

REVIEW

Harnessing the immune system in the battle against breast cancer

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Abstract

Breast cancer is the most prevalent malignancy in women and the second most common cause of cancer-related death worldwide. Despite major innovations in early detection and advanced therapeutics, up to 30% of women with node-negative breast cancer and 70% of women with node-positive breast cancer will develop recurrence. The recognition that breast tumors are infiltrated by a complex array of immune cells that influence their development, progression, and metastasis, as well as their responsiveness to systemic therapies has sparked major interest in the development of immunotherapies. In fact, not only the native host immune system can be altered to promote potent antitumor response, but also its components

can be manipulated to generate effective therapeutic strategies. We present here a review of the major approaches to immunotherapy in breast cancers, both successes and failures, as well as new therapies on the horizon.

Keywords: adoptive transfer, breast cancer, cancer vaccine, cytokines, immunomodulation, immunotherapy, monoclonal antibody, oncolytic virotherapy.

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Introduction

Over the past half century, advancements in our understanding of breast cancer biology have transformed the current landscape of disease management, leading to improvements in early detection strategies, development of breast-conserving surgery techniques, utilization of cytotoxic chemotherapy regimens for the treatment of both local and metastatic disease, engineering of targeted therapies against the hormone pathway and human epidermal growth factor receptor 2 (HER2/neu), and employment of hormonally directed therapies as a preventive measure [1]. This evolution in breast cancer management has led to a one-third reduction in mortality since the year 1990 [2,3], yet breast cancer remains the most prevalent malignancy in women and second most common cause of cancer-related death worldwide [4,5]. Disease-related mortality stems primarily from primary (*de novo*) or secondary resistance to available systemic therapies. A number of novel approaches are being pursued to prevent or circumvent mechanisms of treatment resistance and hopefully improve long-term survival. The recognition of the role of the immune microenvironment in tumor biology has opened the door to an exciting era in oncology

in which immune components are manipulated to harness the inherent ability of the host's immune system to combat malignancy. In this review, immunotherapy approaches for the treatment of breast cancers will be explored with a focus on both successes and failures as well as new therapies on the horizon.

Immune microenvironment in breast cancer

Breast tumors are complex systems comprising two primary components: the cancer cells typically derived from malignant transformation of mammary ductal or lobular cells, and the surrounding stromal compartment composed of a variety of normal host cells (e.g., fibroblasts, immune cells, and cells of the vasculature) and extracellular matrix molecules that are conscripted to provide a biochemical and structural milieu supportive of tumor development, progression, and metastasis [6–10]. One major class of stromal host cells, the immune infiltrate, has garnered considerable attention for its exploitability in the treatment of many malignancies, including breast cancer [11].

Cancer-related inflammation has long been recognized by early pathologists, and in 1863 was even postulated to play a causative role in oncogenesis [12]. Although malignancy-associated inflammation is no longer considered a driver of tumorigenesis, chronic inflammation increases cancer risk and plays a major role in disease pathophysiology [11,13–15]. In breast malignancies, this immune infiltrate has been well studied. Immunohistochemical analyses in the early-to-mid-twentieth century showed a correlation between high levels of mononuclear immune cell infiltration and better prognosis, particularly in the medullary carcinoma histopathologic subtype of breast cancer [16,17]. Immunophenotyping studies in the 1980s further characterized breast tumor immune infiltrates, identifying cells derived from both myeloid (innate) and lymphoid (adaptive) lineages, including T lymphocytes (primarily CD8⁺ and CD4⁺ cells), macrophages, natural killer (NK) cells, and B lymphocytes [18,19].

Tumor-infiltrating lymphocytes (TILs) are one of the most prominent groups of immune cells in breast cancers [20] and consist of predominantly CD8⁺ cytotoxic T lymphocytes, CD4⁺ T helper lymphocytes, NK cells, and FOXP3⁺ T regulatory cells (Tregs) [21–23]. High levels of CD8⁺ T-cell infiltration into breast tumors have been correlated with improved outcomes [24–27], due in part to cancer cell-directed cytotoxicity. Similarly, high levels of NK-cell infiltration are associated with improved prognosis in breast cancer [28] by mediating CD8⁺ cytotoxic T-lymphocyte activity through the production of proinflammatory cytokines such as interferon (IFN)- γ [29–31].

In contrast, high levels of Tregs, which play pivotal roles in immune tolerance by suppressing T-cell activation in the normal physiologic state and killing or inactivating effector T cells in highly inflammatory states [32,33], have been shown to promote tumor progression and are associated with poor prognosis [34,35]. Furthermore, depletion of Tregs in preclinical models by blocking the interleukin-2 pathway has been shown to inhibit mammary tumor growth, likely by reprogramming the microenvironment from an immunosuppressive one to one that is amenable to other immunotherapies (e.g., checkpoint inhibitors) [36,37]. This is supported by patient data showing that reduction in FOXP3⁺ regulatory T cells following neoadjuvant chemotherapy is associated with pathologic complete responses [24].

The role of CD4⁺ T helper lymphocytes in breast cancer is less clear, as they have been noted to have both tumor-promoting and tumor-inhibiting properties, depending on their cytokine expression profile and microenvironmental context. Proinflammatory (Th1) CD4⁺ T lymphocytes, which typically express IFN- γ , have been shown to promote cytotoxic T-lymphocyte activity [38]. In contrast, anti-inflammatory (Th2) CD4⁺ T lymphocytes, which classically express interleukin-4 (IL-4), have been shown to promote metastasis in preclinical models [39,40]. Similarly, the Th17 subset of CD4⁺ helper T cells commonly infiltrates ER⁻ and triple-negative malignancies and is associated with poor prognosis [41] owing to its

promoting effects on cancer cell proliferation, metastasis, and chemoresistance [42,43].

Myeloid lineage cells may comprise up to 80% of the immune cell infiltrate in breast tumors [44], and consist primarily of macrophages, as well as smaller numbers of neutrophils, immature granulocytes, and myeloid-derived suppressor cells. In a classification scheme akin to that of T helper lymphocyte polarization, these macrophages and neutrophils are similarly classified as type 1 or 2 based on analogous cytokine profiles [21]. Breast tumor-associated macrophages (TAMs) are typically M2-polarized, expressing and responding to cytokines such as IL-4, and have been shown to promote angiogenesis [45], cancer cell intravasation into blood vessels [46], diminish responses to cytotoxic chemotherapies [39,47–49], and facilitate metastasis. High levels of TAM infiltration are associated with poorer outcomes [50,51]. In addition to TAMs, other myeloid lineage immune cells have also been identified in the breast tumor immune infiltrate. Mast cells promote angiogenesis in breast tumors and metastatic lesions [52], but their relationship with prognosis varies based on breast cancer subtype and tumor stage [53]. Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells that promote an immunosuppressive tumor microenvironment through multiple mechanisms, including expression of Th2-type cytokines, production of reactive oxygen species, and the metabolism of arginine [54].

The role of the B-lymphocyte infiltrate in breast malignancies is less understood. Studies in medullary carcinoma of the breast, in which high levels of B cell infiltration into tumors are associated with better outcomes, suggest that B lymphocytes undergo clonal proliferation and affinity maturation within primary tumors against both host- and tumor-directed antigens, which ultimately lead to activation of apoptosis [55,56]. These data are further supported by studies in intraductal carcinomas, in which similar results were observed [57,58].

Immunotherapy in breast cancer

The recognition of an inflammatory infiltrate in solid tumors and our growing understanding of the varied functions of tumor inflammation have raised the question of whether the immune system can be effectively exploited to promote tumor destruction. This led to the birth of the field of cancer immunotherapy, which is broadly defined as any therapeutic approach that utilizes a specific component of the immune system or enhances or augments the intrinsic host immune response to treat malignancy [59]. Two fundamental principles have been recognized as guiding the development of cancer immunotherapy. They are as follows: (1) both the innate and adaptive immune systems play an important role in cancer cell immunosurveillance and destruction by utilizing the same mechanisms in play during infection with foreign pathogens and (2) oncogenesis and progression

occurs through the selection and outgrowth of cancer cells with low immunogenicity and the generation of an immunosuppressive microenvironment [60]. Therapeutic strategies have therefore focused on activating both innate and antibody-mediated immune responses to induce cancer cell death. These technologies include cytokines and growth factors, immunomodulatory agents, alteration of the tumor microenvironment, passive immunization with monoclonal antibodies, bispecific and multispecific antibodies, checkpoint inhibitors, vaccines, oncolytic viruses, and adoptive T-cell transfer [61,62] (Table 1).

Cytokines and growth factors

Cytokines and growth factors are secreted or membrane-bound proteins produced by both innate and adaptive immune cells in response to a stimulus (e.g., a pathogen or cancer cell). They exert pleiotropic effects on components of the immune system by binding to specific cytokine receptors on many different effector cells, initiating signaling pathways to modulate cell trafficking, survival, proliferation, maturation, and function, thereby promoting or inhibiting tumor-directed responses while maintaining immunologic homeostasis and self-tolerance. These molecules can also exert effects on cancer cells, contributing to their proliferation, invasiveness, intravasation, metastasis, and chemoresistance [63–66]. Activating or inhibiting these signaling pathways has been a major focus in immunotherapy research.

Cytokine therapy is a therapeutic strategy that was first recognized in the late 1800s when inoculation of highly virulent streptococcal cultures was shown to induce remission in patients with inoperable, metastatic sarcoma [67]. Later successes using systemic IL-2 for the treatment of metastatic renal cell carcinoma and metastatic melanoma [68,69] paved the application of cytokine therapy to other malignancies. However, in breast cancer, systemic cytokine treatment has been less successful for the treatment of breast cancer. IFN α was the first cytokine noted to have a potentially beneficial effect in the treatment of breast cancer. In 1980, Gutterman et al. administered partially purified IFN α derived from human buffy coat preparations to 17 patients with recurrent, metastatic breast cancer and noted 7 patients had tumor regression with 6 patients achieving partial remission as defined by >50% objective decrease in tumor size [70]. A subsequent Phase II study in patients with recurrent metastatic breast cancer who had not received cytotoxic salvage chemotherapy was conducted to determine the efficacy of similarly derived, partially purified IFN α preparations as monotherapy, and it was confirmed that systemic cytokine administration was indeed capable of inducing a partial objective response in 5 of 23 patients with breast cancer and a measurable response in 6 of 23 patients [71]. However, subsequent Phase II trials utilizing purified, recombinant IFN α did not yield significant tumor responses in the treatment of metastatic breast cancers [72,73]. Studies with systemic

administration of other recombinant interferons were similarly unsuccessful in breast cancer [74–76], likely owing to the lack of other cytokines and chemokines present in the original preparations. The addition of IL-2 to IFN therapy has also been ineffective [77].

Limiting factors in the successful application of cytokines include tachyphylaxis with subsequent administrations, ineffective stimulation of T-cell-mediated tumor-directed responses, and significant dose-limiting side effects with systemic therapy, including overwhelming fatigue and severe cytokine release syndromes. Strategies for improving immune activation and decreasing the systemic effects of cytokine therapy are underway in preclinical models and early-phase clinical trials. These approaches include intra-tumoral injection of cytokines [78], combination of cytokine therapy with systemic therapy [79,80], gene therapy with adenovirus vectors and oncolytic viruses expressing cytokines and chemokines under the direction of tissue-specific promoters [81,82], tumor-targeted super-antigen therapy utilizing components of bacterial toxins [83], and cytokine-antibody fusion molecules (reviewed [84]).

Systemic administration of growth factors has similarly found limited use for inducing remission of breast cancer. However, in the management of chemotherapy-induced toxicities, growth factors, particularly granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are routinely used for the prevention of neutropenia [85,86]. Another growing niche for growth factors in breast cancer therapy is as adjuvants to other immunotherapies, such as cancer-directed vaccines.

Disruption of both cytokine and growth factor signaling pathways has also been a major area of immunotherapy research. Neutralizing antibodies against cytokines and certain chemokines have been shown to be effective in preclinical models and are currently in early-phase clinical trials with results forthcoming [87].

Immunomodulatory agents

Immunomodulatory agents are chemicals capable of altering immune responses, angiogenesis, and cancer cell proliferation. The earliest immunomodulatory agents focused on generating potent innate immune responses to stimulate lasting cytotoxic CD8⁺ T-cell effector functions through the activation of Toll-like receptors (TLRs). TLR agonists are nonspecific molecules capable of binding these pattern-recognition receptors, thereby activating transcriptional pathways regulated by nuclear factor (NF)- κ B and activating complement pathways, opsonization, and phagocytosis, inducing apoptosis and stimulating cytotoxic T-cell responses [88].

Preclinical animal studies in the 1960s and 1970s showed that administration of polyadenylic-polyuridylic acid (poly A:U), a double-stranded RNA polynucleotide that stimulates TLR3, after mastectomy in spontaneous tumor and orthotopic

Table 1. Strategies for activation of the immune system against breast cancer.

Class	Mechanism	Examples
Cytokines	Bind to cytokine receptors to initiate cell signaling pathways and stimulate immune cell trafficking and effector function	Interleukin-2 Interleukin-12
Growth factors	Increase number of circulating granulocytes	G-CSF GM-CSF
Toll-like receptor agonists	Bind TLRs to activate antigen-presenting cells (dendritic cells) to upregulate expression of cytokines and co-stimulatory molecules to attract and stimulate effector immune cells (cytotoxic T lymphocytes)	Polyadenylic-polyuridylic acid (Poly A:U) Polyinosinic-polycytidylic acid (Poly I:C)
Immune checkpoint inhibitors	Antibody to CTLA-4, PD-1, or PD-L1 molecules releases T cells from inhibitory signals, thereby unleashing cytotoxic T-cell activity	Ipilimumab (CTLA-4 antibody) Nivolumab (PD-1 antibody) Pembrolizumab (PD-1 antibody) Atezolizumab (PD-L1 antibody) Avelumab (PD-L1 antibody) Durvalumab (PD-L1 antibody)
Bispecific, multispecific antibodies	Simultaneously interact with a cancer-specific epitope and stimulatory molecule(s) on effector cell(s)	HER2/CD3 bispecific antibodies
Adoptive cell transfer	Infusion of T cells stimulated or engineered to have antitumor effector functions	Chimeric antigen receptor (CAR) T cells expressing HER2/neu
Oncolytic viruses	Viruses with specific tropism for cancer cells that induce cancer cell death and activate tumor-directed immune responses	JX-594 (pexastimogene devacirepvec) vaccinia poxvirus expressing GM-CSF
Vaccines	Active immunization against tumor-specific antigens	Nelipepimut-S vaccine against HER2/neu

Therapeutic strategies to harness the innate and adaptive immune system against breast cancer cells include nonspecific immune system stimulation with cytokines, growth factors, and Toll-like receptor agonists, release of T cells from inhibitory PD-L1 signals, use of antibodies to transmembrane tyrosine kinase receptor HER2 to tag HER2+ breast cancer cells for immune-mediated destruction, stimulation of T cells within the HER2+ breast tumor microenvironment, active vaccination or *in vitro* reprogramming of T cells against HER2/neu, and injection of oncolytic viruses. See text for details.

G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HER2, human epidermal growth factor receptor 2; TLR, Toll-like receptor.

transplant models of breast cancer significantly delayed relapse [89,90]. Furthermore, administration of poly A:U to newborn mice that developed spontaneous mammary tumors resulted in reduced incidence of malignancy [91]. This agent entered clinical trials in France during the 1980s [92–94] as an adjuvant therapy in surgically resectable breast cancer and showed significantly improved overall survival in node-positive patients. Head-to-head studies comparing adjuvant cytotoxic therapy with cyclophosphamide, methotrexate, and fluorouracil (CMF) to adjuvant poly A:U demonstrated a significantly improved disease-free survival and reduced incidence of metastasis in patients with limited disease (i.e., 1–3 positive nodes) with positive TLR3-expression [94]. Similar results were obtained when a combination of adjuvant locoregional radiation and poly A:U therapy was compared to adjuvant chemotherapy with CMF [95,96]. Despite these promising results, adjuvant poly A:U has not been approved for

the treatment of breast cancer, likely owing to its applicability to a very small population of women with limited disease. Another TLR3 agonist, polyinosinic-polycytidylic acid (poly I:C), has been shown to induce tumor necrosis [97], reduce metastases [98], and induce long-lasting CD8+ T-cell responses in combination with CpG oligodeoxynucleotides (a TLR9 agonist) [99] in preclinical models. The use of poly I:C has been limited owing to significant toxic effects, specifically severe cytokine release syndromes.

Cytokine-release syndromes with the systemic administration of TLR agonists have largely limited their use as monotherapy in clinical scenarios. However, research into modifying delivery and structural components to reduce their side-effect profiles and their use as immunotherapeutic adjuvants is ongoing. Several TLR agonists, including monophosphoryl lipid A and OK-432 (TLR4 agonists) [100], poly ICLC (TLR3

agonist that is a compounded mixture of poly I:C and poly-lysine), and CpG oligodeoxynucleotide (TLR9 agonist), are being studied as vaccine enhancers. They are also being studied as adjuncts to systemic or radiation therapy [99,101], as well as local therapeutics, for example, topical imiquimod (TLR7 agonist) as therapy for skin metastases in breast cancer [102].

Other immunomodulatory agents inhibit immunosuppressive molecules. For example, indoleamine 2,3-dioxygenase (IDO) is an enzyme that metabolizes tryptophan to produce metabolites that stimulate the expansion of Treg populations, thereby suppressing cytotoxic T-cell activity [103]. In a transgenic model of breast cancer, indoximod (an inhibitor of IDO) in combination with cytotoxic systemic therapy induced tumor regression [104]. These studies have resulted in the initiation of clinical trials utilizing indoximod with cytotoxic therapy for the treatment of breast cancer [105,106].

Altering the tumor microenvironment

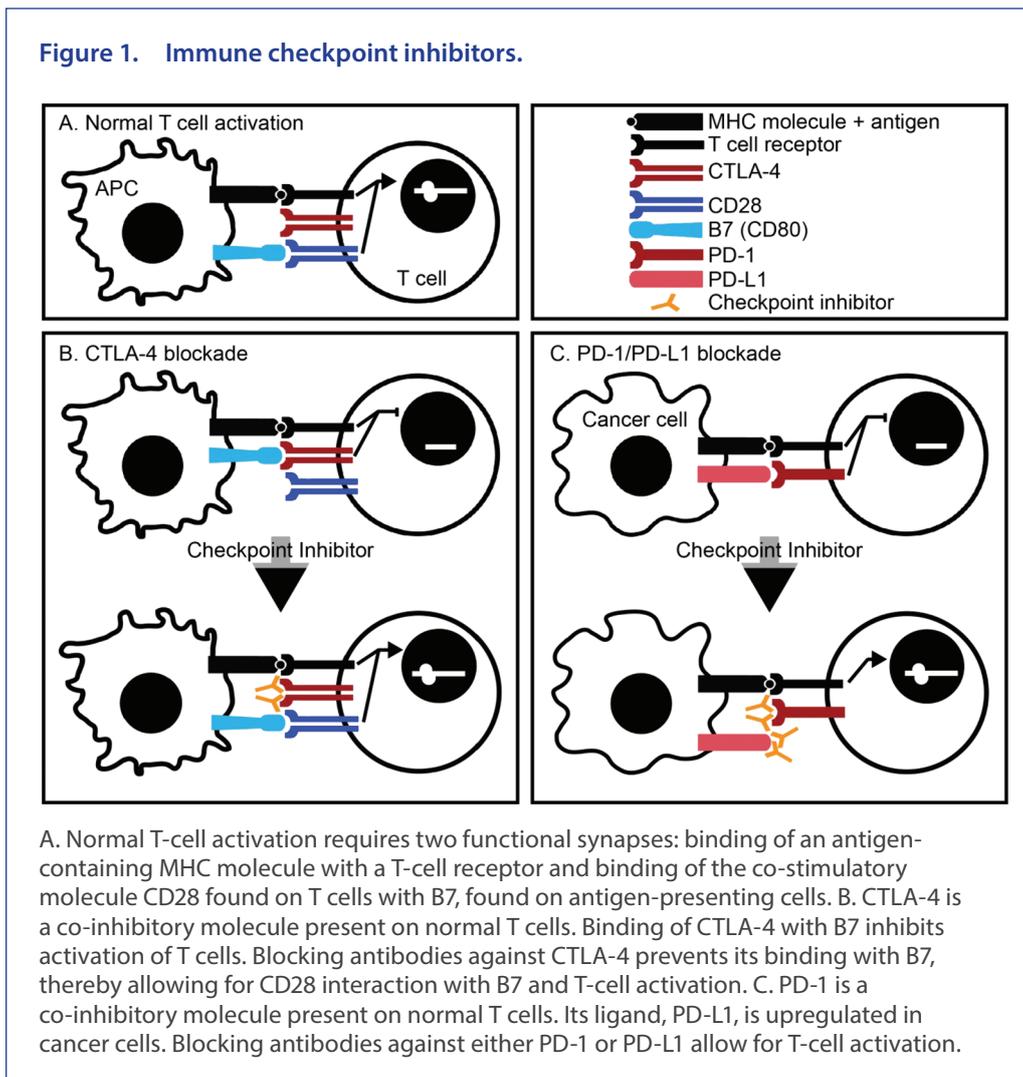
The complexity of the tumor microenvironment and the many roles its constituents play in breast cancer development, progression, metastasis, and response to chemotherapeutic agents has altered the tumor microenvironment a particularly exciting area of research. Efforts to target the immune-stroma include those aimed at preventing recruitment, altering polarization, inhibiting activation, and depleting certain tumor-infiltrating immune cells that may contribute to tumor pathogenesis. TAMs are of particular interest, in part because they can be polarized by Th2 cytokines to adopt an M2 phenotype with tumor-promoting, immunosuppressive functions [107]. Moreover, they comprise a significant proportion of tumor-infiltrating leukocytes in breast cancers, correlating with poor prognosis [108,109]. Colony-stimulating factor-1 (CSF-1) is a regulatory growth factor crucial for macrophage survival, proliferation, and effector functions [110], and neutralizing this molecule or its cognate receptor CSF-1R has been a particularly attractive approach to targeting TAMs. Indeed, blocking antibodies against CSF-1R leads to the depletion of TAMs and FOXP3⁺ Treg populations, an increase in the ratio of CD8⁺:CD4⁺ T cells, and delays in tumor growth in TAM-rich tumor models [111]. Neutralizing antibodies against CSF-1 in combination with paclitaxel effectively reduces metastatic burden in a mouse model of luminal B breast cancer in part by augmenting CD8⁺ T-cell activity [109]. There are currently three monoclonal antibodies targeting CSF-1R in early-phase trials: emactuzumab administered alone or in combination with paclitaxel (NCT01494688) or in combination with the PD-L1 inhibitor atezolizumab (NCT02323191) for unspecified advanced solid tumors, LY3022855 as monotherapy for unspecified treatment-refractory solid tumors (NCT01346358) or specifically for treatment-refractory breast or prostate cancer (NCT02265536), and AMG 820 as monotherapy in unspecified advanced solid malignancies (NCT01444404).

Preventing myeloid cell recruitment is another strategy for interfering with TAM function. Studies in murine models of breast cancer have shown that the interaction between the chemokine CCL2 (C-C motif chemokine ligand 2) and its receptor CCR2 (C-C chemokine receptor 2) promotes recruitment of TAMs into tumors and metastatic sites, promoting tumor angiogenesis, cancer cell extravasation, and metastasis, and inhibiting CCL2/CCR2-mediated recruitment delays relapse, improves survival, and decreases metastatic burden [49,112,113]. Despite promise as a therapeutic strategy in preclinical models, CCL2 antagonism has had limited success. The anti-CCL2 monoclonal antibody carlumab, while generally safe in a Phase I trial for advanced solid malignancies [114], ultimately did not demonstrate *in vivo* ability to block the CCL2/CCR2 pathway or exert antitumor effects in metastatic castrate-resistant prostate cancer, resulting in suspension of further planned studies with carlumab [115]. Similar results were obtained when an antibody against CCR2 was used in patients with advanced malignancy and bony metastases [116].

Immune checkpoint Inhibition

The detection of immune checkpoints that prevent autoimmunity when normal immune responses are triggered, the identification of specific cell types and molecules involved in this process, and the understanding of roles that each of these cells and molecules play in promoting an immunosuppressive tumor microenvironment have led to the development of blocking monoclonal antibodies aimed at improving the host immune response to cancer. By antagonizing these molecules, potent anticancer T-cell responses may be reactivated. This class of therapies, called immune checkpoint inhibitors, has revolutionized the treatment of various malignancies, including malignant melanoma, renal cell carcinoma, non-Hodgkin's lymphoma and smoking-associated non-small-cell lung cancer [117].

Immune checkpoint inhibitors function by interfering with two separate T-cell inhibitory pathways: cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1). Traditionally, activation of T-cell responses requires two functional synapses between antigen-presenting cells (APCs) and T cells: (1) binding of an antigen-containing major histocompatibility complex (MHC) molecule with a T-cell receptor (TCR) and (2) binding of the APC cell surface molecule B7 to the T-cell co-stimulatory molecule CD28. These two interactions result in upregulation of IL-2 expression by the activated T cell, leading to proliferation and stimulation of effector function. CTLA-4 and PD-1 are cell surface molecules expressed by T cells that are structurally homologous to CD28, but which act as co-inhibitory molecules when bound to their respective ligands. CTLA-4 acts a co-inhibitory signal to prevent IL-2 expression by T cells when complexed with B7 on an APC. PD-1, which is expressed on a wide variety of cells that include



leukocytes and parenchymal cells, binds to its ligands PD-L1 and PD-L2 on APCs. Expression of CTLA-4 depends on potency of the immunologic trigger, while expression of PD-1 depends on the duration of the immunologic response and is associated with T-cell exhaustion. Increased expression of CTLA-4 and PD-L1 in tumor tissues allows for the evasion of tumor-directed T-cell responses [118,119] (Figure 1).

There are currently six immune checkpoint inhibitors with FDA-approved indications for clinical use: the CTLA-4 blocking antibody ipilimumab, the PD-1 blocking antibodies nivolumab and pembrolizumab, and the PD-L1 neutralizing antibodies atezolizumab, avelumab, and durvalumab. While there are very rare indications for the use of checkpoint inhibitors in breast cancer treatment, there is evidence to suggest that these therapies may be effective, particularly in breast cancer subtypes with large immune infiltrates, including HER2⁺ and TNBC. These data include increased mutational rates resulting in a larger burden of tumor-associated neoantigens [120], recruitment of tumor-infiltrating lymphocytes [121], and PD-L1 expression [122,123]. Indeed, several early-phase trials are underway with checkpoint inhibitors in

monotherapy (KEYNOTE-012 [124] and KEYNOTE-086 [125] evaluating pembrolizumab for TNBC, KEYNOTE-028 evaluating pembrolizumab for PD-L1⁺ ER⁺ HER2⁻ breast cancer [126], and the JAVELIN trial evaluating avelumab for locally advanced or metastatic breast cancers) or in combination with cytotoxic therapy (nanoparticle albumin-bound paclitaxel with atezolizumab [127–129] and the I-SPY-2 trial evaluating paclitaxel with or without pembrolizumab for 12 weeks followed by doxorubicin and cyclophosphamide for 8–12 weeks [130]). Preliminary results from early-phase trials with pembrolizumab [125] or atezolizumab [131] monotherapy for TNBC indicate that patients who initially respond to therapy have durable treatment responses. It does, however, appear that these responses are dependent on high levels of tumor-infiltrating lymphocytes [125,131], and this response wanes with increasing exposure to systemic cytotoxic chemotherapy [132] (Table 2).

Other immune checkpoints have also been identified, including two inhibitory signals that are co-expressed by activated T cells and upregulated with PD-1 on chronically stimulated tumor-infiltrating lymphocytes – lymphocyte activation gene 3 (LAG3)

Table 2. Clinical trials utilizing immune checkpoint inhibitors for the treatment of breast cancer.

Trial	Inhibitor	Therapeutic strategy	No. pts.	ORR	Median time to response (Range)	Median DoR (months)	Toxicities
KEYNOTE-012 Phase I [124]	Pembrolizumab	Single agent in advanced PD-L1+ TNBC	32	18.5%	17.9 weeks (7.3 to 32.4 weeks)	-	Common: arthralgia, fatigue, myalgia, Nausea 15.6% ≥grade 3 toxicity
KEYNOTE-028 Phase I [126]	Pembrolizumab	Single agent in ER+, HER2-, locally advanced or metastatic disease, ECOG 0-1, and failure or inability to receive standard therapy	25	12% (95% CI, 2.5-31.2)	8 weeks (8.7 to >44 weeks)	-	Common: nausea, fatigue, arthralgia, anorexia, mucositis, pruritus, rash, blurred vision 16% ≥grade 3 toxicity
KEYNOTE-086 Phase II [125]	Pembrolizumab	Single agent in advanced PD-L1+ triple negative breast cancer	170	5% regardless of PD-L1 expression	-	-	12% ≥grade 3 toxicity
KEYNOTE-173 Phase Ib [209]	Pembrolizumab	Neoadjuvant with chemotherapy for locally advanced TNBC (Cohort A: nab-paclitaxel followed by doxorubicin and cyclophosphamide Cohort B: nab-paclitaxel and carboplatin followed by doxorubicin and cyclophosphamide)	Cohort A: 10 Cohort B: 10	Cohort A: 80% prior to surgery Cohort B: 100% prior to surgery	-	-	Myelosuppression: Cohort A: 3/10 Cohort B: 4/10 Cohort A: 80% ≥grade 3 toxicity Cohort B: 100% ≥grade 3 toxicity
I-SPY 2 Phase 2 [130]	Pembrolizumab	Invasive breast cancer with neoadjuvant 12 weeks of paclitaxel with or without pembrolizumab followed by 4 cycles of doxorubicin and cyclophosphamide	69	HR+/HER-: 7/25 (28.0%) compared to 13/88 (14.8%) controls TNBC: 15/21 (71.4%) compared to 16/83 (19.3%) controls	-	-	-
JAVELIN Phase Ib [129]	Avelumab	Metastatic breast cancer refractory to therapy or with progression after standard-of-care therapy	168	3.0% (overall) 5.2% (TNBC)	-	-	13.7% ≥grade 3 toxicity
NCT01375842 Phase Ia [131]	Atezolizumab	TNBC	63	10% (95% CI, 5-17%)	-	-	-
IMpassion130 Phase Ib [127]	Atezolizumab	Combination with nab-paclitaxel in metastatic TNBC treated with ≤3 prior lines of therapy	32	42% (95% CI, 22-63%)	-	21 (3 to 26+)	Common: neutropenia

[133] and T-cell immunoglobulin and mucin domain 3 (TIM-3) [134] – and a co-stimulatory receptor in the TNF family of receptors called OX40 that is capable of promoting CD8⁺ T-cell proliferation and activity, inhibiting generation of Tregs, and expanding memory CD4⁺ T-cell populations [135]. Early-phase clinical trials with these agents are underway and have been reviewed elsewhere [136].

Bispecific and multispecific antibodies

In addition to releasing the brakes on T-cell responses, antibodies can be engineered to directly activate potent tumor-directed responses at the site of the target. One such tool is the bispecific antibody (BsAb), which simultaneously interacts with cancer cell-specific epitopes and a stimulatory molecule on an effector cell population. This molecule creates an immunologic synapse capable of activating a potent innate or adaptive immune response [137]. In breast cancer, several iterations of BsAbs have been generated. HER2 is particularly attractive as the target for the tumor-oriented specificity and is frequently used [138,139]; however, other targets have also been employed, including epithelial cell adhesion molecule (EpCAM) [140,141] and epithelial growth factor receptor (EGFR) [142]. The initial effector cell target was the macrophage CD64 (Fcy receptor I) to activate macrophage-mediated killing. Early-phase clinical trials using these antibodies were largely unsuccessful for several reasons, including the need for systemic cytokine or growth factor administration to boost macrophage responsiveness in some instances and the associated side-effect profile of these enhancing therapies, induction of autoantibodies against the administered BsAbs, and a lack of objective clinical response [143–146].

Although macrophage activation has had a limited effect on tumor-directed immunity in humans, T-cell activation has been a more promising area of research. Newer generations of BsAbs have effector cell specificities targeting CD3 on T cells [147] and elicit potent CD8⁺ cytotoxic T-cell activity both *in vitro* and *in vivo* in preclinical models of breast cancer [139,148–151]. Furthermore, pre-stimulating and arming T cells with BsAbs [152], as well as combining them with checkpoint inhibitors, are promising areas of further research.

Another approach to immune activation has been the use of multispecific antibodies, which are capable of binding to three or more epitopes and have also been utilized to activate tumor-directed immune responses. For example, the trifunctional monoclonal antibody ertumaxomab, which recognizes HER1, CD3, and Fcy receptor types I and III, has been shown to induce lysis of human breast cancer cells *in vitro* [153] and is capable of eliciting a strong immune response in humans as evidenced by early-phase clinical trials [154].

Vaccines and oncolytic viruses

Cancer vaccines are a form of active immunization against tumor-specific antigens designed to stimulate immune

responses directed against cancer cells, rather than prevent disease as in the canonical sense of the term ‘vaccine’. Many different approaches have been utilized to generate cancer vaccines, including whole tumor cells, whole cell lysates, peptides, glycosylated antigens, nucleic acids, and viruses [155,156]. When these vaccines are delivered, they elicit innate immune responses resulting in phagocytosis and processing of antigens by APCs. APCs insert these antigens into major MHC class I molecules to trigger T-cell-mediated cytotoxicity or MHC class II molecules to trigger T helper cell responses and humoral immunity. Adjunct molecules can also be used to induce more potent and durable responses [157,158].

In breast cancer, the most commonly employed epitope is HER2. While many different platforms have been used to generate anti-HER2 vaccines, the most promising is NeuVaxTM, a synthetic peptide analogue of HER2 called nelipepimut-S, which is capable of binding to CD8⁺ cytotoxic T cells and is administered with the immune adjuvant GM-CSF [159]. In Phase I and II trials, nelipepimut-S in combination with GM-CSF following surgical intervention with adequate lymph node dissection and completion of neoadjuvant or adjuvant radiation or chemotherapy improved disease-free survival [160], although results from a Phase III trial were less optimistic, with the study being suspended for futility following an interim safety and futility analysis. Several other trials with nelipepimut-S are in progress, including combination therapy with trastuzumab for ER⁻/PR⁻, lymph node-negative HER2⁺ or lymph node-positive HER2⁺ disease following standard-of-care therapy (NCT01570036), in high-risk HER2⁺ breast cancer populations (NCT02297698), and in ductal carcinoma *in situ* (NCT02636582). Other driver mutation-targeted vaccines currently in early-phase trials include AVX901, a virus-like replicon particle based on an attenuated Venezuelan equine encephalitis virus engineered to express HER2 [161] and INO-1400, a synthetic DNA vaccine targeting the hTERT oncogenic protein with or without a plasmid expressing IL-12 (NCT02960594). Vaccines against carbohydrates, which are expressed in a wide variety of solid tumor types, including breast cancer, are also being studied. One such example is MAG-Tn3, a multiple antigenic glycopeptide vaccine conjugated with tetanus toxoid that targets the universal carbohydrate tumor antigen Tn (α -D-N-acetylgalactosamine linked with serine or threonine), which is highly expressed in breast tumors [162]. It is currently in Phase I evaluation for patients with localized breast cancer at high risk for relapse (NCT02364492). Another example is OPT-822/OPT-821, a synthetic glycoprotein containing the universally expressed tumor carbohydrate Globo H bound to the carrier protein keyhole limpet hemocyanin (KLH). In an international, randomized, double-blind, placebo-controlled Phase II/III trial, patients who mounted an IgG response detectable at titers of $\geq 1:160$ to the vaccine showed significant progression-free survival (HR, 0.71 [95% CI, 0.52–0.97] $P=0.029$) and interim overall survival (HR, 0.57 [95% CI, 0.33–0.97] $P=0.04$ for OS) [163].

Oncolytic viruses are genetically engineered viruses with cancer cell-specific tropism resulting in the infection and destruction of cancer cells while sparing host tissues. These viruses induce cancer cell death through multiple mechanisms, including virus-mediated cancer cell cytotoxicity, infection of surrounding endothelial cells resulting in destruction of tumor vasculature, and activation of tumor-directed immune responses [164,165]. Oncolytic virus-based vaccines that are approved for other malignancies are now in early-phase trial for the treatment of breast cancer. These include talimogene laherparepvec (T-vec), approved for use in locally recurrent, non-resectable melanoma, and JX-594 (pexastimogene devacirepvec), which has an FDA orphan drug designation for hepatocellular carcinoma. T-vec is a herpes simplex virus-1 (HSV-1) with deletions of ICP34.5 and ICP47, virulence factors that play critical roles in viral replication and inhibition of host immune responses, and engineered to produce GM-CSF [166]. It is currently in clinical trial as monotherapy for the treatment of locally recurrent breast cancer (NCT02658812), as neoadjuvant therapy in TNBC (NCT02779855), and in combination with the PD-L1 inhibitor atezolizumab for metastatic TNBC (NCT03256344). JX-594 is a replication-competent vaccinia poxvirus with deleterious mutations in its viral thymidine kinase gene, resulting in tropism for cancers with overexpression of thymidine kinase, and which expresses human GM-CSF to stimulate antitumor immune responses [167]. It is currently in trial as a combination therapy with metronomic cyclophosphamide in advanced breast cancer in France (NCT02630368).

Adoptive transfer of immune cells

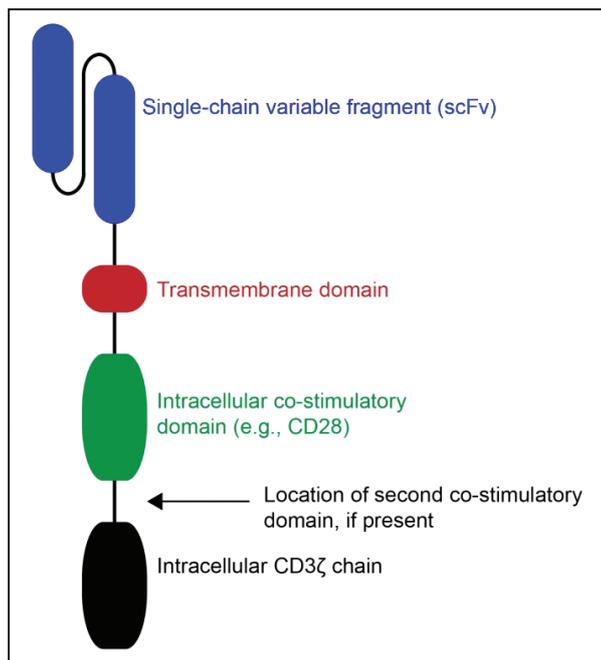
Adoptive transfer is the collection and *ex vivo* manipulation of immune cells to stimulate antitumor activity, with reinfusion of these cells back into patients [168]. Evidence that adoptive transfer had potential as an effective anticancer therapy was first shown in syngeneic murine tumor transplant models in 1955 [169], with subsequent successes in rodent sarcoma and lymphoma models in the 1970s [170–172]. However, it was a series of studies in the early 1980s that provided the groundwork for the development of adoptive transfer technologies. Peripheral blood lymphocytes (PBL) collected from healthy donors and grown in the presence of PBLs from patients with malignancy were capable of lysing both previously cultured and freshly harvested human cancer cells [173]. Similar results were obtained using PBLs collected from patients with malignancy [174] and tumor-infiltrating leukocytes [175] expanded *ex vivo* with IL-2. Adoptive transfer of PBLs collected from cancer patients that were expanded *ex vivo* (lymphokine-activated killer cells) and coadministered with IL-2 resulted in regression of metastatic disease and improved survival in preclinical models of metastatic melanoma and colon adenocarcinoma [176–178], providing the impetus for further research and initiation of clinical trials [179–181].

After early clinical successes were observed in melanoma and renal cell carcinoma with the combination of lymphokine-activated killer (LAK) cells and IL-2, efficacy of this regimen in other malignancies was evaluated, including breast. However, this therapeutic strategy ultimately yielded minimal responses in advanced breast tumors, as well as most other epithelial malignancies evaluated [182,183]. *In vitro* three-dimensional cell culture models of breast cancer data suggested that the lack of response to adoptive transfer therapies was due to decreased ability of immune cells to infiltrate and adhere to tumors and was also related to the fact that environmental signals inhibited the proliferation of infiltrating leukocytes. These data provided a basis for understanding the lack of clinical efficacy [184,185].

While adoptive transfer of PBLs and TILs expanded *ex vivo* has largely remained unsuccessful in breast cancer, studies focusing particularly on TILs have shifted toward identifying and expanding effector cells in TIL populations that recognize specific, immunogenic, non-synonymous mutations that can be used for adoptive transfer. A particularly effective strategy for identifying these cells relies on the use of whole exome sequencing to detect clonal mutations in whole tumor tissue samples, inducing expression of oligopeptides bearing these mutations by immune cells (typically B cells or dendritic cells) *ex vivo*, coculturing these antigen-presenting cells with TILs, and sorting activated lymphocytes by flow cytometry for confirmation of cytotoxic activity and clonal expansion [186,187]. A recent case report showed that combining this strategy with pre-transfer lympho-depleting chemotherapy and a single dose of the immune checkpoint inhibitor pembrolizumab was capable of generating durable antitumor immunologic responses and measurable regression in a patient with metastatic, chemotherapy-refractory ER⁺, HER2⁻ breast cancer [188].

A more common approach to adoptive therapy technologies that is rapidly becoming a major area of research is the genetic engineering of T cells capable of producing highly specific and potent antitumor responses. Techniques for rapidly generating T-cell specificity that allows for immediate initiation of downstream cell signaling cascades and effector functions, so-called armed T cells, are the transduction of retroviruses or lentiviruses encoding bispecific and multispecific antibodies [139,149,150] and chimeric antigen receptors (CARs). Chimeric antigen receptors are genetically engineered hybrid T-cell receptors typically consisting of a single-chain variable fragment (scFv) of the antibody-based B-cell receptor to confer antigen specificity plus the T-cell receptor CD3 ζ transmembrane and intracellular signaling domains linked to one or more intracellular co-stimulatory domains (Figure 2) [189]. While many breast cancer-specific antigens have been identified, HER2 is again the most frequently targeted antigen for CAR design. T cells armed with HER2-specific CARs demonstrate potent *in vitro* T-cell-mediated cancer cell cytotoxicity and are capable of inducing regression of tumors in rodent mammary tumor models [190–192]. As these T-cell responses typically wane with time, other approaches have been utilized to

Figure 2. General structure of chimeric antigen receptor.



The second generation chimeric antigen receptor (pictured) comprises three primary components: a single chain variable fragment (scFV) that recognizes a specific tumor antigen (e.g., HER2), an intracellular co-stimulatory domain (commonly CD28), and the intracellular CD3 ζ chain. Third generation CARs may have a second co-stimulatory domain between the second generation co-stimulatory domain and the CD3 ζ chain (typically 4-1BBB or OX40).

prolong T-cell activity. For example, transposon technology has been used to express a HER2 CAR in Epstein–Barr virus (EBV)-specific cytotoxic T lymphocytes, which proliferate in response to chronic host infection with EBV, thereby affording continued *in vivo* T-cell expansion while redirecting effector activity toward HER2⁺ cancer cells [193]. There are currently two early-phase clinical trials assessing safety and efficacy of adoptive transfer of autologous HER2 CAR-expressing T cells in breast cancer patients (NCT01935843, NCT02547961).

Adoptive transfer of other immune cell populations, particularly NK cells and dendritic cells (DCs), has also been an active area of research. NK cells act through multiple mechanisms, including receptor-mediated cytotoxicity, ADCC, activation of death receptor-mediated apoptotic pathways, and expression of cytokines that promote adaptive immune responses [194]. NK cells collected from circulating peripheral blood mononuclear cells (PBMCs) in breast cancer patients exert potent cancer cell-directed cytotoxicity *in vitro* and significantly reduce cancer cell engraftment and growth in murine xenograft models of breast cancer metastasis [195]. NK cells can also be modified to express tumor antigen-directed CARs,

leading to potent receptor-mediated cytotoxicity. For example, a modified NK-92 cell line derivative that expresses a CAR with an scFv targeting HER2 and the CD3 ζ and CD28 co-stimulatory signaling domains is capable of potent and selective killing of cancer cells expressing HER2 *in vitro* and, upon adoptive transfer, is capable of homing to orthotopic HER2⁺ murine mammary tumors and reducing metastasis formation in murine models of pulmonary metastasis [196]. Another modification of the NK-92 cell line designed to simultaneously express the cytokine IL-15 and a CAR with an scFv targeting EpCAM and the CD3 ζ and CD28 co-stimulatory signaling domains was also shown to selectively kill breast cancer cells *in vitro* [197].

Dendritic cells are the primary innate immune cell population responsible for activating adaptive T-cell-mediated cytotoxic and humoral responses. They are typically derived from the *ex vivo* differentiation of PBMCs, bone marrow-derived monocytes, or CD34⁺ hematopoietic stem cells with GM-CSF and IL-4 with or without TNF- α . In breast cancers, several mechanisms have been employed to load DCs with antigens, including infusion with whole tumor lysates [198], incubation with oligopeptides homologous to portions of known tumor antigens [199,200], transduction of viral vectors engineered to express tumor antigens [201,202], and fusion of activated DCs with cancer cells [203]. Despite their ability to activate T cells *in vitro* and promising evidence in murine breast cancer models, DC-based immunotherapies in monotherapy have been largely unsuccessful in early-phase trials [199,200,202].

Strategies employed to enhance the effects of adoptive transfer with DCs (so-called DC vaccines) are combining DC infusion with cytokine-induced killer cells (CIKs) and depleting Treg populations. CIKs are a heterogeneous population of MHC nonrestricted lymphocytes, expanded *in vitro*, that typically express both CD3 and CD56, but also include small subsets of CD3⁻, CD56⁺ NK cells and CD3⁺, CD56⁻ T cells. All three of these subsets are activated by DCs, and those CIKs with dual expression of CD3 and CD56 are capable of both NK and antigen-specific T-cell activity when activated [204]. Indeed, in a Phase I/II trial, combining autologous transfer of *ex vivo*-selected and expanded DC/CIK with high dose chemotherapy improved both progression-free and overall survival in patients with metastatic breast cancer [205]. Promising results have also been seen with daclizumab, a monoclonal antibody directed against CD25, that downregulates expression of both CD25 and FOXP3 in Tregs, resulting in reprogramming of these immune cells and allowing for priming and boosting of CD8⁺ T-cell activity when combined with DC-based adoptive transfer in patients with metastatic breast cancer [37].

Challenges and future perspectives

Breast tumors are infiltrated by a diverse array of immune cells that shape and influence the progression of this heterogeneous group of malignancies. Increasing understanding of the basic biological mechanisms of this complex immune

microenvironment and the recognition that components of the immune system and their effector functions can be augmented and potentiated to effectively target and destroy breast tumors has driven the rapid development and evolution of breast cancer immunotherapies.

Increasing sophistication of immune system-based technologies has resulted in enhanced specificity, leading to better side-effect profiles and improved outcomes. However, several challenges remain. Tumors have consistently found methods to adapt and develop resistance against cytotoxic chemotherapeutics, and emerging evidence indicates that this is true for immunotherapies as well [206]. Given the complexities of the immune system, combination therapies will need to be employed to circumvent immune-mediated resistance. These strategies include simultaneously combining immunotherapy with cytotoxic agents, augmenting both the innate and adaptive immune systems, tandem immunotherapies, and combining immune checkpoint inhibition with DC-based vaccines [207], among others. Combinatorial methods will necessarily need to be approached

with caution, as global activation of the immune system has the potential for serious adverse effects, including severe cytokine release syndromes. Therefore, reliable mechanisms for efficiently and specifically activating or disinhibiting multiple tumor-directed responses *in vivo* will need to be further cultivated. Finally, while many of these approaches are technically feasible, immunotherapies are often cost prohibitive and will require development of streamlined, high-throughput technologies, particularly for those therapies that rely on adoptive transfer.

Despite these challenges, rapid advances in breast cancer immunotherapies are showing significant promise for the treatment of many subtypes, including TNBCs. In TNBCs, TIL infiltration correlates positively with response to immune checkpoint inhibitors, and these responses are long lasting [131,208]. As this response appears to be more effective when immunotherapy is administered as part of first-line therapy [126] and with the identification of biomarkers for response to immunotherapy, strategies harnessing the immune system may alter the landscape of current approaches for TNBCs.

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