to HER-2 by DTH. The serum GM-CSF pharmacokinetics were similar to those observed with cyclophosphamide, doxorubicin, and vaccination, suggesting that cyclophosphamide may be altering immune priming in a manner that maintains peak levels of GM-CSF at day 2 across serial vaccination cycles. Early exploratory analyses revealed an overall survival 40 months (Emens, unpublished data) compared with the historical overall survival of 13–24 months in similar patients who received standard Trastuzumab alone [22, 23]. Based on the acceptable safety profile and the chemotherapy dose finding study described previously, we are now actively conducting a similarly designed study of vaccination sequenced with cyclophosphamide at 200 mg/m^2 with weekly Trastuzumab in patients with high-risk HER-2$^+$ breast cancer and no evidence of disease. The safety of the regimen and the promising clinical data together provide support for testing the vaccine with Trastuzumab and the optimal dose of cyclophosphamide 200 mg/m^2 in a larger randomized study designed to rigorously demonstrate meaningful clinical activity of cyclophosphamide-modulated vaccination on a Trastuzumab backbone.

Targeting distinct elements of the tumor microenvironment to promote tumor rejection

The preclinical and clinical work described above aims to integrate tumor vaccines with established cancer drugs that work through effects on the transformed cancer cell itself. In addition, both cyclophosphamide and Trastuzumab modulate the tumor microenvironment. Low dose cyclophosphamide abrogates the suppressive activity of systemic and intratumoral Tregs [5], whereas Trastuzumab facilitates antibody-dependent cytotoxicity (ADCC) [24] and inhibits angiogenesis through modulating vascular endothelial growth factor (VEGF) production [21]. The immune-based activity of DC101, a monoclonal antibody that targets the VEGF receptor 2 (VEGFR2) found on tumor-associated endothelial cells was also explored [25]. This antibody is known to disrupt the tumor-associated vasculature [26]. Treating non-tolerant NT tumor-bearing FVB/N mice with DC101, but not control IgG, induces tumor regression during active antibody treatment [27]. Notably, this regression continues to complete tumor clearance after the DC101 antibody treatment is stopped. Corresponding to this effect, FVB/N mice treated with DC101, but not control IgG, develop HER-2-specific T cells even though they have not been vaccinated. Furthermore, T cell depletion studies demonstrated that DC101 activity is partially T cell dependent, with the residual antitumor effect likely due to its direct anti-angiogenic activity. This pattern is reflected in *neu*-N mice, where although the growth of existing NT tumors is stabilized by DC101, the tumors do not regress [25]. Adding DC101 to either the control vaccine 3T3GM or the targeted vaccine 3T3neuGM delays tumor outgrowth to the same extent, again reflecting the direct anti-angiogenic activity of DC101 in this tolerant setting. Sequencing vaccination with cyclophosphamide and doxorubicin in the setting of DC101 treatment unmasks the T cell-dependent activity of DC101 in *neu*-N mice by allowing the vaccine to work and results in a tumor free survival rate of about 70 %. Other anti-angiogenic drugs, including monoclonal antibodies specific for VEGF, and the multi-kinase inhibitors sorafenib and sunitinib, can also modulate immunity [4]. VEGF-specific monoclonal antibody therapy can enhance dendritic cell function by functioning as a sink for the inhibitory cytokine VEGF [27], sunitinib can inhibit myeloid-derived suppressor cells and Tregs [28], and sorafenib can shift the phenotype of macrophages from the protumorigenic phenotype M2 to the antitumor phenotype M1 [29]. These findings together suggest that targeted cancer drugs represent a rich resource of immunomodulators. Recent data demonstrating enhanced T cell recognition of melanoma cells in the context of BRAF inhibitor treatment in provide further support for this concept [30].

Conclusions

Cancer immunotherapy has finally taken its place in the clinic alongside the more traditional treatment strategies of surgery, chemotherapy, endocrine manipulation, and radiation therapy, but we still clearly have lots of work to do. The series of preclinical and clinical studies described here illustrates one approach to developing combinatorial immunotherapies, incorporating relevant murine models of breast cancer, innovative clinical trial design, and the collection of clinical samples that facilitate the dissection of mechanisms of immune priming and response as they are shaped by drug-vaccine interactions in cancer patients themselves. Simultaneously, detailed elucidation of the pathways that control cancer growth and progression and are specific to each cancer type—or even subtype—has yielded a large number of promising targeted drugs with unique activities that will also intersect with the host antitumor response. Trastuzumab, a humanized monoclonal antibody in common use for the treatment of early and late stage HER-2-expressing breast cancer, is one of the first examples of a highly active targeted agent specific for a pathway indispensible for oncogenesis, and that also has clear immune-

modulating activity. Rapidly emerging data suggests that targeted agents distinct from monoclonal antibodies—tyrosine kinase inhibitors and other small molecules—will also have immune-modulating activity. The challenge before us lies in using innovative strategies that effectively marry immune-based strategies with pathway-specific agents to create the ultimate personalized medicine and generate therapeutic synergy powerful enough to cure cancer.

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