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REVIEW

Immunotherapy for the treatment of breast cancer

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ABSTRACT

Introduction: Breast cancer is the most common cancer as well as the first cause of death by cancer in women worldwide. Although routine treatment improves the outcome of early stage breast cancer patients, there is no effective therapy for the disseminated disease. Immunotherapy has emerged as a powerful therapeutic strategy for the treatment of many cancers. Although traditionally conceived as a non-immunogenic tumor, breast cancer is now considered a potential target for immunotherapy.

Areas covered: In this review, the authors discuss different immunotherapeutic strategies that are currently being tested for the treatment of breast cancer: These strategies include: (i) blockade of immunological checkpoints, (ii) antitumor vaccines, (iii) regulatory T cell blockade, (iv) adoptive T cell transfer therapy, (iv) adoptive immunotherapy with monoclonal antibodies, and (v) combination of immunotherapy with chemotherapy.

Expert opinion: A growing body of evidence indicates that immunotherapeutic strategies can benefit a larger cohort of breast cancer patients than hitherto anticipated. Since breast tumors entail multiple mechanisms to impair antitumor immunity, the immunological characterization of individual tumors and the selection of suitable combinations of chemotherapeutic and immunotherapeutic approaches are required to achieve significant clinical benefit in these patients.

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1. Introduction

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed every year, which represents about 25% of all cancers in women [1]. Routine treatment consists of surgical resection followed by chemotherapy, radiotherapy, or hormone therapy [2]. Conventional therapies are not specific and often elicit severe side effects, including nausea, vomiting, loss of appetite, fatigue, immune suppression, anemia, and even impaired cognitive function that interferes with the quality of life [3]. In addition, hair loss secondary to chemotherapy may lead to anxiety and can affect the patient's sense of self and identity [4]. Although the response to this treatment is relatively good in early stages of the disease, many patients suffer relapses. Considering the limitations of traditional therapy, research efforts are focused on the development of novel therapeutic strategies that are more specific and lead to durable responses.

In recent years, immunotherapy has emerged as a powerful therapeutic approach for the treatment of many cancers [5]. Immunotherapeutic strategies stimulate the immune system to detect and eradicate disseminated tumor cells. Due to the specificity of the antitumor immune response and the possibility of generating antitumor immunological memory, the field of immunotherapy has grown exponentially over the last few decades. Unfortunately, since breast tumors were traditionally thought to be poorly immunogenic, the use of immunotherapy was long considered inappropriate to treat breast cancer patients. However, mammary tumors can be infiltrated with immune cells and the level of tumor infiltrating T lymphocytes (TILs) positively correlates with good prognosis in breast cancer patients, particularly in triple-negative (TNBC) and human epidermal growth factor receptor 2 (HER2)-positive early breast cancer [6]. Several factors seem to be responsible for the recruitment of lymphocytes within breast tumors, among them, it is known that high endothelial venules (HEV) interact with blood vessels and contribute to lymphocyte infiltration. In patients with invasive breast cancer, high density of tumor HEVs correlates with lower risk of relapse and with longer metastasis-free and disease-free rates, as well as better overall survival rate [7]. A higher number of TILs in pretreatment biopsies also correlates with better response to neoadjuvant therapy [8]. It is important to mention that different subtypes of mammary tumors differ on the level of TILs. Triple-negative tumors, which are negative for estrogen (ER) and progesterone (PR) receptors and lack HER2 gene amplification, exhibit the highest level of TILs [9]. It has been hypothesized that the genomic instability of these tumors leads to the generation of neo-antigens that can be readily detected by the immune system [9]. Hormone receptor-expressing tumors have been associated with lower immunogenicity. However, patients with HER^+ tumors also exhibit a significant infiltration of TILs [10].

Although lymphocytes can indeed infiltrate breast tumors [9], different mechanisms can impair antitumor specific T cell

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Article highlights

- Breast tumor parenchyma can readily be infiltrated by lymphocytes. The grade of lymphocytes infiltration varies according to breast cancer type, being triple negative tumors the most infiltrated tumors.
- Immunosuppressive mechanisms are present in breast tumors, such as immunological checkpoints, Tregs, MDSCs, TAMs, IDO.
- Breast cancer is an excellent candidate for immunotherapeutic approaches that block immunosuppressive cells and pathways.
- Since a mechanism of tumor immune escape involves the up-regulation of immunological checkpoints, the modulation of these ligand-receptor interactions has shown promising results, and can be additionally incorporated to traditional therapies.
- Antitumor vaccines are now being reconsidered as a powerful tool for the treatment of breast cancer. They provide high specificity with good toxicological profile. Their use is being encouraged in combination with additional therapies.
- Adoptive transfer involves the extraction of peripheral T lymphocytes from patients, their expansion *ex vivo* and the reinfusion into the patient to target tumor cells.
- Chemotherapy can render tumors more immunogenic and hence boost the efficacy of immunotherapeutic strategies.
- Further studies are needed to fully understand the impact of immunotherapy approaches on the survival of breast cancer patients.

This box summarizes key points contained in the article.

responses (Figure 1). The molecular analysis of breast tumors has shown a robust upregulation of immunomodulators [11]. A prevalence of regulatory cytokines characterizes the tumor microenvironment, such as IL-10 or TGF-β, which skew the immune response to a less efficacious Th2 or regulatory T (Treg) phenotypes [12]. TILs can also be inactivated within the tumor mass by tryptophan deprivation, which impairs T cell proliferation and activation [13]. Indoleamine-2,3-dioxygenase (IDO), the enzyme responsible for the conversion of tryptophan to kynurenine, is present in many solid tumors including breast cancer [14]. The depletion of tryptophan and the increase in immunosuppressive kynurenine metabolites inhibit effector T cell proliferation, increase T cell anergy and apoptosis, and induce the expansion of Tregs [15]. However, the prognostic value of IDO in breast cancer patients remains controversial. Soliman et al. found that the overall survival was better in ER^+ patients with high IDO expression [14], meanwhile metabolomic and molecular approaches have demonstrated that IDO promotes tumor progression and predicts poor patient survival [16]. Immunological checkpoints cytotoxic t lymphocyte antigen 4 (CTLA-4, CD152) and programmed cell death protein 1 (PD-1) are inhibitory T cell receptors that are upregulated in breast tumors, allowing tumor escape and favoring the development of metastasis [17–19]. PD-1 ligand PD-L1 is present in breast cancer specimens and has been recently associated with histological grade and negative hormone receptor status [20]. High PD-L1 expression was reported to be positively correlated with Treg infiltration in breast cancer patients and concomitant high levels of both markers were detected in tumors with the worst prognosis. Tumor infiltrating Tregs constitute an important therapeutic target, as they seem to suppress effector T cell function in a dose-dependent manner [21,22]. A recent report indicates that meta-analysis of over 8500 breast tumor samples shows significant association between higher tumor infiltrating Tregs and poor prognosis in terms of overall survival [23].

We will review the main immunotherapeutic strategies evaluated in preclinical and clinical trials for the treatment of breast cancer patients (Table 1), which are summarized in Figure 1.

2. Treating breast cancer with immunotherapy

2.1. Blockade of immunological checkpoints

2.1.1. CTLA-4

CTLA-4 is an inhibitory coreceptor expressed in the surface of T cells that inhibits their proliferation and activation. CTLA-4 competes with CD28, a molecule that provides costimulatory signals required for T cells activation, for the binding of B7-1 (CD80) and B7-2 (CD86) present in antigen-presenting cells (APCs). While CD28 expression is constitutive, CTLA-4 is inducible in conventional T cells. However, CD80 and CD86 exhibit higher affinity for CTLA-4 than for CD28 [24,25]. Several studies have shown high CTLA-4 expression in tumor specimens and peripheral mononuclear cells, as well as elevated circulating levels of soluble CTLA-4 in breast cancer patients [26–28], which are associated with poor prognosis [25,29]. Thus, CTLA-4 is an attractive therapeutic target to improve antitumor immunity in breast cancer patients. Ipilimumab is the first fully humanized mAb against CTLA-4 that was approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma in 2011. A number of clinical trials are now evaluating the safety and efficacy of CTLA-4 blockade in breast cancer patients, mainly in combination with additional immunological checkpoint blockade or with cytotoxic strategies. A phase I trial is currently recruiting patients with locally advanced or metastatic HER2-negative breast cancer to be treated with ipilimumab in combination with anti-PD-1 mAb nivolumab and a histone deacetylase inhibitor that inhibits tumor cell growth (entinostat, NCT02453620). Combination of ipilimumab and nivolumab is also being tested in early-stage breast cancer following tumor cryoablation (NCT02833233). The safety and tolerability of another anti-CTLA4 mAb, tremelimumab, are being assessed in patients with advanced breast cancer as a single therapeutic option or in combination with anti–PD-L1 immune checkpoint inhibitor durvalumab (NCT02527434, NCT01975831), and hypofractionated radiotherapy (NCT02639026). It is important to notice that the clinical response to immunological checkpoint blockade can exhibit unique dynamics since, unlike traditional anticancer agents, its antitumor efficacy can often be observed after an initial phase of tumor progression [30].

Although CTLA-4 blockade constitutes a valuable approach for the treatment of breast cancer patients, its immune-related toxicity remains a concern. Since CTLA-4 limits T cell activation and clonal expansion, its blockade lowers the threshold required for T cell activation. Thus, it is frequently associated with severe autoimmune and immune-related side effects, such as colitis, dermatitis, uveïtis, and hypophysitis, which indeed correlate with clinical responses [31]. If when side effects arise, mAb treatment is interrupted and the patient receives corticosteroids, the

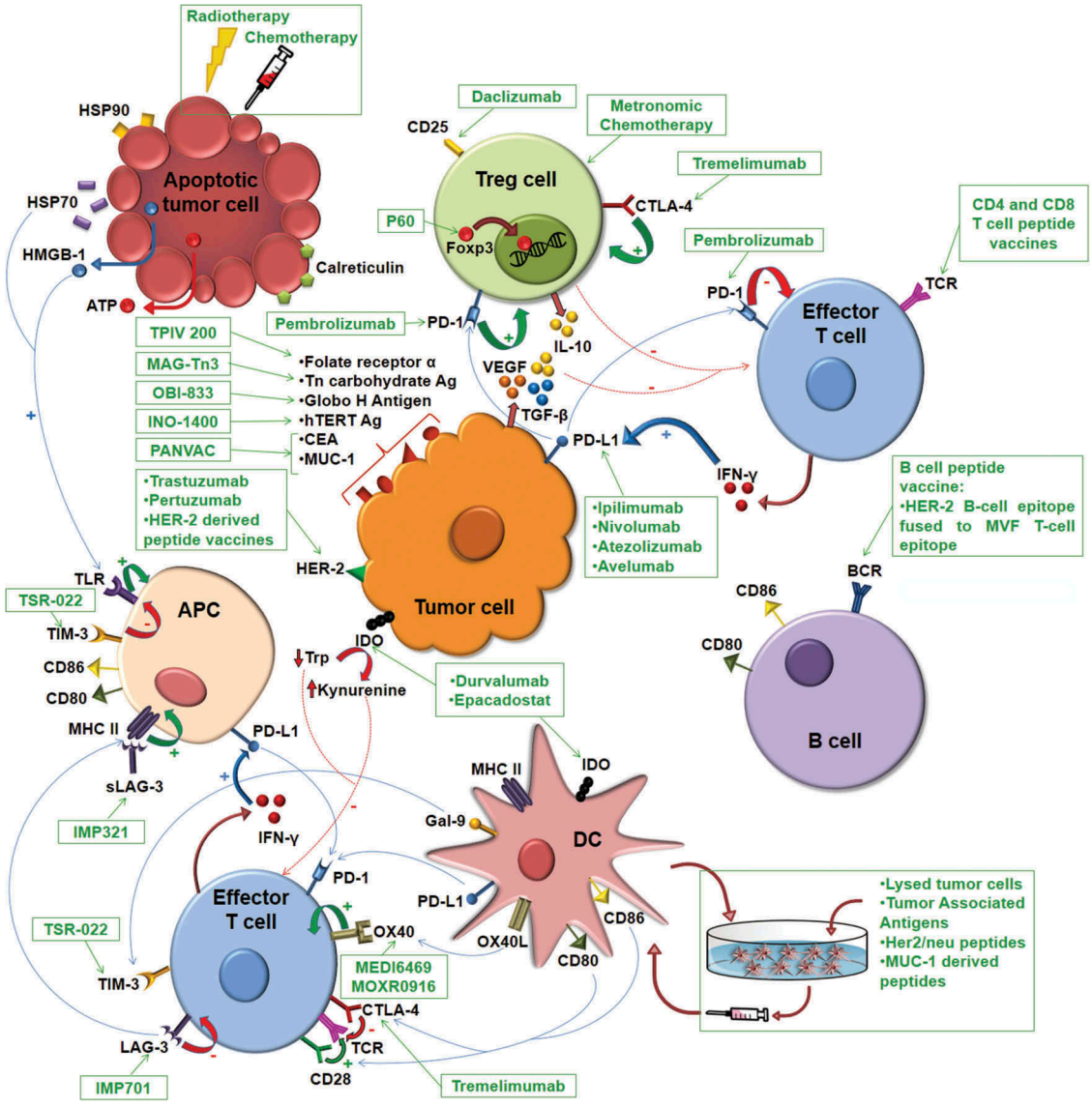

Figure 1. Mechanisms of tumor immunological escape and therapeutic targets that can improve antitumor immunity. Full color available online.
Tumor cells develop multiple immunosuppressive mechanisms that inhibit antitumor immunity, such as cytokines that inhibit effector T cell responses, and IDO, which reduces the availability of tryptophan and produces T cell cytotoxic metabolites. The presence of TILs in the tumor microenvironment is associated with the local production of IFN-gamma, which induces the expression of immunological checkpoints. Several immunological checkpoints are present in breast tumors, including CTLA-4, PD-1, TIM-3 and LAG-3, which can be targeted using specific mAbs. Since Tregs are also recruited into the tumor microenvironment, *metronomic chemotherapy*, Treg-depleting mAbs and Foxp3-blocking peptide P60 can improve the immune response induced by immunotherapeutic strategies, i.e. antitumor vaccines. Although breast cancer was traditionally considered to be not immunogenic, these cells express a myriad of tumor associated antigens, i.e. Folate receptor α, Tn carbohydrate Ag, Globo H hexasaccharide 1 Ag, hTERT Ag, CEA, MUC-1, HER2, that can be targeted with antitumor vaccines and mAb-based therapies. In addition, standard therapies can boost antitumor immunity as they induce immunogenic cell death, which leads to the exposure and release of pro-inflammatory molecules, such as calreticulin, HSP90, ATP, HSP70 and HMGB1, which stimulate DC maturation through TLR signaling. Therapeutic strategies for all these targets are indicated in green boxes. Arrows show interactions between molecules. Red arrows indicate inhibitory pathways, while green arrows mark stimulatory signals.

outcome is usually good. However, this immunosuppressive treatment counteracts the antitumor effect of immunotherapy. Local administration of anti-CTLA-4 mAbs has been explored in preclinical models to reduce the toxicity observed following its systemic administration [32]. Subcutaneous injection of a low-dose slow-release formulation of anti-CTLA-4 mAb close to the tumor led to a 1000-fold decrease in circulating levels of mAbs when compared to systemic administration. Local treatment induced the expansion of tumor antigen-specific T cells and elicited antitumor efficacy with reduced risk of autoimmunity and immune-related toxicity in tumor-bearing mice [32]. This approach could be explored to reduce the toxicity of mAbs against immunological checkpoints in breast cancer patients.

2.1.2. PD-1

PD-1 is an inhibitory TCR coreceptor that binds to B7-H1 (PD-L1) and B7-DC (PD-L2) to inhibit T cell function in nonlymphoid and lymphoid organs, respectively [33]. PD-L1 is expressed in hematopoietic and non-hematopoietic cells, including endothelial and tumor cells, and its expression is upregulated in response to inflammatory signals, such as IFNs and TNF-alpha, that are locally produced by TILs [33]. PD-1

Table 1. Immunotherapy clinical trials in breast cancer patients.

Immunotherapy	Approach	Action	Phase	Reference
Immunological checkpoint blockade	Nivolumab+Ipilimumab+Entinostat (histone deacetylase inhibitor)	PD-1 and CTLA-4 inhibition+ antineoplastic drug	I	NCT02453620
	Nivolumab+Ipilimumab+ cryoablation	PD-1 and CTLA-4 inhibition+ cryoablation	Pilot	NCT02833233
	Tremelimumab	CTLA-4 inhibition	I	NCT02527434
	Durvalumab + Tremelimumab	CTLA-4 receptor and PD-1 ligand inhibition	I	NCT01975831
			II	NCT02527434
	Durvalumab + Radiotherapy	PD-1 ligand inhibition+ cytotoxicity	I	NCT02639026
	Pembrolizumab	PD-1 receptor inhibition	I	NCT01848834
			III	NCT02555657
	Pembrolizumab+Epacadostat	PD-1 ligand inhibition+ IDO inhibition	I	NCT02178722
	Atezolizumab+Nab-paclitaxel	PD-L1 ligand inhibition+cytotoxicity	II	NCT02530489
	Atezolizumab + CPI-444	PD-L1 blockade+inhibition of adenosine-mediated immunosuppression	I	NCT02655822
	Avelumab	PD-L1 blockade	I	NCT01772004
	TSR-022+PD-1 blockade	TIM-3+PD-1 blockade	I	NCT02817633
	IMP321+Paclitaxel	Soluble LAG-3+ chemotherapy	I	NCT00349934
			II	NCT02614833
	MEDI6469+Radiation	OX-40 stimulation+ cytotoxicity	I/II	NCT01642290
	MOXR0916+atezolizumab+ bevacizumab	OX-40 stimulation+PD-L1 blockade+VEGF neutralization	I	NCT02410512
Therapeutic vaccines	Folate Receptor Alpha (FRα) peptides + GM-CSF	Cytotoxic T-lymphocyte (CTL) response and macrophages activation	II	NCT02593227
	MAG-Tn3	Tn carbohydrate antigen recognition	I	NCT02364492
	OBI-833	CTL response against Globo H-expressing tumor cells	I	NCT02310464
	GP2+GM-CSF vs. AE37+GM-CSF	CTL response against HER2-expressing tumor cells	II	NCT00524277
	NeuVax+GM-CSF	CTL response against HER2-expressing tumor cells	III	NCT01479244
	MVF-HER2 peptides	B and T cell response against HER2-expressing tumor cells	I	NCT01376505
	INO-1400 + INO-9012	TERT inhibition+IL-12	I	NCT02327468
	Personalized polyepitope DNA	CTL response against autologous tumor antigens	I	NCT02348320
	PANVAC	CTL response against tumor cells expressing MUC-1 and CEA	II	NCT00179309
	HER-loaded DC vaccine	Immune response against HER2-expressing tumor cells	I	NCT02063724
	Trastuzumab+NeuVax+GM-CSF	Cytotoxicity in HER2-expressing tumor cells	II	NCT01570036; NCT02297698
Adoptive t cell therapy	Chimeric T cell receptors (CAR)	c-Met inhibition	I	NCT01837602
	Immune cells engineered	Overexpressed mesothelin protein recognition	I	NCT02414269
	T cells engineered	NY-ESO-1, MAGE-A4, PRAME, survivin, and SSX markers recognition	I	NCT02239861

engagement to its ligands inhibits T cell activation, cytokine secretion, proliferation, and survival [34,35]. Although the expression of PD-1 and its ligands in breast cancer was largely ignored, this has recently gained great attention. PD-1 expression has been readily detected in TILs in breast cancer patients and is also associated with poor prognosis [36]. PD-L1 is upregulated in patients with breast cancer. PD-L1 overexpression has been detected in 20–40% of breast tumors, when compared to normal breast samples [19,37] and is associated with features of poor prognosis, i.e. higher grade and proliferation rate. PD-L1 expression has been also detected in circulating metastatic cells of breast cancer patients [37–39]. PD-1 and PD-L1 are now considered therapeutic targets for the treatment of breast cancer, and thus, mAbs that block their function have been tested in these patients. Pembrolizumab is a high-affinity, highly selective, humanized monoclonal IgG4-k antibody against PD-1 that was tested as single agent in KEYNOTE-012, a phase I clinical trial in heavily pretreated patients with advanced TNBC (NCT01848834) [40]. PD-L1 expression was detected in ~60% of the patients. Pembrolizumab efficacy was assessed in 27 patients and led to an overall response rate of 18.5% and one patient with complete response. The efficacy of pembrolizumab in TNBC patients is comparable to the results observed in head and neck and gastric cancer cohorts of KEYNOTE-012. The safety of this treatment protocol was similar to that reported in melanoma patients with Grade 3–5 and side effects developed in 15% of the patients. These included anemia, aseptic meningitis, lymphopenia, headache, and pyrexia and immune-mediated adverse effects were individual cases of grade 3 colitis, grade 3 hepatitis, and grade 2 hypothyroidism. It is worth to mention that one patient died because of disseminated intravascular coagulation, presumably caused by pembrolizumab treatment. Therefore, the dose and frequency of administration should be adjusted in order to improve the safety profile. Although this was a relatively small study, these results are promising and the efficacy of this agent is being assessed in the randomized phase III KEYNOTE-119 study (NCT02555657).

Considering that TNBC is more likely to be infiltrated with TILs and to express the PD-1/PD-L1 system, this type of tumor has gained more attention than other subtypes for the treatment with mAbs against checkpoint inhibitors. However, hormone sensitive breast tumors can also be targeted with these therapies. Pembrolizumab is being evaluated in a phase I trial in patients with PD-L1^{+}/ER^{+}/HER2^{-} locally advanced or metastatic breast cancer that underwent several lines of prior treatment [41]. In this trial, PD-L1 expression was detected in 20%

of the 248 tumors assessed, a much lower rate than that observed in KEYNOTE 012, a difference that seems to be related to the type of breast tumor evaluated in each trial [19]. The overall response rate in 25 patients treated was 12%, with another 16% showing stable disease. Considering that these patients had not responded to 3–5 prior lines of therapy, these results are encouraging and suggest that immunological checkpoint inhibitors should not be restricted to patients with TNBC.

Atezolizumab, a humanized mAb that blocks PD-L1 was evaluated in combination with paclitaxel in 32 TBNC patients that were treated with up to 3 prior lines of systemic therapy [42]. Grade 3–4 neutropenia was observed in 40% of the cohort, but no treatment-related deaths were reported. Antitumor efficacy was reported in 70% of these patients that was independent of PD-L1 status. $CD8^+$ T cell response was not impaired in these patients by concomitant chemotherapy. Although this is a small study, the results reported indicate that this is a tolerable approach with promising antitumor activity. This combination strategy is currently being evaluated in a phase II clinical trial (NCT02530489) [42].

Avelumab (MSB0010718C) is a fully human anti-PD-L1 IgG1 antibody that is being evaluated in the phase Ib trial JAVELIN (NCT01772004), which recruits patients with different types of breast cancer [43]. Stable disease was achieved in 40/168 patients (23.8%). Although further analysis is needed in order to determine which characteristics correlate with a better outcome, the response seems to be related to the expression of PD-L1 in tumor infiltrating immune cells. It is important to remark that although the safety profile was acceptable for most of the cohort with Grade 3–4 toxicities reported in less than 14% of the patients, 2 treatment-related deaths were reported. These observations indicate that although the blockade of immunological checkpoints is a very promising therapeutic strategy for breast cancer patients, especially those that do not have other therapeutic choices left, these are therapies that still need to be improved.

Checkpoint inhibition can also be combined with inhibitors of immunosuppressive molecules present in the tumor microenvironment. As such, IDO inhibitor Epacadostat, which increases and restores the proliferation and activation of effector T cells and inhibits tumor-associated Tregs, is currently being assessed in combination with pembrolizumab in TNBC patients (NCT02178722). Atezolizumab is being evaluated in combination with CPI-444, another new immunotherapy drug that binds to adenosine A2A receptors expressed on the surface of immune cells, including T lymphocytes, NK cells, macrophages, and dendritic cells. This drug prevents tumor-released adenosine binding to A2A receptors on immune surveillance cells, thereby abrogating adenosine-induced immunosuppression in the tumor microenvironment (NCT02655822). Although combination therapies can improve antitumor efficacy, these cocktails can also worsen the chance of immune-related side effects, which should be closely monitored.

2.1.3. Other immunological checkpoints

T cell Immunoglobulin Mucin-3 (TIM-3) is an inhibitory receptor expressed by Tregs and exhausted T cells and NK cells in the context of chronic inflammatory processes, such as cancer [44]. Co-expression of TIM-3 with PD-1 has been reported in exhausted TILs in many tumors, including breast cancer [45], and concomitant blockade of these two pathways has shown therapeutic benefit in preclinical cancer models [46]. Thus, double blockade could prove useful to improve antitumor immunity. In fact, a humanized mAb that inhibits TIM-3, TSR-022, is being currently under clinical investigation in patients with advanced solid tumors alone or in combination with PD-1 blockade (NCT02817633). TIM-3 is also upregulated in tumor-associated macrophages and DCs in response to immunosuppressive molecules present in the tumor microenvironment, such as IL-10, VEGF, and Arginase I [47]. TIM-3 has been involved in the inability of tumor-associated DCs to respond to damage-associated molecular patterns released by tumor cells during immunogenic cell death, i.e. HMGB1 [47]. These findings support the combination of traditional cytotoxic approaches, such as radiotherapy and chemotherapy, with TIM-3 blockade for synergistic antitumor efficacy.

LAG-3 is an inhibitor receptor expressed on the cell membrane of T lymphocytes and NK cells that restricts effector function and increases the immunosuppressive function of Tregs [48]. This molecule is a CD4 homologue associated to the CD3/TCR complex, which upon binding to major histocompatibility complex class II (MHC II) inhibits the expansion of activated T cells, suppressing immune responses. Combination of LAG-3 blockade with specific antitumor vaccination increases activated $CD8^+$ T cells in the tumor and disrupts the tumor parenchyma [49]. Another report showed that LAG-3 and PD-1 function synergistically promote tumor escape, which suggests that a combination therapy for both targets could achieve better outcome [50]. Since LAG-3 was detected to be co-expressed with PD-1 in 15% of the patients with TNBC [51], combined blockade of LAG-3 and PD-1/PD-L1 could improve antitumor immunity in this subset of TNBC patients. A blocking mAb that neutralizes LAG-3 (IMP701) is being clinically tested by Novartis in cancer patients. Contrary to its membrane-bound form, a soluble form of LAG-3 (sLAG-3) is an endogenous high affinity ligand for MHCII that induces the maturation and activation of APCs, improving antitumor immunity. In breast cancer patients, circulating levels of sLAG-3 were observed to be positively correlated with disease-free and overall survival rates in patients with hormone-sensitive tumors [52]. IMP321 is a recombinant sLAG-3-Ig fusion protein that enhances antitumor immunity [53]. IMP321 can be combined with chemotherapy and has proven a useful vaccine adjuvant. NCT00349934 is a phase I study which evaluated IMP321 therapeutic value in patients with metastatic breast cancer who were also receiving paclitaxel. Results showed that first-line treatment with this chemo-immunotherapeutic strategy led to a sustained increase in the number and activation of monocytes, DCs, NK cells, and long-lived cytotoxic effector-memory $CD8^+$ T cells [54]. This treatment showed an acceptable toxicity profile without clinically significant local or systemic IMP321-related adverse events. Clinical benefit was observed in 90% of the 30 patients evaluated. This study suggests that targeting LAG-3 could improve antitumor immunity without higher risk of toxicity. A phase IIb clinical trial is currently recruiting participants (NCT02614833).

OX40 (CD134) is an immunological checkpoint with costimulatory activity expressed primarily on $CD4^+$ T cells, and at a lesser extent in activated effector $CD8^+$ T cells, Tregs, and NK cells [55]. OX-40 is upregulated upon TCR engagement and enhances proliferation and effector function, as well as memory T cell development [56]. Although OX-40 activation also induces the expansion of Tregs, it seems to promote an exhausted phenotype in this cell population [57]. OX-40 ligand (OX-40L) is upregulated in APCs in response to TLR activation and proinflammatory cytokines. Since the availability of endogenous OX-40L has been proposed to be a limiting factor in OX-40 signaling in T cells [56], OX40 agonists have been developed to overcome immune tolerance in cancer [58]. Agonistic anti-OX40 mAb or OX40L-Fc fusion protein has demonstrated antitumor efficacy and immunological memory in preclinical cancer models [59]. Since costimulatory signaling through OX-40 generates optimal cytotoxic lymphocytes (CTLs), OX-40 agonists are ideal tools to boost antitumor immunity in combination with traditional cytotoxic treatments, as well as with active immunotherapeutic strategies or blockade of co-inhibitory receptors [59]. A phase I/II clinical trial combines MEDI6469, an agonist anti-OX40 mAb, with stereotactic body radiation for patients with advanced metastatic breast cancer who have failed prior hormone or chemotherapy (NCT01642290), showing not severe adverse effects so far [60]. Combination of MOXR0916, a humanized agonist anti-OX40 mAb, with PD-L1 blockade using atezolizumab and VEGF neutralization with bevacizumab is being evaluated in a clinical trial in patients with advanced solid tumors (NCT02410512). This dose-escalation trial showed that this treatment was well tolerated, without dose limiting toxicity or severe immune-related adverse effects [61]. These findings warrant further clinical evaluation to determine the clinical benefits of these therapeutic strategies.

Overall, targeting immunological checkpoints with specific antibodies is a very promising approach for the treatment of breast cancer patients, in particular, for TNBC. However, it is important to balance the risk of these therapeutic strategies, as some of them can lead to severe adverse effects in some of the patients. More research is needed in order to better predict which patients will benefit from each of these therapies so as to reach a balanced risk: benefit ratio.

2.2. Antitumor vaccines

The goal of therapeutic antitumor vaccines is to achieve highly specific antitumor cellular immune responses. The efficacy of antitumor vaccines is mostly dependent on antigen-specific CTLs to detect and eradicate disseminated cancer cells [62]. Additionally, primary T cell responses can be accompanied by the induction of memory T cells, which mediate long-term antitumor immunological memory to impair recurrences [63]. The identification of mutated and aberrantly expressed self-tumor antigens could allow the development of personalized therapeutic vaccination strategies or adoptive transfer protocols to enhance antitumor immunoreactivity.

There are many different types of antitumor vaccines depending on the immunogenic source, i.e. whole tumor lysates, antigenic peptides overexpressed by tumors, DNA, RNA, and viral vaccines. Several approaches are currently being evaluated in breast cancer patients:

2.2.1. Peptide vaccines

Immunogenic peptides are short aminoacidic sequences derived from tumor antigens that bind to MHC molecules in APCs and induce CTL responses to detect and kill tumor cells expressing the corresponding antigen. Candidate peptides that target $CD8^+$ or $CD4^+$ T cells are those that can potentially be presented by MHC-I or MHC-II, respectively. In the cells, these peptides are generated by proteolysis of endogenously synthesized proteins in the cytosol, loaded onto MHC molecules in the endoplasmic reticulum, and presented on the cell surface for surveillance by $CD4^+$ and $CD8^+$ T cells [64]. One crucial step in the development of peptide vaccines targeting $CD8^+$ or $CD4^+$ T cells is the choice of the correct peptide, which has to be presented in the context of the MHC complex and to stimulate T cell proliferation. New techniques are being developed to overcome the current limitations of peptide screening [65].

Folate receptor alpha (FR alpha) is present in 80% of TNBCs and its expression is significantly associated with worse disease-free survival [66]. TAPIMMUNE company developed a peptide vaccine containing five immunogenic peptide epitopes of the human folate receptor alpha in order to prevent breast tumor recurrences. This vaccine is intradermally injected with GM-CSF as vaccine adjuvant to generate a cytotoxic T lymphocyte response against FR alpha-overexpressing tumor cells. This vaccine is being tested in combination with cyclophosphamide in treating breast cancer patients that have already received the standard care (NCT02593227).

Aberrant glycosylation processes in tumor cells lead to the expression of neo-carbohydrate antigens, such as the tumor-associated Tn carbohydrate antigen that is overexpressed in breast carcinoma. MAG-Tn3 is a fully synthetic vaccine based on three consecutive Tn moieties that are linked to a $CD4^+$ T cell epitope to induce anti-Tn antibody responses. The vaccine also contains the universal helper tetanus toxoid-derived peptide TT830-844, which can bind to various MHCII molecules. MAG-Tn3 vaccination generates the production of tumor-specific anti-Tn glycosidic antibodies, which results in antibody-dependent cell cytotoxicity against Tn-expressing tumor cells in preclinical models of cancer. This approach is currently being tested in breast cancer patients (NCT02364492) [67].

OBI-833 vaccine targets the Globo H hexasaccharide 1 antigen, which is a tumor-associated antigen present in breast tumors. This antigen is conjugated to DT-CRM197, a mutated form of diphtheria toxin to increase immunogenicity. A clinical trial to evaluate the safety and tolerability of escalating doses of this vaccine is ongoing in patients with metastatic breast cancer. A secondary aim of this trial evaluates the humoral immune responses following administration of this vaccine (NCT02310464).

HER2 is a member of the epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinases and is essential for normal cell growth and division. Its abnormal expression is linked to cancerous processes and, thus, has become an important biomarker and therapeutic target, especially in breast cancer. It is overexpressed in approximately 25–30% of breast cancer patients and associated with increased

tumor biological activity, being indicative of unfavorable evolution of this disease [68]. HER2-derived peptide vaccines are a very attractive therapeutic strategy for the treatment of patients with $HER2^+$ mammary carcinomas. There are very interesting results from patients that receive HER2-derived peptide vaccination in order to prevent recurrences. GP2 is a human leukocyte antigen (HLA)-A2-restricted immunogenic peptide derived from the HER2 protein. In a phase I/II trial, HLA-A2+ patients *with any level of HER2 expression* that rendered disease free after standard treatment were treated with GP2 and GM-CSF (NCT00524277). Vaccination was well tolerated and demonstrated clinical benefit in HER2-overexpressing tumor-bearing patients, none of which developed recurrences at 34 month follow up [69]. NeuVax is another HER2-derived peptide vaccine that binds to HLA-A2 and HLA-A3 molecules, which are encountered in 60–75% of the population [70]. This vaccine has been administered with GM-CSF as adjuvant to reduce the risk of recurrences in breast cancer patients. Early phase clinical trials demonstrated that NeuVax induces peptide-specific T cell expansion and anti-HER2 immunological memory even in patients with low levels of HER2 expression. The disease-free survival at 60 months was significantly improved by the vaccination (94.7% vs. 80.2% in the control group) with minimal toxicity [70]. The PRESENT phase III clinical trial is currently evaluating the efficacy of NeuVax in patients with low-to-intermediate HER2 expression (NCT01479244). Since peptide vaccines fully activate an adaptive antitumor response and trigger antitumor immunological memory, these strategies lead to sustainable clinical responses. Thus, they could yield superior clinical benefit than antibody-mediated targeting of HER2 with trastuzumab or pertuzumab, as these are passive immunotherapeutic approaches that do not lead to immunological memory [70]. In addition, while peptide vaccines seem to be efficacious in HER2+ tumors regardless the level of antigen expression, *Ab*-based strategies elicit antitumor effects in up to 20–30% of the breast tumor patients with the highest expression of HER2 [70]. Further clinical research is required to determine whether antibody-mediated or cell-mediated targeting of HER2 lead to better clinical outcomes with optimal toxicity profiles.

Another therapeutic approach less studied for breast cancer is the use of peptide vaccines targeting B cell epitopes [71]. These peptides activate tumor-specific B cells, and as a consequence, a humoral response is triggered, eliciting high-affinity antipeptide antibodies. This therapy is very attractive because it could potentially replace the administration of humanized mAbs, which would in turn be endogenously produced upon B cell epitope vaccination. Kaumaya et al. developed chimeric peptide vaccines using HER2 B cell epitope combined with the promiscuous T cell epitope of measles virus fusion protein (MVF) to achieve B and T cell stimulation [72]. A phase I clinical trial in patients with metastatic cancer showed that these vaccines were able to elicit a specific antibody response in 62% of the patients without severe adverse effect [73]. A vaccine that combined two HER2 B cell epitopes MVF-$HER2^{597-626}$ and MVF-$HER2^{266-296}$ emulsified with normuramyl-dipeptide is being tested in a phase I clinical trial for patients with HER2 metastatic tumors (NCT01376505). More research is still needed to evaluate the overall survival of patients receiving this therapy.

Peptide vaccines are very attractive immunotherapeutic approaches, as these preparations are reproducible and easy to synthesize. These vaccines have proved safe and effective at inducing sustained antitumor immunity. However, the MHC restrictions of these peptides limit the use of each vaccine to a subset of patients that express certain HLA molecules [74]. This limitation can be overcome by the use of multiple peptides that bind to different MHCI molecules, i.e. HLA-A1, A2, A3, or A11, and can be prepared as a stable mixture to be administered to patients that express one or more of these MHC molecules [75]. Nevertheless, the MHC subtype of the patient should be assessed in order to optimize the success of the peptide vaccination. Although the goal of administering peptide vaccines is to target MHCI molecules in APCs, when peptides are systemically injected, they can bind to non-professional APCs, which could lead to tolerance due to suboptimal costimulation. In addition, the half-life of peptides is very short upon *in vivo* injection, which can also lead to suboptimal antigen presentation, reducing the efficacy of the vaccine [75]. The fact that peptide vaccines possess a single immunogenic region minimizes cross-reactions and adverse effects, which are more likely when using whole antigens or tumor lysates [74]. However, targeting one or only a few tumor antigens could conduct to immunoediting, and the recurrence of a tumor that lacks the targeted antigen [76] or the outgrowth of an MHCI negative tumor [77].

2.2.2. DNA vaccines

DNA vaccine INO-1400 consists of a plasmid encoding the human telomerase reverse transcriptase (hTERT), a tumor-associated antigen, which is found in 85% of cancer cells, containing two immunogenic mutations. Upon intradermal vaccination with INO-1400, hTERT protein is expressed and activates the immune system to mount a cytotoxic T cell response against telomerase-expressing tumor cells, which may result in tumor cell death. INO-9012, a plasmid DNA vaccine encoding the human proinflammatory cytokine interleukin-12 (IL-12), activates the immune system by inducing NK cells, promoting interferon-gamma production and generating cytotoxic T cell responses against tumor cells. Clinical trial NCT02327468 is currently evaluating INO-1400 alone or in combination with INO-9012, which are delivered intramuscularly followed by electroporation once a month in breast cancer patients who are at high risk for recurrence.

Personalized polyepitope DNA vaccine is a strategy for breast cancer patients with persistent TNBC following neoadjuvant chemotherapy. This personalized vaccine is generated using the patient's own cancer cells to prepare naked plasmid DNA vaccines. The hypothesis of this proposal is that personalized polyepitope DNA vaccines are safe for human administration and able of produce measurable $CD8^+$ T cell responses to mutant tumor-specific antigens. To receive this vaccine, patients must have received chemotherapy prior to having surgery and still present some rest of the tumor remaining in the breast (NCT02348320).

PANVAC is a complex antitumor vaccine that consists of two viral vectors, i.e. recombinant vaccinia virus and recombinant fowl poxvirus, which encode for tumor-associated antigens mucin 1 (MUC-1) and carcinoembryonic antigens (CEA), as well as T cell costimulatory molecules, i.e. B7.1, intracellular adhesion molecule-1 and leukocyte function-associated antigen-3. After subcutaneous injection in combination with recombinant GM-CSF, this vaccine stimulates specific cytotoxic T lymphocyte responses in murine models of cancer [78]. An early phase trial in patients with metastatic breast cancer (NCT00179309) has shown that monthly administration of this vaccine in combination with docetaxel is well tolerated and doubles the progression-free survival when compared to chemotherapy alone (7.9 vs. 3.9 months) [79]. Although this was a relatively small study, these results are encouraging and warrant a larger randomized study.

2.2.3. DC vaccines

Another approach is the use of dendritic cell (DCs) vaccines, which can be loaded with tumor lysates [80], tumor antigens [81], or total tumor RNA [82]. There are several steps in the preparation of these vaccines: (i) precursor cells are isolated from the patient's peripheral blood, (ii) precursors are differentiated *ex vivo* into DCs, (iii) DCs are loaded with tumor lysates, peptides or RNA, (iv) antigen-loaded DCs are matured and activated with adjuvants, i.e. toll-like receptor (TLR) agonists, and (iv) DC vaccines are readministered into the patient. Until now, the only DC-based vaccine approved by the FDA is Sipuleucel-T (Provenge, Dendreon Corp., Seattle, WA) for the treatment of metastatic prostatic cancer [83].

DCs can be loaded with tumor associated antigens, *ex vivo* or *in vivo*. DCs pulsed with HER2/neu peptide [84] are being tested in an ongoing phase I clinical trial (NCT02063724). Among others, there are studies using DC vaccines loaded with MUC-1-derived peptides [85] or p53 peptides [86]. Although these approaches using specific peptides are promising, the possibility of generating tumor resistance by selection of an antigen negative population among the tumor cells could lead to resistance. Instead of individual peptides, several studies have used apoptotic tumor cells or have fused DCs with tumor cells. Breast cancer patients who received autologous DCs fused to tumor cells showed antitumor immune responses. However, this response was not enough to induce tumor regression [80,87].

The selection of appropriate DCs adjuvants will have a direct impact on their phenotype, the relative level of costimulatory molecules, the levels and profile of cytokine production and the half-life of the cell upon administration. Immature DCs have high capacity to capture, transport and process antigens and, when an inflammatory signal arrives, they undergo maturation [88]. It has been proposed that combination of different TLR to activate DCs can increase effector antitumor immunity [89]. Activation of TLRs allows the functional maturation to immunogenic DCs and the priming of naive T cells, and therefore is crucial at coupling innate and adaptive immunity [90]. However, the election of multiple adjuvants needs caution, as we have found that dual activation of TLR9 and TLR7/8 in murine and human DCs inhibits DC activation [91].

Although antitumor vaccines induce immunity in breast cancer patients, their efficacy has been lower than anticipated. The efficacy of antitumor vaccines could be improved by reducing tumor-associated immune dysfunction. It has been postulated that the endogenous immune response is insufficient to stop tumor progression due the presence of Tregs, Bregs, and MDSCs, as well as by the suppressor molecules present in the tumor microenvironment [92]. Thus, combination of antitumor DC vaccines with strategies that target Tregs or immunological checkpoints could improve antitumor immunity. In fact, combined activation of OX-40 and blockade of CTLA-4 enhanced the antitumor efficacy of HER-2 vaccines in breast tumor models, increasing TILs and upregulating IFN-γ production in $CD8^+$ and $CD4^+$ T cells [59] promoted DC maturation and proliferation and enhanced the efficacy of DC vaccines loaded with tumor antigen in murine models of breast cancer [93]. Since the clinical benefit of immunological checkpoint mAbs seem to depend on the preexistence of $CD8^+$ T cells in the tumor microenvironment [94], antitumor vaccination-mediated recruitment of TILs optimizes the response to checkpoint blockade. In turn, a persistent CTL response to antitumor vaccines is allowed by the blockade of immunological checkpoints, which inhibit T cell exhaustion. Thus, antitumor vaccines initiate an antitumor immune response that is then sustained by immunological checkpoint blockade [95]. These combination strategies remain to be validated in breast cancer patients.

2.3. Regulatory T cell blockade

Tregs are crucial at maintaining the peripheral immune tolerance [96]. Tregs have been involved in the immunological escape of tumors and their resistance to immunotherapeutic approaches. These cells have the ability to induce apoptosis and inhibit proliferation and maturation of antitumor effector T lymphocytes [96]. It has been described that cancer patients have increased levels of circulating Tregs compared with healthy donors. Moreover, circulating Tregs increase during tumor progression [21]. The presence of high proportion of Tregs among TILs, and a low ratio between $CD8^+$ T cells and Tregs have been associated with negative prognosis in breast cancer patients [22]. Due to their role in tumor immunological escape, Tregs have been the target of several therapeutic strategies to improve antitumor immunity.

Metronomic chemotherapy, which is the prolonged administration of low doses of conventional chemotherapeutic drugs, has been shown to deplete Tregs in cancer patients [97]. Extensive research has focused on the metronomic administration of cyclophosphamide (CP). Low doses of CP administrated orally to cancer patients result in lower circulating levels of Tregs [97]. A clinical study involving 12 patients with metastatic breast cancer showed that CP led to reduced levels of Tregs and increased levels of effector T cells for 4–6 weeks. However, the number of Tregs returned to basal levels even when CP administration was not finished [98]. Considering the good safety profile of CP metronomic therapy, this strategy is a very attractive option as maintenance

treatment for patients who underwent standard chemotherapy as first line of treatment. TNBC patients that received low doses of CP for 1 year exhibited increased survival compared to patients without maintenance therapy [99]. Dual metronomic chemotherapy with CP and other chemotherapeutic drugs, i.e. methotrexate or carboplatin, has been shown to improve the efficacy of antitumor vaccines in patients with metastatic breast cancer [100]. Although the occurrence of acute side effects in patients receiving metronomic chemotherapy is reduced when compared to standard chemotherapy [101], prolonged metronomic chemotherapy may lead to the accumulation of high total doses of chemotherapeutic drugs, which increases the risk of developing secondary malignancies, such as leukemia [102].

The systemic administration of daclizumab, a mAb against CD25, the *interleukin*-2 (IL-2) receptor [103] was able to reduce the number of Tregs in patients with metastatic cancer expressing carcinoembryonic (CAE) antigen [104]. Another strategy was the use of fusion proteins that combine IL-2 with diphtheria toxin, which reduced the number of Tregs in patients with metastatic renal cell carcinoma [105]. Administration of a humanized antibody anti-CD25 efficiently depleted circulating Tregs in patients with metastatic breast cancer and increased antitumor immunity induced by a peptide vaccine [106]. However, it is not clear whether this antibody depletes Tregs o inhibits their function. It is worthy to mention that an important disadvantage of this approach is that anti-CD25 mAbs can also deplete effector T cells, which transitory express CD25 during their activation [107,108].

Another strategy that can more specifically block Treg function is to target Foxp3, a transcription factor that is expressed by Tregs and is required to achieve its immunosuppressive function [109]. P60, a cell penetrating peptide that binds and inhibits Foxp3 has been developed to block Tregs [110]. P60 enters the cell, binds Foxp3 and inhibits its nuclear translocation. Mice-bearing CT26 tumors that were immunized with peptide AH1, a CT26 cell-associated peptide, and treated daily with P60 peptide exhibited stronger anti-AH1 peptide immunity [110]. We found that the efficacy of therapeutic DC vaccines loaded with tumor cell lysates was increased when it was used in combination with P60 peptide treatment in preclinical models of breast cancer [111]. It is important to consider that all the immunological approaches that involve Treg blockade entail a risk of immune-related side effects and autoimmunity that needs to be addressed before its translation to clinical oncology.

2.4. Adoptive T cell transfer therapy

Adoptive T cell transfer therapy (ACT) involves the purification of T cells from the patient, which are then genetically modified or chemically treated *ex vivo* to enhance their activity, followed by their reintroduction into the patient with the goal of inducing antitumor immunity (Figure 2). The first report of adoptive transfer of lymphocytes into mice was in 1955 to study the immunological response to tumor transplants [112]. The understanding of T cell function and the mechanisms that modulate their activation and maturation has led to the development of different techniques to successfully expand these cells *ex vivo*. The initial strategy was to digest or disaggregate tumor samples to isolate TILs. Then, TILs could be stimulated with different tumor cell lines, with autologous tumor cells or with tumor lysates. It was demonstrated that in one single sample of tumor there are different populations of

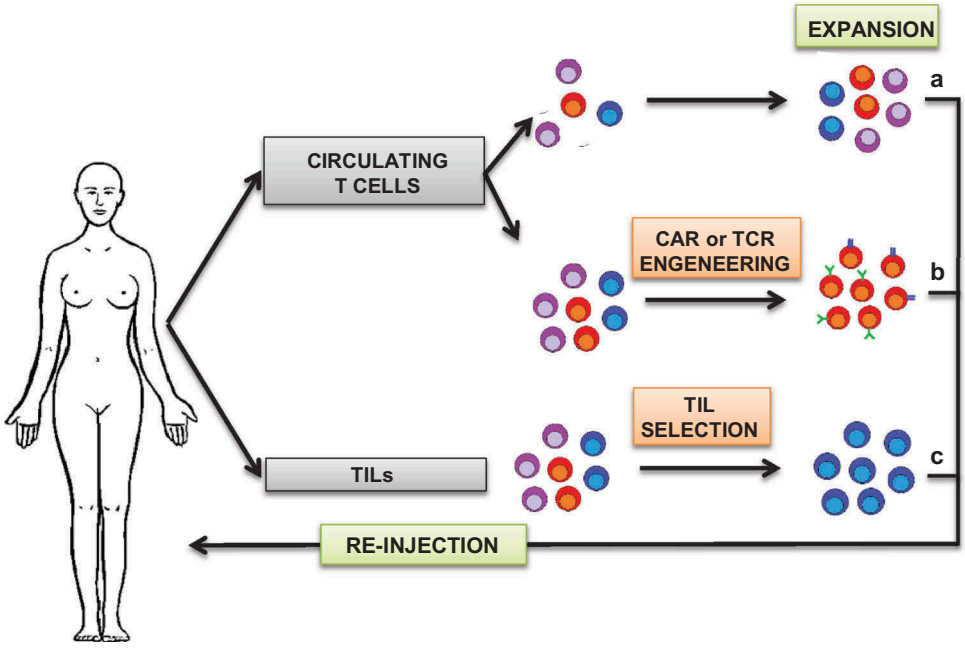

Figure 2. Adoptive T cell transfer therapies.
Circulating T cells or tumor infiltrating lymphocytes (TIL) are collected from the patient`s blood or tumor, respectively. For a non-specific treatment (a) T cells are expanded *ex vivo* and re-injected followed by the administration of high doses of IL-2. For specific antitumor treatment: (b) T cells are engineered to express chimeric antigen receptor (CAR) or T cell receptor (TCR) against specific tumor associated antigens (TAA), then expanded and re-injected into the patient, or (c) TILs are selected against specific tumor cells, expanded and re-injected.

lymphocytes with distinct antigenic specificities and phenotypically diverse populations [113]. Although in metastatic melanoma, clinical trials revealed that the administration of TILs conditioned *ex vivo* leads to tumor regression [114], this treatment have not proved clinical benefit in breast cancer patients [115].

An alternative strategy is the extraction of peripheral T lymphocytes to expand them *ex vivo* and re-infuse them into the patient in combination with the systemic administration of high doses of IL-2. Although this is a nonspecific immunotherapy because it does not utilize specifically enriched antigen-specific T cells, a single infusion of costimulated autologous T cells in the early posttransplant period accelerated the numerical and functional recovery of T cells in patients with advanced myeloma [116] and non-Hodgkin lymphoma [117]. Studies in breast cancer mouse models [118] and in metastatic breast cancer patients [119] revealed that the infusion of autologous T cells that were cocultured *ex vivo* with autologous DCs loaded with tumor lysate is feasible and well tolerated. This treatment led to an increase in tumor-specific memory T cells in peripheral blood in 44% of the patients, who exhibited a significantly longer median survival than nonresponders.

The advances in molecular biology and genetic engineering have led to the development of two new types of ACT. One of them is based on the genetic engineering of T cells that express a chimeric antigen receptor (CARs) composed of an antibody binding domain that target tumor antigens fused to T cell signaling domains. The second strategy consists on the genetic engineering of T cells with an antigen-specific T cell receptor α and β chains (αβTCR). In each case, T cells redirect their specificity to target tumor antigens. The major difference between them is the dependence on the expression of MHC on target cells. CARs are independent of MHC, since the associated antibody recognizes and binds tumor antigens on the cell surface. αβTCR-based antigen recognition is MHC restricted, i.e. target cells must have intact antigen processing and presentation and the treatment population must share a MHC allele. An advantage of TCR gene engineering is that it allows targeting both cell surface and intracellular antigens [120]. Diverse targets were proposed to target breast tumors, i.e. MUC-1-specific CAR T cells [121], dual targeting using CAR T cells against HER2 and MUC1 [122], or CAR T cells directed against folate receptor alpha, which are potent killers of TNBC cells *in vitro* and inhibit tumor growth in immunodeficient mice [123].

To improve the efficacy of this therapy, a lot of effort is invested in the recognition of tumor regression antigens, i.e. antigens which recognition results in tumor control, and predictive biomarkers [124]. On this last point, the next-generation TCR sequencing is a tool that could help characterize T cell infiltration and define predictive biomarkers [125]. ACT is a highly personalized treatment and the selection of the different approaches will depend on each case and the capability to afford the high cost of the treatments. These complex therapies require new developments for each patient, with weeks of cell culture, skilled manhours, and patient preparation [114].

2.5. Adoptive immunotherapy

HER2-positive breast cancers have been extensively targeted with antibody-based therapeutic strategies. Trastuzumab, the first recombinant humanized IgG monoclonal antibody specifically created to target tumor cells that overexpress HER2, received FDA approval in 1998. Trastuzumab binds and blocks HER2 pathway on tumor cells, leading to an inhibition of the MAPK and PI3K/Akt pathways, which suppresses tumor growth and proliferation [126]. In addition, tumor cells opsonized with trastuzumab can be recognized and killed through antibody-dependent cellular cytotoxicity by NK cells [127]. Addition of trastuzumab to chemotherapy significantly improves progression-free survival and overall survival and has become standard of care in HER2+ breast cancer patients [128].

Pertuzumab is a humanized monoclonal antibody IgG type that was originally approved to be used in combination with trastuzumab and chemotherapy in patients with HER2+ metastatic cancer. In the CLEOPATRA clinical trial [129], treatment with pertuzumab combined with trastuzumab and docetaxel showed a statistically significant reduction of 38% risk of death in patients with metastatic breast cancer compared with those who received standard therapy [130].

Trastuzumab emtansine (TDM-1) is the first of a new class of drugs called 'antibody-drug conjugate,' which combines the efficacy of a monoclonal antibody (trastuzumab) with the cytotoxic power of chemotherapy (emtansine). The trastuzumab component of the conjugate binds specifically HER2, which is internalized and the chemotherapeutic agent is released within the cell, so that is not only more effective but it is also well tolerated, reducing the incidence of adverse effects. Approval of TDM1 was based on the results of the EMILIA study, an international phase III clinical trial in which HER2+ locally advanced or metastatic breast cancer patients exhibited longer survival and experienced fewer adverse events than those that received standard second-line treatment [131].

2.6. Immunotherapy and chemotherapy

Chemotherapy has been traditionally considered to exert an immunosuppressive effect [132] and its efficacy has been thought to rely only on a direct cytotoxic effect on tumor cells. However, in recent years a growing body of evidence demonstrated that chemotherapy can trigger immunological changes. It has been described that chemotherapy can lead to immunogenic cell death, which increases the availability of proinflammatory molecules in the tumor microenvironment. During immunogenic cell death, a number of alterations in the composition of the plasma membrane occur, leading to exposure in the cell surface of calreticulin and heat-shock protein 90 (HSP90) and to the release of intracellular proinflammatory molecules, i.e. HSP70, ATP and nuclear protein high mobility group box 1 (HMGB1) [133]. These molecules act as TLR ligands, inducing activation of DCs in the tumor microenvironment, facilitating cross-priming and tumor-specific T cell clonal expansion [134]. Ligation of TLR4 by HMGB1 has been proposed to play an important role in the efficacy of chemotherapy, as breast cancer patients with TLR4 loss-of-function alleles have shown poor prognosis after adjuvant chemotherapy [135]. In addition, a recent study in over 1,700 breast tumor specimens indicates that the presence of HMGB1 together with signs of

autophagy were positive predictors for longer survival in breast cancer patients treated with anthracycline-based adjuvant chemotherapy [136].

Many chemotherapeutic agents commonly used to treat breast cancer seem to exert immune mediated antitumor effects. For example, in breast cancer patients taxanes and docetaxel administration increased intratumor T cell infiltration [137,138]. In addition, the presence of IFN-γ-producing TILs, as well as a higher ratio of $CD8^+$ TILs vs. Tregs predicts favorable responses to chemotherapy in breast cancer patients [135]. In preclinical tumor models, doxorubicin induces the expression of costimulatory molecules in circulating $CD4^+$ T cells [139] and decreases the content of immunosuppressive TAMs within MMTV-neu mammary tumors [140]. Cyclophosphamide treatment has been shown to increase antigen presentation and cytokine secretion in endogenous DCs and to partially inhibit the suppressor activity of Tregs in mice [141]. In the breast cancer tumor model 4T1, early *gemcitabine* treatment significantly inhibited tumor growth, reduced splenomegaly, and significantly decreased the proportion of MDSCs in the spleen [142]. More importantly, breast cancer patients who received paclitaxel or docetaxel showed increase in serum IFN-gamma, IL-2, IL-6, GM-CSF levels and enhancement in circulating NK and LAK activity, while both agents led to decreased circulating levels of acute phase cytokines IL-1 and TNF-alpha [137]. This is very important when designing strategies that combine chemotherapeutic drugs and immunotherapy, since IFN-gamma upregulates PD-L1 expression in tumors, rendering them sensitive to immunological checkpoint blockade. In fact, it has been recently reported that chemotherapy triggers T cell infiltration in tumor models that otherwise lacked TILs, and sensitizes them to immunological checkpoint inhibition, leading to durable therapeutic responses [143]. These findings indicate that immunological checkpoint blockade could benefit a larger cohort of patients than hitherto anticipated.

3. Conclusion

In recent years, immunotherapy has emerged as a powerful tool to fight against breast cancer. Several clinical trials are undergoing to evaluate multiple immunotherapeutic strategies, such as the use of monoclonal antibodies that block immunological checkpoints or that target tumor associated antigens, the administration of antitumor vaccines or engineered autologous T cells. Combination of these novel strategies seem to be boosted rather than inhibited when combined with routine chemotherapy. Thus, immunotherapeutic strategies, which have been traditionally neglected for the treatment of breast cancer patients, arise as new and versatile tools for the treatment of this disease.

4. Expert opinion

Breast cancer has traditionally been considered a nonimmunogenic tumor and, thus, the development and application of immunotherapeutic approaches for this disease have been delayed for years. However, a robust body of evidence has shown that the lack of immune responsiveness is functional and seems to rely on the mechanisms of immunological escape that these tumors develop. Breast tumors exhibit high levels of CTLA-4, PD-1 and PD-L1, IDO, and tumor infiltrating Tregs, MDSCs and TAMs, all of which constitute therapeutic targets that could improve antitumor immunity and that are indeed exploited as therapeutic targets for other tumors.

The fact that breast tumors exhibit many immunosuppressive mechanisms opens the windows to combine therapies that synergize, i.e. antitumor vaccines and Treg inhibition or blockade of immunological checkpoints. Considering that the repertoire of immunosuppressive mechanisms seem to vary between different patients and that each therapy is costly and could entail adverse effects due to the suppression of systemic immunological tolerance, it is important to characterize which therapeutic approaches could help each patient. Thus, research needs to also focus on the detection of biomarkers that could predict efficacy of each treatment or therapeutic cocktail. In addition, characterization of the set of tumor antigens present in each individual tumor specimen will be required to optimize the construction of personalized, more robust and specific antitumor cellular therapies, i.e. vaccines and ACT. Targeting multiple antigens it is crucial when using these strategies, not only to improve the initial rejection of the tumor, but also to prevent antigen loss due to selection pressure and increase the chance of developing immunological memory to protect the patient against metastasis and tumor recurrence.

Although chemotherapy was traditionally considered immunosuppressive and inappropriate to use in combination with immunotherapy, extensive research has proved this dogma wrong and has shown that chemotherapy improves the efficacy of many immunotherapeutic approaches in cancer patients. This reminds us that science cannot be ruled by dogmas, but by empiric evidence. Fortunately, undergoing clinical trials are testing the combination of chemo and immunotherapy, which could not only improve antitumor immunity, but could also help reduce the doses or the length of chemotherapy to diminish the severity of side effects associated to this treatment.

It is worth to mention that immune cells from cancer patients could exhibit different phenotypes than those from healthy donors. It has been shown that endogenous DCs and T cells from patients with cancer cannot perform their effector functions correctly due to the characteristic immunosuppressive milieu. This issue can be overcome allowing DC or T cell expansion and activation *ex vivo*. However, it remains controversial whether leukocytes obtained from these patients are optimal for DC and T cell expansion and more important, whether these cells would be optimal to develop antitumor immunity. Although this issue could be surpassed by preparing these vaccines using PBMCs from compatible healthy donors, these questions need to be assessed.

In summary, extensive evidence demonstrates that breast cancer is an excellent candidate for immunotherapy and encourages the prompt evaluation of immunotherapeutic approaches that are already used in other types of cancer without further delay.

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