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Breast cancer immunobiology driving immunotherapy: vaccines and immune checkpoint blockade

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Abstract

Breast cancer is immunogenic, and infiltrating immune cells in primary breast tumors convey important clinical prognostic and predictive information. Furthermore, the immune system is critically involved in clinical responses to some standard cancer therapies. Early breast cancer vaccine trials have established the safety and bioactivity of breast cancer immunotherapy, with hints of clinical activity. Novel strategies for modulating regulators of immunity, including regulatory T cells, myeloid-derived suppressor cells and immune checkpoint pathways (monoclonal antibodies specific for the cytotoxic T-lymphocyte antigen-4 or programmed death), are now available. In particular, immune checkpoint blockade has enormous therapeutic potential. Integrative breast cancer immunotherapies that strategically combine established breast cancer therapies with breast cancer vaccines, immune checkpoint blockade or both should result in durable clinical responses and increased cures.

Keywords

breast cancer immunity; breast cancer vaccine; clinical trials; immune checkpoint blockade; immune tolerance; immunotherapy; tumor microenvironment

Concerted efforts to optimize the discrete components of standard therapy for breast cancer (surgery, radiation therapy, chemotherapy and endocrine therapy) have generated small improvements in clinical outcomes. In the aggregate, these incremental gains have resulted in significant improvements in overall survival for those afflicted with breast cancer. More recently, targeted therapies that improve quality of life and delay disease progression in patients with metastatic breast cancer have emerged; these include trastuzumab, pertuzumab, lapatinib, T-DM1 and everolimus. Trastuzumab-based chemotherapy also confers a small survival advantage in advanced disease. The incorporation of trastuzumab into standard

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adjuvant breast cancer therapy decreases the risk of relapse for those newly diagnosed with the disease by 50% [1]. These very recent successes highlight the potential for further gains in overall survival as additional targeted drugs are effectively incorporated into adjuvant therapy.

The genomic revolution has substantially enhanced our understanding of breast cancer biology. While breast cancers were historically divided into those that are hormone-dependent (expressing the estrogen receptor-α [ER] or progesterone receptor [PR]) and those that are not (ER- and PR-negative), current gene profiling illuminates breast cancer as a heterogeneous collection of disease subtypes [2]. These include the various intrinsic subtypes (luminal A, luminal B, HER-2-overexpressing, basal-like and claudin-low) and a normal breast-like group. Each of these has a distinct prognosis and natural history. While these detailed molecular subtypes do not yet drive clinical practice, at least three distinct subtypes are treated differently according to the current standard of care. Breast tumors that express ER and/or PR are typically treated with endocrine therapy as one component of therapy; those that overexpress HER-2 are typically treated with trastuzumab (and other HER-2-directed therapies in advanced disease), and those that fail to express ER, PR and HER-2 – triple negative and many basal-like breast tumors – are typically treated with traditional cytotoxic chemotherapy. Defining the genes driving the biology of each of these molecularly discrete breast tumor subtypes has the clear potential for generating new strategies for highly effective targeted therapy, including immunotherapy.

Breast cancer & the immune system

Breast cancer & immune suppression

The immune system can play a dual role in breast cancer, both promoting tumorigenesis through inflammatory pathways that also suppress adaptive immunity and preventing tumor formation through active immune surveillance. Consistent with this duality, some breast cancer patients display clear evidence of immune suppression. They have lower absolute numbers of lymphocytes [3] and Vα24Vβ1 natural killer T (NKT) cells [4] in the peripheral blood, with decreased STAT1 signaling and IFN-γ production in both lymphocytes and NK cells [5]. $CD3^+CD8^+$ cytotoxic T cells derived from breast cancer patients display a significant downregulation of the T-cell receptor (TCR)-ζ chain and cell surface CD28, and a significant upregulation of CD95 (FAS) [6]. Patients with breast cancer have increased numbers of both $CD4^+CD25^+$ regulatory T cells (Tregs) [7] and myeloid-derived suppressor cells (MDSC) [8] within the peripheral blood, and elevated serum arginase levels [9]. Dendritic cells (DCs) within the peripheral blood and tumor-draining lymph nodes of breast cancer patients are both diminished in number and are dysfunctional, with low cell-surface MHC Class II and B7 (CD86) molecules, and lower IL-12 secretion [10,11]. Tumor-infiltrating T lymphocytes (TILs) and mature DCs correlate with lymph node involvement and tumor grade, and the presence of plasmacytoid DCs is associated with shorter disease-free and overall survival [12]. Breast cancer metastasis to sentinel lymph nodes is associated with maturation arrest and apoptosis of DCs, and poor co-localization of DCs with $CD8^+$ T cells [13]; these effects may be due in part to tumor-derived TGF-β[14]. More recently, a chronic inflammatory signature was reported to be uniformly present in all breast cancers, regardless of subtype [15]. In the setting of persistent chronic inflammation, dynamic interactions between host and genomically-damaged breast epithelial cells support continued tumor progression and metastasis. In preclinical breast cancer models, IL-4-expressing $CD4^+$ T lymphocytes indirectly promote invasion and metastasis of breast cancers by directly regulating tumor-associated $CD11b^+Gr1^-F4/80^+$ macrophages (TAMs) [16]. These TAMs in turn enhance metastasis through activation of epidermal growth factor receptor signaling in breast tumor cells. In addition, tumor-infiltrating Tregs stimulate breast cancer metastasis by secreting receptor activator of nuclear factor κ-B ligand to stimulate the

spread of receptor activator of nuclear factor κ-B^+ breast tumor cells [17]. Breast cancers can downregulate the expression of cell-surface MHC Class I molecules and decrease expression of LMP2 and LMP10 subunits within the endogenous antigen-processing pathway [18]. Notably, HER-2 signaling can be associated with downregulation of the peptide transporter associated with antigen processing, LMP2, LMP10 and the proteasome activator proteins, PA28α/PA28β and tapascin [19]. The majority of breast cancers express COX2, which results in high intratumoral levels of prostaglandin E_2 (PGE_2) and an inhibitory environment for DC and T-cell function [10]. PGE_2 can induce FoxP3 expression in T cells within the tumor microenvironment, thereby transforming incoming effector T cells to create local Tregs that shut intratumoral immune responses down [20].

Breast tumor cells also express several molecules involved in immune checkpoint regulation. CD40 is expressed on the surface of breast tumor cells, and appears to play a role in apoptosis as well as immune regulation [21]; its impact on infiltrating immune cells remains unclear. B7-H1 (programmed death ligand-1 [PD-L1]) is expressed within breast tumors by both malignant mammary epithelial cells and by TILs, particularly in tumors with high-risk features (high grade, lack of ER and PR expression, HER-2 expression, large tumor size) [22]. B7-H1 expression appears to be associated with high levels of cell proliferation (Ki67 high) [23]. B7-$H1^+$/PD-1^+ T lymphocytes and Tregs coinfiltrate the tumors of patients with high-risk breast cancer [24]. B7-H1 expression is associated with phosphoinositide-3 (PI3) kinase activation, and tumor cell resistance to immune-mediated attack can be alleviated by PI3 kinase inhibition [25]. Doxorubicin downregulates cell surface B7-H1 and upregulates expression of B7-H1 in the nucleus of breast cancer cells by a PI3 kinase-dependent process [26]. In addition, over 50% of breast cancers express B7-H4 [27–29], a cell surface protein that both inhibits anti-tumor T-cell responses and promotes neoplastic transformation [28]. B7-H4 expression increases in proportion and intensity with tumor progression, and high levels of B7-H4 expression are associated with decreased levels of TILs [30].

Breast cancer can be immunogenic

Notably, the presence of immune cells in breast cancers can also exert an anti-tumor effect, and predict a favorable response to cancer therapy. Different types of infiltrating immune cells have distinct prognostic and predictive significance. DCs, M1 macrophages, Th1 $CD4^+$ T cells, cytotoxic $CD8^+$ T cells and NK cells protect against tumor growth, whereas M2 macrophages, MDSCs, neutrophils, Th2 $CD4^+$, Th17 $CD4^+$ and $FoxP3^+$ $CD4^+$ T cells promote tumor growth. The influence of these immune cells depends on their intratumoral and peritumoral cellular distribution, their architecture, and the overall immune context and histology of the breast tumor [31]. Poor prognostic factors (lack of ER and PR expression, high tumor grade, and lymph node involvement) are associated with a significantly higher $CD3^+$, $CD8^+$ and $FoxP3^+$ cellular infiltrate prior to chemotherapy [32]. Chemotherapy results in a dramatic decrease in $FoxP3^+$ cellular infiltrate, with an increase in cytotoxic T-cell markers (TiA1 and granzyme-B) in patients with a pathologic complete response (pCR) to chemotherapy [32]. MHC class I expression on tumor cells and intratumoral Tregs can predict relapse-free survival after adjuvant chemotherapy [33]. The percentage of intratumoral lymphocytes is a significant predictor of pCR, with the pCR of lymphocyte-infiltrated breast cancers being about 40%, and the pCR response of lymphocyte-poor breast tumors being about 5% [34]. Lymphocyte-rich ER-negative breast cancers respond better to neoadjuvant anthracycline-based chemotherapy regardless of HER-2 status, with pCR rates of 74% compared with 31% in lymphocyte-rich versus lymphocyte-poor patients, respectively [35]. Adjuvant anthracycline-based therapy is associated with greater disease-free survival only in patients whose tumors had high levels of intraepithelial $CD3^+$ T lymphocytes [35]. High $CD8^+$ and low $FoxP3^+$ T cell infiltrates after chemotherapy are

significantly associated with better disease-free and overall survival, outperforming classical predictive factors [36]. Interestingly, a score derived from a combination of the CD8:FoxP3 ratio and pathological staging identifies a subgroup of patients with a long-term overall survival of 100% [36]. The total number of $CD8^+$ TILs within tumors and TILs present in distant tumor-associated stroma are both associated with better patient survival [37].

Integrated molecular profiling of breast cancers has shown that the strongest predictor of a favorable disease outcome is a gene signature reflecting a high Th1 and cytotoxic T lymphocyte response relative to a Th2 response [15]. Basal-like (most frequently triple negative) breast tumors displayed high expression of Th2 genes and a low Th1/Th2 gene ratio. Interleukin signaling pathways were preferentially activated by *in situ* or invasive breast cancers compared to normal healthy breast tissue, with STAT4 signaling representing the largest single pathway difference. Tumorassociated high endothelial venules (HEV) are located specifically within lymphocyte-rich areas of solid tumors, including breast cancers [38]. The density of tumor-associated HEV within the tumor stroma is a strong predictor of infiltration by $CD3^+$ and $CD8^+$ T cells as well as B cells [38]. Tumor-specific cytotoxic T-cell responses tend to develop in breast tumors that are well differentiated, express the ER, have low levels of proliferation, and contain high levels of intratumoral IFN-α and low levels of intratumoral TGF-β [39]. Primary breast tumors with high densities of tumor-associated HEVs also have increased naive, central memory and activated effector memory T cell infiltrates and upregulated T-helper type 1 and $CD8^+$ cytotoxic T-cell genes. Tumor-associated HEV were independently associated with a lower risk of disease relapse and longer disease-free and overall survival. In contrast, tumor-specific antibody (largely IgM) responses were detected in about 50% of patients, and were associated with advanced tumor stage, lack of tumor-specific T-cell responses, and increased intratumoral TGF-β and decreased intratumoral IFN-α.

Immunity & breast cancer therapy

Rapidly growing literature illustrates a role for the immune system in the clinical response to some standard systemic breast cancer therapies. As discussed above, adjuvant anthracycline-based therapy was associated with greater disease-free survival only in patients whose tumors had high levels of intraepithelial $CD3^+$ T lymphocytes [35]. In contrast, there was no association between intratumoral $CD3^+$ T cells and outcome in patients treated with cyclophosphamide, methotrexate and 5-fluorouracil [35]. Consistent with this, individuals with breast cancer who carry a specific mutation of the TLR-4 have a higher risk of relapse after adjuvant treatment with anthracycline-based chemotherapy than those who are wild-type for TLR-4 [40]. Additionally, standard anthracycline-based chemotherapy is associated with an increase in MDSCs within the peripheral blood [8]. A number of other breast cancer therapies also engage the immune system. Early breast cancers treated with preoperative paclitaxel demonstrate new immune cell infiltrates within the tumor at the time of surgery [41]. The aromatase inhibitor letrozole has been shown to reduce intratumoral $FoxP3^+$ Tregs, with a greater decrease in $FoxP3^+$ T cells observed in responding patients after 6 months of neoadjuvant aromatase inhibitor therapy [42]. Zolendronate promotes the activity of Vγ9Vδ2 T cells in patients with breast cancer [43,44]. Patients treated with trastuzumab develop tumor-specific $CD4^+$ T lymphocytes both within the peripheral blood [45], and within the primary breast tumor itself [46].

Tumor-specific effector T cells

The clinical potency of the breast cancer-specific immune response is determined by the scope of the T cell repertoire available for recruitment, and the magnitude of active immune tolerance and suppression [47]. T cells with the highest recognition efficiency (avidity) for breast tissue-specific antigens are typically deleted centrally during thymic education, or

peripherally under conditions of high antigen load (widely metastatic breast cancer). The central processes of thymic deletion and selection, complemented by peripheral deletion, result in a T-cell repertoire that is optimized for fighting infectious disease and avoiding autoimmune disease, but that is suboptimal for fighting cancer due to a relatively lower efficiency of tumor antigen (self antigen) recognition [48–50]. Despite these deletion mechanisms, high-avidity T cells specific for self antigens, including tumor antigens, can escape thymic deletion to take up residence in the periphery [51–54]. Two major backup systems keep high-avidity autoreactive cells in check, thereby maintaining immunologic homeostasis under normal conditions. These two back-up systems are cell-based (including a variety of immunoregulatory cells), and molecule-based (including the immunologic checkpoint system). Because they abrogate existing protective anti-tumor responses to developing neoplasms, manipulating these layers of regulation can augment natural or therapeutically induced anti-tumor immunity.

Regulatory cells & tumor immunity

The cell-based regulatory system includes two major classes of regulatory cells: $CD4^+CD25^+FoxP3^+$ Tregs and MDSCs, which in mice are $GR\text{-}1^+CD11b^+$. Naturally occurring Tregs represent about 5–10% of peripheral $CD4^+$ T lymphocytes in healthy individuals [55]. These cells express the immune checkpoint molecule cytotoxic T lymphocyte antigen-4 (CTLA-4), the glucocorticoidinduced tumor necrosis factor receptor, and the transcription factor FoxP3, and secrete IL-10 and TGF-β. This cell phenotype can be induced within the tumor microenvironment, or in response to vaccination, effectively transforming effector T cells into suppressive Tregs. MDSCs have more recently emerged as important regulators of immunity, providing a negative feedback mechanism for curtailing the normal immune response, but inhibiting antitumor immunity [56]. They accumulate in the context of active inflammation, and expand in patients with cancers that secrete proinflammatory mediators, such as VEGF and granulocyte macrophage-colony stimulating factor (GM-CSF). They are a heterogeneous group of myeloid-derived cells, and phenotypic markers of these cells in humans have not been well defined. Certain metabolic enzymes, indoleamine 2,3-deoxygenase (IDO) and arginase (ARG) are expressed by intratumoral myeloid-derived cells and, in the case of IDO, by tumor cells themselves [57,58]. IDO and ARG inhibit immune responses by depleting local amino acid levels essential for T cell function within the tumor microenvironment. IDO and ARG represent promising therapeutic targets for enhancing anti-tumor immunity, as inhibiting their activity promotes the intratumoral inflammatory response.

Immune checkpoint regulation & tumor immunity

The genomic revolution has dramatically advanced our understanding of the molecular control of the tumor-specific immune response. While the immune response is initiated by the recognition of antigen in the context of MHC by the TCR, the ultimate magnitude and quality of the response is determined by the summation of positive and negative signals delivered by cell surface immune checkpoint molecules [59]. These immune checkpoint pathways regulate both the magnitude of T-cell activation by antigen presenting cells, and T-cell activity within the tumor microenvironment. Cell surface receptors and ligands that mediate inhibition of the immune response are frequently overexpressed within the tumor microenvironment by tumor cells themselves, resulting in active abrogation of activated infiltrating T cells, or lack of activation of resident T cells due to an activation sequence limited to signaling through the MHC:Ag:TCR interaction. Conversely, three distinct classes of positive and negative signaling molecules are present at the interface between antigen-presenting cells and T cells. It is the summation of signals emanating from these receptors that determines the ultimate strength of T-cell activation, and drives the differentiation of naïve T cells into primary effector T cells and effector memory T cells. The classical B7

accessory molecules, B7-1 (CD80) and B7-2 (CD86), interact with CD28 and CTLA-4 early in T-cell activation to transmit activating and inhibiting signals respectively. Here, the receptor that promotes T-cell activation, CD28, is expressed on naive and resting T cells, and the receptor that antagonizes T-cell activation, CTLA-4, is upregulated after T-cell activation to provide feedback inhibition of the immune response. Newer checkpoint molecules within the B7 family, members of the PD-1 family of coreceptors for example, further toggle T-cell signaling toward activation or inhibition early in the activation process. After primary T cell-activation, other types of immune checkpoint molecules are recruited to the immunologic synapse to refine the character, quality, and durability of the ultimate T-cell response. The inducible costimulator (ICOS) and CD40 pathways promote Th2 responses and T cell-dependent humoral immunity, whereas the OX40/4-1BB pathways promote the activation of $CD4^+$ and $CD8^+$ T cells. A third major influence on T-cell activation within the tumor microenvironment is delivered by soluble cytokines. These distinct types of signaling, along with a summary of the clinical development status, are summarized in Table 1.

Breast cancer vaccines

Breast tumor antigens & vaccine platforms

The development of effective breast cancer vaccines for both therapy and prevention depends in part on the identification of *bona fide* breast tumor antigens that function as tumor rejection targets. Many endogenous proteins induce tumor-specific T cells, but these T cells do not induce tumor regression or rejection. This lack of T-cell efficacy could be due to intratumoral counter-regulatory measures that shut these cells down, heterogeneous expression of tumor antigens within the primary breast tumor or its metastases, or evasion of immunity by downregulation of the tumor antigen itself within breast tumors. The immunization strategy likely to have the highest efficacy will employ a vaccine platform that incorporates multiple antigens, includes antigens essential for cellular transformation, and includes proven tumor rejection targets. New information revealed by the genomic and proteomic classification of breast tumors should hasten the identification of new breast tumor antigens that play central roles in breast cancer initiation, progression and metastasis, and that also represent targets for effective immune-mediated rejection. The HER-2 protein is just such an example, and the clinical success of trastuzumab and pertuzumab, both monoclonal antibodies specific for HER-2, defines HER-2 as the first truly validated target for breast cancer immunotherapy.

A number of breast tumor antigens have been described, and HER-2, carbohydrate antigens, and mucin-1 (MUC)-1 have received the greatest attention as antigens for vaccine formulation. Breast cancer patients develop low levels of T cells and antibodies specific for both HER-2 and MUC-1 [60–62], suggesting that it may be possible to amplify these baseline immune responses to a therapeutically relevant level with well designed vaccination protocols. As an alternative to antigen-specific vaccination with peptides or protein subunits, vaccine platforms derived from cell extracts or whole tumor cells themselves represent an inherently polyvalent immunization strategy. Cell-based vaccines thus have the advantage of delivering multiple antigens, increasing the likelihood of including the most potent immune rejection target antigens and decreasing the likelihood of immune escape by the tumor due to the evolution of antigen- specific loss variants.

Distinct vaccination platforms engage different aspects of the anti-tumor immune response (Table 2). The $CD8^+$ T-cell response has long been considered the primary determinant of immune-mediated tumor rejection. Therefore, many of the first cancer vaccine strategies focused on inducing tumor-specific $CD8^+$ T cells, largely through immunizing with short peptide antigens that bind to MHC Class I molecules. Although this strategy can induce

$CD8^+$ T cells, these T-cell responses are typically weak and short-lived. It is now clear that engaging the $CD4^+$ helper T-cell response is critical for maximizing tumor immunity, as it optimizes both the $CD8^+$ T-cell response and also supports the humoral anti-tumor immune response [63]. Newer peptide-based vaccines include T helper peptide epitope mixtures, or utilize long peptides that effectively deliver both $CD4^+$ and $CD8^+$ T epitopes in the same peptide. Finally, a large body of data now support the idea that a coordinated immune response involving $CD8^+$ T cells, humoral immunity and innate immune effectors (NK cells, macrophages, neutrophils, eosinophils and basophils) functioning in concert most effectively mediates tumor rejection [64,65]. The breast cancer platforms tested to date recruit these arms of the immune response with varying degrees of effectiveness.

Breast cancer vaccine trials

A large number of early phase breast cancer vaccine trials have been conducted, and are summarized in Table 3. Most available clinical data evaluate vaccines that target HER-2 or carbohydrate antigens such as MUC-1.

Clinical trials targeting HER-2

Vaccine platforms targeting HER-2 that have been evaluated clinically include peptide-based vaccines, protein-based vaccines, plasmid DNA-based vaccines, DC-based vaccines and vaccines that deliver HER-2 in a viral or bacterial platform. The HER-2 directed vaccine in most advanced clinical testing is the HER-2-specific peptide vaccine being developed by the US Military Institute Clinical Trials Group. In follow up to an early clinical trial testing the vaccine in patients with breast and ovarian cancer [66], they first conducted two complementary clinical trials testing the HER- 2-specific peptide epitope E75 (HER-2 p369–377) plus GM-CSF given intradermally to previously treated, disease-free, lymph node-positive breast cancer patients (a dose-escalation study), or lymph node-negative breast cancer patients (a dose optimization study) [67–70]. These two trials combined enrolled a total of 186 HLA-A2 or -A3 patients, vaccinating 101 patients and following the remaining 85 patients as controls. Toxicities were minimal, and the immune response to vaccination was dose-dependent. At a median follow up of 20 months, the recurrence rate was 5.6% in vaccinated patients compared to 14.2% in the controls ($p = 0.04$). With further follow up, the difference in recurrence rate lost significance as immunity waned. The use of booster inoculations in 53 patients vaccinated on the original trial was then evaluated. E75-specific immunity remained elevated in 94.4% of patients at 6 months postvaccination compared with 48% of patients over 6 months from vaccination ($p = 0.002$); booster vaccination was safe and able to restimulate E75 immunity in those patients who had lost immune responses [71]. Additional analyses from these trials showed that the E75 vaccine was able to induce intra and interantigenic spreading in vaccinated patients [72], decreased peripheral blood Tregs and serum TGF-β, and increased memory $CD4^+$ and $CD8^+$ T cells [73,74]. Interestingly, analyses also showed that HLA-A3 and HLA-A2 patients responded similarly to the vaccine (an HLA-A2-binding HER-2 epitope) [75], and that breast cancer patients with HER-2-negative tumors developed higher levels of immunity than breast cancer patients with HER-2-overexpressing tumors [76]. The E75 peptide vaccine is currently being tested in a Phase III clinical trial, the PRESENT study, to definitely determine its ability to prevent disease relapse in patients with early stage HER-2-negative breast cancer. The US Military Institute Clinical Trials Group has tested other HER-2-derived peptide vaccines in small clinical trials with evidence of both safety and bioactivity; these vaccines target the transmembrane HER-2 epitope GP2 [77], and the HER-2/*neu* peptide epitope p776–790 modified with the addition of Ii-Key, a four amino acid LRMK modification that enhances binding to MHC class II [78,79].

The Tumor Vaccine Group at the University of Washington (USA) has tested a variety of HER-2 peptide and protein vaccines in patients with HER-2-overexpressing breast cancers [80–86]. This series of clinical trials demonstrated that patients with breast cancer who were vaccinated with HER-2-derived peptides containing both MHC class I and II epitopes and GM-CSF developed robust, durable $CD8^+$ T-cell responses by ELISPOT [81], and HER-2/*neu*-specific $CD4^+$ T-cell immunity as reflected by new delayed-type hypersensitivity (DTH) specific for HER-2 [82]. In contrast, vaccination with an HLA-A2-binding peptide derived from HER-2, p369–377, plus GM-CSF results in short-lived peptide immunity in patients with breast cancer [83]. Similar to observations in the US Military Institute Trials, immune responses induced by the HER-2 peptide vaccine that activated both $CD4^+$ and $CD8^+$ T cells displayed epitope spreading within the HER-2 protein and to other antigens not directly included in the vaccine [84,85]. Vaccination with HER-2 protein encompassing the HER-2 intracellular domain was similarly immunologically active [86].

A vaccine composed of DCs that were activated *in vitro* with IFN-γ and bacterial lipopolysaccharide to become highly polarized DC1-type DCs that secrete high levels of IL-12p70 (IL-12p70) and pulsed with HER-2 HLA Class I and II peptides has been tested in two separate clinical trials of patients with ductal carcinoma *in situ* (DCIS) [87,88]. In 13 patients with DCIS, the vaccine was injected intranodally to supply both antigenic stimulation and a synchronized preconditioned burst of IL-12p70 directly at the site of T cell activation. Vaccination resulted in a phenotypic inversion of HER-2-specific tetramer-binding $CD8^+$ T cells from $CD28^{low}CTLA4^{high}$ to $CD28^{high}CTLA4^{low}$, and high rates of vaccine-induced IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells. In addition, seven of 11 evaluable patients showed markedly decreased HER-2 expression in the surgical tumor specimens with measurable decreases in residual DCIS, consistent with vaccine-induced immunoediting at the tumor site. In the second study, 27 patients with DCIS were immunized with high levels of durable HER- 2-specific $CD4^+$ and $CD8^+$ T-cell immunity [88].

Clinical trials targeting carbohydrate antigens & MUC-1

Breast cancer vaccines that target carbohydrate antigens and MUC-1 have been most extensively tested clinically. MUC-1 is a complex tumor antigen comprised of a protein that is aberrantly glycosylated by transformed cells from secretory tissues, including the breast epithelium. Therefore, MUC-1-related epitopes include both peptide antigens unmasked by altered glycosylation patterns and aberrant glycosylation products themselves. Vaccines composed of MUC-1 epitopes conjugated to keyhole limpet hemocyanin (KLH) can result in antigen-specific humoral immune response but fail to elicit MUC-1-specific T cells [89–94]. Giving a low dose of cyclophosphamide intravenously 3 days prior to vaccination results in higher antibody titers and longer median survival than vaccination alone in early trials [95]. A multicenter Phase III clinical trial of 1028 women with metastatic breast cancer randomized women to receive cyclophosphamide plus a MUC-1 carbohydrate epitope conjugated to KLH (experimental arm) or cyclophosphamide plus KLH alone (control arm); no overall survival benefit emerged [96]. Concomitant hormone therapy was given to 34% of trial participants, and a trend toward improved time to progression and overall survival was observed in these patients. This hypothesis-generating, *post hoc* analysis suggests that combined therapy with a MUC-1-based vaccine and antiestrogen therapy could be beneficial.

Clinical trials evaluating targeted immunomodulators in breast cancer

A new era of targeted immunotherapy has arrived with the successful clinical development of two monoclonal antibodies that modulate immune checkpoint pathways [97,98]. The first monoclonal antibody, ipilimumab (Yervoy^R, Bristol-Myers Squibb, NY, USA), blocks the

negative activity of the immune check-point molecule CTLA-4. This drug was approved for use by the US FDA based on an overall survival benefit for both untreated and treatment-refractory metastatic melanoma patients [99,100]. Ipilimumab has a unique toxicity profile, and its use is complicated by clinically significant autoimmune breakthrough events, including autoimmune colitis, hypophysitis, hepatitis, and thyroiditis. Notably, the emergence of autoimmune breakthrough events seems to correlate with clinical response to therapy.

The second monoclonal antibody, BMS-936558 (formerly MDX-1106) blocks the negative activity of the immune check-point molecule PD-1. It was tested first in a Phase I study of 39 patients with advanced metastatic melanoma, colorectal cancer, castrate-resistant prostate cancer, non-small-cell lung cancer or renal cell carcinoma, with a good safety profile and evidence of clinical activity [101]. Further testing has shown a high response rate in patients with different advanced stage cancers, including non-small-cell lung cancer (18%), melanoma (28%) and renal cell cancer (27%) [102]. Many responses were durable, and lasted greater than 1 year. Early analyses suggest that the expression of PD-L1 (B7-H1) by the tumor was predictive for response to anti-PD-1 therapy. This clinical activity is higher than that observed for ipilimumab at the same stage of testing, and BMS-936558 appears to have a much better safety profile than ipilimumab. A Phase I trial testing these BMS-936558 and ipilimumab in combination is in progress. Further validating the PD-1 pathway in cancer, a monoclonal antibody, BMS-936559, specific for PD-L1, has recently been reported to have clinical activity, with evidence of an acceptable safety profile and durable clinical responses in patients with advanced non-small-cell lung cancer, renal carcinoma, melanoma and ovarian cancer [103]. Other immune checkpoint modulators in active clinical testing include additional antibodies specific for PD-1 (MPDL3280A, MK-3475, CT-011 and AMP-224), and BMS-663513, a monoclonal antibody specific for CD137 (4-1BB) in Phase II testing in melanoma. Finally, an agonist anti-OX-40 antibody is under evaluation in prostate cancer [104].

Reports evaluating the activity of immune checkpoint modulators in breast cancer are beginning to emerge (Table 4). The first clinical trial tested tremelimumab, a fully human monoclonal antibody specific for CTLA-4, in combination with exemestane in patients with metastatic breast cancer [105]. Most treatment-related adverse events were mild to moderate diarrhea and pruritus (about 44%), and constipation and fatigue (about 23%). The best overall response was stable disease for at least 12 weeks in 42% of patients (11/26). Most patients developed increased peripheral $CD4^+$ and $CD8^+$ T cells expressing ICOS, and a marked increase in the ratio of $ICOS^+$ T cells to $FoxP3^+$ Tregs. The second study tested a recombinant, soluble LAG-3Ig fusion protein, IMP321, in 30 patients with metastatic breast cancer [106]. This agent binds with high avidity to MHC Class II, resulting in activation of antigen-presenting cells (APCs), which then prime antigen-experienced memory $CD8^+$ T cells. IMP321 was given subcutaneously every 2 weeks the day after days 1 and 15 of standard weekly paclitaxel chemotherapy (80 mg/m^2) for 6 cycles. There was a sustained increase in the number and activation of APCs, and an increased percentage of NK cell and durable cytotoxic effector-memory $CD8^+$ T cells. No clinically significant local or systemic IMP321-related adverse events were noted. Progression-free survival was 90% at 6 months, arguing for further Phase II and III testing of this agent.

Currently, there are at least three active clinical trials evaluating immune checkpoint modulation in breast cancer patients. The first is a clinical trial of preoperative cryoablation or ipilimumab alone, or given together in patients with early stage, resectable breast cancer (NCT01502592). The other two are clinical trials of BMS 936558 (formerly MDX-1105) or MPDL3280A, both monoclonal antibodies specific for PD-L1 in patients with multiple advanced cancers, including breast cancer (NCT00729664 and NCT01375842).

Expert commentary

What have we learned?

These early and late stage clinical trials teach us several lessons about how to best move toward a successful strategy for breast cancer immunotherapy [107]. First, tumor vaccines alone are safe, with side effects generally limited to local injection site reactions and systemic flu-like symptoms. Even early stage clinical trial designs should therefore focus more on clinical response and clinically relevant immunologic correlates. Second, many vaccines induce measurable tumor-specific antibody and/or anti-tumor $CD8^+$ T-cell responses, but these responses typically have little to no impact on tumor growth. There are at least two reasons for this. First, engaging only one component of the immune system ($CD8^+$ T cells or antibody-specific responses for example) is almost certainly inadequate for an effective therapeutic response. Vaccination strategies should therefore recruit some combination of multiple immune effector mechanisms, including $CD4^+$ and $CD8^+$ T cells, antibody-secreting B cells, and innate immune effectors. Second, immune suppressive mechanisms within the tumor microenvironment inhibit the activity of vaccine-induced immune effectors. This argues for immunotherapies that combine vaccines with immunologically active standard cancer therapies, or with targeted immunomodulators and/or immune check-point inhibitors. It is fairly clear that breast cancer vaccines as a single therapeutic agent are unlikely to be clinically effective, particularly in advanced disease.

In particular, metastatic breast cancer presents several daunting challenges to the efficacy of immune-based therapy. First, immune tolerance becomes progressively more entrenched with disease progression. Second, the magnitude of the vaccine-induced immune response is highly likely to be outmatched by the sheer number of disseminated breast tumor cells. Third, patients with Stage 4 disease require treatment, and standard breast cancer drugs can either promote or antagonize the anti-tumor immune response [108]. Thus, the interactions of these drugs with the immune system require a scientifically rational approach to combining standard breast cancer treatment and vaccine therapy. Certain chemotherapy agents can abrogate the influence of intratumoral Tregs and MDSC [109]. Targeted immunomodulators specific for immunosuppressive cytokines and immune checkpoints are likely to be very effective, with activity alone and particularly in combination with vaccination.

Given these barriers, many immunotherapy strategies are likely to be most effective in the setting of minimal residual disease. Thus, clinical trials should be geared toward patients with the lowest burdens of disseminated disease, including metastatic breast cancer patients with minimal to no evidence of disease, and early stage breast cancer patients at high risk for recurrence after standard adjuvant breast cancer therapy. For those patients with larger burdens of disease, breast cancer vaccines should be combined with established drugs in patients with measurable metastatic disease, with the goals of reducing the tumor load to equalize magnitude of the disease and the immune response, and of alleviating the effects of immune tolerance and suppression. Incorporating drugs that alter the immunologic milieu (cyclophosphamide and paclitaxel) or that target breast cancer biology (endocrine therapy and trastuzumab) is required to enhance vaccine activity in advanced disease. Overall, altering both the context in which immunotherapy for breast cancer is undertaken (the patient population) and the context in which immune effectors exert their power (the tumor microenvironment) [110] will maximize its likelihood of success. Clinical trials that rationally sequence multiple immunomodulatory drugs with breast cancer vaccines require integrating a thorough understanding of immune tolerance and the immunobiology of breast cancer patients, and knowledge of the pharmacodynamic interactions between the immune response and the distinct drugs that comprise the immunotherapeutic intervention.

The first steps toward the future

The first clinical trials strategically integrating breast cancer vaccines with chemotherapy, trastuzumab, and/or the small molecule tyrosine kinase inhibitor lapatinib have already been reported (Table 4). The Tumor Vaccine Group at the University of Washington tested their HER-2-specific T helper peptide-based vaccine given in the setting of standard trastuzumab therapy in 22 patients with metastatic HER-2-positive breast cancer [111]. Concurrent therapy was well tolerated, with 15% of patients displaying an asymptomatic decline in left ventricular ejection fraction below the normal range during combination therapy. HER-2-specific immunity was significantly boosted and maintained with vaccination, with epitope spreading within the HER-2 protein and to other tumor antigens noted. The magnitude of survival was inversely correlated with serum TGF-β levels, and at a median follow up of 3 years from the first vaccination, median overall survival had not yet been reached.

The group at Duke tested a HER-2 protein vaccine (dHER-2, a recombinant protein that includes the extracellular domain and a portion of the intracellular domain of HER-2) and the adjuvant AS15 given concurrently with daily lapatinib (1250 mg/day) in 12 women with metastatic, trastuzumab-refractory HER-2-overexpressing breast cancer [112]. There was no evidence of cardiotoxicity and HER-2-specific antibody and T cells were induced in 100% and 8% of patients, respectively.

Our group has completed two clinical trials of combinatorial immunotherapy with an allogeneic, cell-based breast tumor vaccine that secretes GM-CSF. The first clinical trial tested the vaccine given alone or in sequence with a range of low doses of cyclophosphamide and doxorubicin in 28 patients with metastatic breast cancer (Table 3) [113]. This intervention is safe, and associated with the induction of $CD4^+$ T cell-dependent HER-2-specific immunity as measured by DTH and antibody levels. Optimal chemotherapy doses (CY: 200 mg/m^2 and DOX: 35 mg/m^2) significantly augmented the low levels of $CD4^+$ T-cell-dependent immunity (antibody levels) induced by vaccine alone. Cyclophosphamide doses over 200 mg/m^2 inhibited both DTH and HER-2-specific antibody responses, illustrating the importance of defining cancer vaccine-drug interactions in patients. Importantly, this study defined a cyclophosphamide dose of 200 mg/m^2 as most optimal for enhancing vaccine-induced immunity compared to doses of 0, 250 or 350 mg/m^2, which were ineffective. Historically, cyclophosphamide doses of 300 mg/m^2 have been used for enhancing immunotherapies in Phase III trials, and this study provides a possible explanation for the lack of vaccine efficacy observed. The second clinical trial tested the vaccine in women with measurable or evaluable HER-2-overexpressing metastatic breast cancer in a single arm, open label study of cyclophosphamide (300 mg/m^2) and standard weekly trastuzumab [114]. 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. Early exploratory analyses revealed a favorable trend in overall survival [Emens LA, Unpublished Data].

Five-year view

In the next 5 years, large multicenter clinical trials will lay the path toward approval of the first therapeutic breast cancer vaccine. In addition, the first steps toward testing a preventive vaccine for breast cancer patients at high risk of developing the disease will be undertaken. The first clinical trials of targeted immune check-point modulation with ipilimumab and antibodies that target the PD-1 pathway specifically in breast cancer patients are already underway, and data from these will be reported. Neoadjuvant clinical trials evaluating the activity of vaccines and immune checkpoint modulators alone or in combination will yield important insights about how to tip the immunobiology of the breast tumor

microenvironment toward tumor rejection. It is likely that the influence of intrinsic breast cancer subtype biology on the efficacy of these agents will begin to emerge, laying the groundwork for personalized breast cancer immunotherapy. In addition, the first clinical trials integrating breast tumor vaccines with targeted immune checkpoint modulators will be conducted, opening the door for integrative breast cancer immunotherapy even for patients with larger disease burdens. Finally, new challenges will undoubtedly be revealed, as novel immune-related toxicities and new mechanisms of immunologic resistance to checkpoint modulation begin to emerge [115].

Information resource

Information on active breast cancer vaccine and immune checkpoint inhibitor clinical trials can be found at www.clinicaltrials.gov

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• of interest

•• of considerable interest

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Key issues

- Breast cancer is immunogenic.
- Endogenous immune responses in breast cancer provide clinically relevant prognostic and predictive information.
- The breast tumor microenvironment is a complex ecosystem where the balance of immunity can be tipped in favor of tumor progression or rejection.
- The efficacy of some standard breast cancer drugs is due, in part, to activation of the immune response.
- Breast cancer vaccines alone have been associated with evidence of vaccine-induced immunity, but little evidence of clinically meaningful activity to date.
- The activity of breast cancer vaccines can be enhanced by strategically combining them with standard cancer drugs that have immune modulating activity.
- Mononclonal antibodies that manipulate immune checkpoint pathways have great clinical promise.
- Integrative immunotherapy that combines standard breast cancer therapies with breast cancer vaccines and/or immune checkpoint modulation has great potential to improve clinical outcomes.
- Clinical trials evaluating immunotherapy in the neoadjuvant setting will yield new insights into the immunobiology of the breast tumor microenvironment, and suggest new immunotherapeutic targets.
- Capitalizing on insights gained from studying immunotherapy in established breast cancer will open the door for preventive breast cancer vaccines in high-risk patients.

Table 1

Diverse positive and negative molecular interactions promote or antagonize T-cell activity.

Co-receptor molecule	T-cell receptor	Signal	Drug	Company
B7 FAMILY				
CD80/CD86	CD28	+	?	?
	CTLA-4	−	Ipilimumab	FDA-approved for melanoma; Bristol-Myers Squibb
			Tremelimumab	Prior Phase III testing melanoma; Medimmune
PDL1/PDL2	?	+		
	PD-1	−	BMS936558	Phase I/11 trials; Bristol-Myers Squibb
			MK3475	Phase I trials; Merck
			CT-011	Phase II trials; Curetech
			AMP-224	Phase I trials; Amplimmune
			MPDL3280A	Phase I trials; Genentech
B7P1	ICOS	+	?	?
B7H3	?	−	MGA271	Phase I trials; Macrogenics
B7H4	?	−	?	?
HVEM	BTLA	−	?	?
Antigen-specific peptide: MHC complex				
MHC:Ag	TCR:KIR	−	?	?
MHC:Ag	TCR:LAG3	−	IMP321	Phase II trials; Immutep
TNF receptor family				
CD137L	CD137	+	BMS663513	Phase I/II trials; Bristol-Myers Squibb
OX40L	OX40	+	Agonist anti-OX40	Phase I trials; Portland Providence Medical Center
CD70	CD27	+	?	?
CD40	CD40L	+	CP-870893	Phase I trials
GAL9	TIM3	−	?	?

?: Not known; ICOS: Inducible costimulator.

Table 2

Breast cancer vaccine platforms.

Platform	Requirement for HLA match	Relative immunogenicity	Toxicity
Peptide + adjuvant	Yes	Low	Low
Protein + adjuvant	No	Medium	Low
Carbohydrate + adjuvant	No	Low	Low
Plasmid DNA	No	Low	Low
Recombinant virus	No	High	Medium
Recombinant bacteria	No	High	Medium
Dendritic cells	Yes	High	Low
Tumor cells	No	Medium	Low
Heat shock protein	No	High	Low

Table 3

Summary of Phase I, II and III breast cancer vaccine trials.

Vaccine	Patient population	n	Antigen-specific immune response	Clinical benefit	Ref.
HER-2 peptide, E75+GM-CSF	Stage IV breast and ovarian cancer		New E75-specific $CD8^+$ T cells and DTH	NR	[66]
HER-2 peptide, E75+GM-CSF	Early stage LN^{pos} and LN^{neg} breast cancer patients	186	E75-specific $CD8^+$ T cells; decreased serum TGF-β and peripheral Tregs; epitope spreading; Increased memory $CD4^+$ And $CD8^+$ T cells; equal bioactivity in HLA-A2 and HLA-A3 patients; greater potency for $HER\text{-}2^{neg}$ breast cancer	Recurrence rate in vaccinated vs control patients, 14.2 vs 5.6%	[67–76]
HER-2 peptide, GP2+GM-CSF	Early stage LN^{neg} breast cancer patients	18	New GP2- and E75-specific $CD8^+$ T cells	NR	[77]
HER-2 peptide, E75: Ii-Key (AE75)+GM-CSF	Early stage LN^{neg} breast cancer patients	15	New AE75-specific $CD8^+$ T cells	NR	[78,79]
HER-2 peptide+, GM-CSF	Stage III/IV $HER\text{-}2^{pos}$ lung, breast, ovarian	64	New HER-2-specific IgG, T cell and DTH	NR	[80–85]
HER-2 ICD, protein+GM-CSF	Stage II/III/IV, $HER\text{-}2^{pos}$ breast and ovarian	29	New HER-2-specific IgG, T cells	NR	[86]
HER-2 DC	DCIS	13, 27	New HER-2-specific $CD4^+$ and $CD8^+$ T cells; inversion of $CD8^+$ T cells from $CD28^{low}CTLA4^{high}$ to $CD28^{high}CTLA4^{low}$	Decreased HER-2 expression and less residual DCIS	[87,88]
HER-2 DC (Lapuleucel-T)	Stage IV $HER\text{-}2^{pos}$ breast cancer	18	New HER-2-specific T-cell proliferation	1 partial response, 3 stable disease >12 months	[117]
HER-2 DC	Stage II/III/IV NED breast cancer	7	New HER-2-specific T-cell responses	100% DFS at >4 years follow-up	[118]
Influenza virosomes+HER-2 peptides	Stage IV $HER\text{-}2^{pos}$ breast cancer	10	New HER-2 peptide and protein-specific antibody in 80%; increased T-cell activity; decreased peripheral Tregs	NR	[119]
NeuGcGM3- VSSP+Montanide™ (SEPPIC, Paris, France)	Stage III/IV breast cancer	21	New vaccine-specific IgA, IgG, IgM	10% stable disease (for 18 and 40 months)	[120]
NeuGcGM3-VSSP+Montanide vs best supportive care	Stage IV breast cancer	79	New vaccine-specific IgG and IgM	Trend toward survival improvement	[121]
GLOBO-H-KLH+QS-21	Stage IV breast cancer	27	New IgM, CDC, ADCC; no IgG	56% 2-year DFS	[89–94]
Phase III THERATOPE: CY+KLH vs CY +STn-KLH	Stage IV breast cancer	1028	New vaccine-specific IgG	No difference	[96]
CEA-MUC-1-TRICOM poxvirus	Stage IV breast and ovarian cancer	26	Inconsistent	Possible clinical benefit in patients with minimal disease	[122]
hTERT peptide+montanide+GM-CSF	Stage IV breast cancer, $HLA\text{-}A2^+$	19	New TILs post-vaccine; Functional hTERT-specific peripheral $CD8^+$ T cells	Improved survival associated with hTERT immunity	[123]

Vaccine	Patient population	n	Antigen-specific immune response	Clinical benefit	Ref.
p53-loaded DCs	Stage IV breast cancer, HLA-A2$^+$ progressive	6	New p53-specific T-cell responses	1/3 of patients with stable disease	[123]
	Stage IV breast cancer, HLA-A2$^+$	26	New p53-specific T-cell responses in 38% of vaccinated patients, serum YKL-40 and IL-6 associated with response	42% with stable disease	[124]
Allogeneic, GM-CSF-secreting breast tumor cells	Stage IV breast cancer	28	Increased HER-2 specific DTH and antibody responses; best chemotherapy doses CY 200 mg/m^2+DOX 35 mg/m^2	NR; factorial response surface design of various chemotherapy doses	[113]
CD80:MDA-MB231	Stage IV breast cancer	30	Inconsistent	No benefit	[125,126]
DC-autologous tumor fusion	Stage IV breast and renal cancer	23	Increased CD4$^+$ and CD8$^+$ T cells	43% of patients with stable disease or partial response	[127]
Survivin 2 peptide ± IFA	Advanced/recurrent breast cancer	14	Increased frequency of survivin-specific T cells in 50% of patients; increased function of survivin-specific T cells in 7% of patients	14% with stable disease	[128]

ADCC: Antibody-dependent cellular cytotoxicity; CDC: Complement-dependent cytotoxicity; CEA: Carcinoembryonic antigen; CY: Cyclophosphamide; DC: Dendritic cell; DCIS: Ductal carcinoma *in situ*; DFS: Disease-free survival; DOX: Doxorubicin; DTH: Delayed-type hypersensitivity; GM-CSF: Granulocyte–macrophage colony-stimulating factor; hTERT: Telomerase; ICD: Intracellular domain; IFA: Incomplete Freund's adjuvant; KLH: Keyhole limpet hemocyanin; LN: lymph node; MUC1: Mucin 1; NED: No evidence of disease; NR: Not reported.

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Table 4

Summary of breast cancer immunotherapy combined with standard breast cancer drugs.

0Patient population	n	Immunotherapy	Breast cancer therapy	Immunologic outcome	Ref.
Stage IV ER^+ breast cancer	26	Tremelimumab	Exemestane	Increased peripheral $ICOS^+$, $CD4^+$ and $CD8^+$ T cells; increased ratio of $ICOS^+$ T cells/$FoxP3^+$Tregs	[105]
Stage IV breast cancer	30	IMP321 (LAG-3Ig)	Paclitaxel (80 mg/m^2)	Increased numbers of activated APC; increased % of NK and long-lived cytotoxic effector-memory $CD8^+$ T cells	[106]
Stage IV HER-2^+ breast cancer	22	HER-2 helper peptides	Trastuzumab	Enhanced HER-2-specific T-cell immunity; epitope spreading; decreased serum TGF-β	[111]
Stage IV HER-2+ breast cancer	12	HER-2 protein (dHER-2)	Lapatinib	HER-2-specific antibody and T-cell responses in 100 and 8% of patients respectively	[112]
Stage IV HER-2^+ breast cancer	20	GM-CSF-secreting, HER-2^+ breast tumor cells plus CY 300/mg^2	Trastuzumab	Enhanced HER-2-specific DTH in about 1/3 of patients	[114]
Stage IV HER-2^+ breast cancer	8	Plasmid HER-2 DNA+GM-CSF+ IL2	Trastuzumab	Delayed induction of HER-2-specific antibody and T-cell responses	[129]

APC: Antigen-presenting cell; CY: Cyclophosphamide; DTH: Delayed-type hypersensitivity; ER: Estrogen receptor; GM-CSF: Granulocyte–macrophage colony-stimulating factor; ICOS: Inducible costimulator; NK: Natural killer.