



# CANCER THERAPY



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# The Era of Immunotherapy in Breast Cancer

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## Inhibitory Immune Checkpoints

The development of chronic infections and cancer is facilitated by a variety of immune subversion mechanisms, such as the production of anti-inflammatory cytokines, induction of regulatory T (Treg) cells and expression of immune checkpoint molecules, including CTLA-4 and PD-1, Lymphocyte-Activation gene-3 (LAG-3), and T-Cell Immunoglobulin-3 (TIM-3) [1,2]. These regulatory immune checkpoints are often enhanced during cancer therefore become a very important therapeutic target in the treatment of cancer and also, have potential to enhance the efficacy of cancer and infectious disease vaccines [3]. Indeed, antibodies binding to CTLA-4, PD-1, or PD-L1 have shown remarkable efficacy, especially in combination therapies [1]. Immune checkpoints have distinct ligands and suppress T-cell function through multiple mechanisms.

CTLA-4 blockade allows for activation and proliferation of more T-cell clones, and reduces Treg-mediated immunosuppression. PD-1 pathway blockade restores the activity of antitumor T cells that have become quiescent. A dual pathway blockade could have a synergistic effect, resulting in a larger and longer lasting antitumor immune response.

### CTLA-4

CTLA-4 was one of the first inhibitory receptors shown to play a role in suppression of T-cell responses [4]. Two signals are

required for T-cell activation: recognition of antigen presented by MHC class I and co-stimulation through CD28 following binding to CD80 (B7-1) or CD86 (B7-2) expressed by Antigen Presenting Cells (APCs). CTLA-4 is structurally similar to CD28 so acts as a false substrate and binds to CD80 or CD86 at a higher affinity than CD28. It has been suggested that CTLA-4 expression interferes with T-cell activation by reducing CD28 co-stimulation [5]. An alternative way for CTLA-4, it can be secreted by T-cells and exerts its inhibitory function independent of cell-cell-interactions. CTLA-4 is constitutively expressed on Treg cells, but can be expressed on effector T-cells following their activation [6,7]. However, unlike Treg cells, which express CTLA-4 on their cell surface, activated T cells mainly express CTLA-4 intracellularly. This differential expression suggests a dual function of CTLA-4. Expression of CTLA-4 by Treg cells serves as a mechanism of Treg cells to suppress excessive T-cell responses. Other mechanisms of CTLA-4-mediated suppression of T-cell activation include an increase in T-cell motility, inhibition of TCR and CD28, and reduction in CD25 expression [8]. It has also been shown that high CTLA-4 expression on CD4+ cells is crucial for the suppressive function of Treg cell [9]. Overexpression of CTLA-4 has been associated with worse prognosis and higher clinical stages in patients with breast cancer [10].

### Clinical Application

The suppression of anti-tumor T-cell responses is largely me-



diated by Treg cells and immune checkpoints, such as CTLA-4. Immune checkpoint inhibitors have been licensed for use in humans with cancer, notably melanoma, and are predominantly monoclonal antibodies against immune checkpoints on immune cells that block the interaction with the respective checkpoint ligands. The primary aim of immune checkpoint blockade is to reduce suppression of effector T cells, especially CD8+ T cells, and thereby improve tumor-specific immune responses. Moreover, immune checkpoint blockade has the potential to enhance the effectiveness of other immunotherapeutic approaches, such as cancer vaccines. CTLA-4 was the first immune checkpoint that was targeted in the treatment of cancer. Preclinical studies demonstrated that blocking of CTLA-4 reduced tumor growth in mouse models of melanoma, colon carcinoma, and many others [11]. The efficacy of CTLA-4 therapy in preclinical models was associated with increased T-cell infiltration into tumors [11]. Based on these findings, antibodies targeting CTLA-4 were tested in clinical trials. One of these antibodies, ipilimumab, was the first checkpoint inhibitor to be approved in 2011 by the U.S. Food and Drug Administration (FDA). It was approved for the treatment of metastatic melanoma and has shown a high survival rate among the patients up to 10 years [12,13].

### PD-1/PD-L1 pathway

The Programmed cell Death-1 receptor (PD-1) is an immune checkpoint inhibitor which is expressed on the surface of immune effector cells such as B cells, T cells and NK cells. PD-1 has two known ligands, PD-L1 (also known as B7-H1 and CD274) and PD-L2 (also known as B7-DC and CD273), which have distinct expression profiles with PD-L1 being expressed on several tumor types [14]. It is activated mainly by PD-L1 which can be expressed by all human cells. PD-1 expression on T-cells is induced by antigen stimulation [3]. In cancer, the expression of PD-L1 is one of the major immune escape mechanisms [15]. PD-1 expressed on T-cells can bind PD-L1 (CD274) and PD-L2 (CD273), typically expressed by APCs or tumor cells. PD-1 ligation interferes with TCR downstream signaling events that suppresses T-cell function and can lead to T-cell exhaustion [16]. The PD-1/PD-L1 checkpoint is a dominant immune pathway operative in the Tumor Microenvironment (TME). PD-1 does not induce cell death directly but reduces cell growth factors as well as survival signals. Activation of PD-1 by PD-L1 or PD-L2 induces down regulation of T-cell activity, reduced cytokine production, T-cell lysis and induction of tolerance to antigens [17]. Tumor expresses PD-L1 as a response to pro-inflammatory cytokines produced by the Tumor-Infiltrating lymphocytes (TILs) [18]. PD-L1 is regulated by Micro-RNAs through binding to the 3' untranslated region resulting in m-RNA translation blockade [19]. PD-L1 expression by tumors has been associated with mechanisms of developing immune resistance. Tumors express PD-L1 as a response to pro-inflammatory cytokines such as IFN- $\gamma$ , IL-4 as well as IL-10 produced by the Tumor-Infiltrating Lymphocytes (TILs) [20]. Therefore, blocking PD-1 signaling can induce an anti-tumor response.

### PD-L1 in cancers

It appears that upregulation of PD-L1 may allow cancers to evade the host immune system such as pancreatic cancer, gastric cancer, ovarian cancer and Inflammatory Myofibroblastic Tumors (IMTs) by creating immunosuppressive tumor microenvironment. Inflammatory myofibroblastic tumors are rare mesenchymal neoplasms that are composed of myofibroblastic cells accompanied by inflammatory infiltrate. A recent study investigated the immune profiles of IMTs, including PD-

L1 expression and all samples revealed that PD-L1 expression is extremely elevated [21]. Non-Small Cell Lung Cancer (NSCLC) is one of the most prevalent cancers and is responsible for a large proportion of all cancer-related deaths. It was proved that PD-L1 expression is relatively high in NSCLC and associated with poor prognosis [22]. Regarding ovarian cancer, PD-L1 was found variably expressed in the cytoplasm and on the cell surface. Moreover, the release of cytokines such as IL-10 and IL-6 from Tumor Associated-Macrophages (TAM) stimulated the expression of PD-L1 on the surface of cancer cells [23]. A study indicated that positive PD-L1 expression was correlated with a poor overall survival outcome in pancreatic cancer patients [24]. PD-L1 expression was markedly increased in gastric cancer patients and correlated with poor prognosis [39]. Multiple solid tumor types including melanoma, Renal cell carcinoma, thymoma and colorectal cancer express PD-L1 to generate an immunosuppressive tumor microenvironment and avoid T-cell cytotoxicity [26]. Moreover, PD-L1 expression is prevalent in many human cancers as head and neck, cervical, hepatocellular carcinoma and acute myeloid leukemia [27,28].

### PD-L1 in Breast cancer

PD-L1 immunohistochemistry was performed on 215 Triple Negative Breast Cancers (TNBCs), it was found that PD-L1 positive breast cancer patients had significantly longer disease-free survival and overall survival compared with PD-L1 negative patients [29]. A study was conducted on breast cancer patients with LAG-3+TILs, it was found that 53% of PD-1+ and 61% of PD-L1+ cases were also positive for LAG-3+TILs. Concurrent infiltration of LAG-3+ and CD8+TILs was significantly associated with increased Breast Cancer-Specific Survival (BCSS) [30]. Six studies that included 7877 cases were selected for the analysis. Higher PD-L1 expression in all cells was related to higher histological grade and lymph node metastasis. Higher PD-L1 expression in tumor cell was related to larger tumor size, estrogen receptor negativity, progesterone receptor negativity, human epidermal growth factor type-2 positivity, and triple negative breast cancer. PD-L1 positivity in all cells was associated with poorer disease-free survival, although it was not significantly associated with overall survival [31]. PD-L1 is expressed by approximately 20% of breast cancers. TNBC and HER2-positive breast cancers expressing higher levels of expression than ER-positive breast cancers (33%, 56% and 11%, respectively) [32]. While the prognostic significance of PD-L1 expression in breast cancer remains unclear, the greater the response to PD-1 and PD-L1 blockade in PD-L1 positive cancers led to the interest in investigating immune checkpoint inhibitors for breast cancer. Furthermore, a study demonstrated that single-cell RNA sequencing analysis suggests that the myeloid lineage is a significant source of HO-1 expression, and is co-expressed with PD-L1/2 in human breast tissues [33]. Recently, a study showed that Crk adapter protein which is involved in cell migration and invasion, promotes PD-L1 expression and immune evasion in triple negative breast cancer [34]. PD-L1 expression levels were significantly higher in the lymphocytes and tumor cells of the lymph node metastasis than in the primary tumors of TNBC [35]. Another study showed that the up-regulation of the transmembrane mucin MUC1-C contributes to immune escape in an aggressive form of breast cancer through the induction of PD-L1 [36]. A study revealed that PD-1/PD-L1 inhibition may increase chemotherapy efficacy by inhibiting the MDR1/P-gp expression in breast cancer cells through MAPK/ERK pathway [37]. Correlation analysis of PD-L1 expression and prognosis in TNBC was performed. PD-L1 expression rate was 34.5% in tumor cells and was 62% in TILs. The

PD-L1 expression in tumor cells was positively correlated with tumor size, ki-67, TILs [38].

### Clinical Application

PD-1 and PD-L1 blocking antibodies have been tested and remarkable responses were observed for patients with melanoma and NSCLC. To date, three antibodies have been approved by the FDA that targeting the PD-1: PD-L1 axis [1]. The first antibody targeting PD-1 pembrolizumab was approved in September 2014 for the treatment of advanced melanoma in patients failing other treatments and later became the first-line treatment [39]. Pembrolizumab is now approved for NSCLC [40]. A second PD-1-blocking antibody “nivolumab” has been approved for the treatment of melanoma, NSCLC and renal cancer [41]. Moreover, “atezolizumab” is an anti-PD-L1 antibody which has been approved by the FDA in May 2016 for the treatment of bladder cancer [42]. A recent study reported that patients that the combination of anti-PD-1 (Nivolumab) with anti-CTLA-4 (ipilimumab) induced significant responses rather than monotherapy [43]. This suggests that CTLA-4 blockade increases the pool of activated T cells in the lymph nodes, but does not prevent immune suppression in the tumor microenvironment whereas PD-1 blocking antibodies prevent T-cell exhaustion at the tumor site. Although checkpoint inhibitors are proving successful in the treatment of certain cancers, they are associated with frequent side effects particularly autoimmune syndromes including, colitis and hepatitis [41]. Blockade of CTLA-4 and PD-1 induced similar adverse effects however high grade adverse effects were less significant in patients treated with pembrolizumab when compared with ipilimumab [44-46].

### Immunotherapy

The idea of exploiting the host's immune system to treat cancer dates back decades and relies on the insight that the immune system can eliminate malignant cells during initial transformation [47]. Immunotherapies against existing cancers include various approaches ranging from stimulating effector mechanisms to counteracting inhibitory and suppressive mechanisms [48]. Strategies to activate effector immune cells include:

a) Vaccination with tumor antigens or augmentation of antigen presentation to increase the ability of the immune system to produce immune response against neoplastic cells. Unfortunately, the general lack of understanding of the mechanisms of immunization, and particularly of the role of Dendritic Cells (DCs) has led to a series of failures of therapeutic cancer vaccines because DCs are known to be the most effective APCs and play a pivotal role in coordinating innate and adaptive immune responses [49-51]. Therapeutic vaccines elicit an immune response against tumor-specific or tumor-associated antigens. Several trials of vaccines are currently enrolling patients: a therapeutic vaccine made from a human bladder cancer cell line that has been irradiated and engineered to express soluble gp96, a chaperone protein being used in phase II of the trial that is currently enrolling patients with high-risk, non-muscle invasive bladder cancer who have completed surgery. A phase I study is testing a fusion protein vaccine with or without the biological therapy sirolimus in patients with a variety of solid tumors, including recurrent and metastatic bladder cancer [52]. Several trials of vaccines, given alone or with other therapies, are currently enrolling breast cancer patients: NeuVax is under investigation to prevent breast cancer recurrence among patients with HER2 1+ and 2+ following surgery. GVAX, a therapeutic vaccine made from breast cancer cell lines irradiated and

engineered to express the immune molecule GM-CSF, is being tested in a phase II trial [53].

b) Oncolytic Virus (OVs) immunotherapy represents a novel form of cancer therapy that employs native or engineered viruses that selectively replicate in and kill cancer cells [54]. OVs are believed to promote antitumor responses mainly through two distinct mechanisms of action: acute tumor debulking owing to tumor cell infection and lysis and induction of systemic antitumor immunity [55]. The increased interest in employing viruses for the treatment of cancer is the fact that the viral genome can be modified to augment antitumor activity and attenuate pathogenicity [56]. Numerous modifications that have been developed include the insertion of promoters that restrict the expression of virulence genes to tumor cells or the deletion of pathogenic genes to limit the growth and lytic activity of viruses to cancer cells [57,58]. Additionally, OVs can be engineered to express specific cytokines that favor immune cell recruitment and activation or to produce co-stimulatory molecules on tumor cells so facilitate the generation of T-cell activating signals [59]. Numerous viruses have been tested as vectors, some are naturally non-pathogenic such as paramyxovirus and picornavirus, and others are genetically manipulated such as herpes simplex virus [60]. The most advanced agent in clinical development is (T-VEC) which has been recently approved by the FDA for the treatment of melanoma resulting in enhancing antigen presentation and increasing oncolytic therapeutic activity [59,61].

c) Adoptive Cell Therapy (ACT) is a promising form of immunotherapy which invests the antitumor properties of lymphocytes to eradicate tumor cells [62]. ACT is an approach where T-cells generally mixtures of CD8+ and CD4+ T-cells grown from metastatic tumor deposits are harvested and expanded prior to adoptive transfer [63]. This approach reverses the functional impairment of the tumor-specific T-cells [64]. Adoptive Cell Therapy in colorectal cancer includes: Tumor-Infiltrating Lymphocytes (TILs) in metastatic digestive tract cancers; T cells engineered to target VEGFR in patients with metastatic cancer; T cells engineered to target MAGE-A3 in patients with metastatic cancer that expresses MAGE-A3; T cells targeting EGFR in patients with advanced cancer, including colorectal cancer; Natural Killer (NK) cells, important innate immune cells, in patients with advanced cancer [65].

d) One approach to trigger antitumor immune responses has been termed “checkpoint blockade”, referring to the blockade of immune-inhibitory pathways activated by cancer cells. CTLA-4, an inhibitory receptor that down-regulates the initial stages of T-cell activation, was the initial target for checkpoint antibodies [66]. Multiple other immune checkpoint pathways that could be the target of novel therapies have been identified such as Lymphocyte Activation Gene 3 (LAG3) and T-cell immunoglobulin 3 (TIM3) [67]. A promising clinical research in breast cancer is the use of immune checkpoint inhibitor which works by targeting molecules that serve as checks in the regulation of immune responses and block inhibitory molecules or activate stimulatory molecules. There are some phase I/II studies that enrolled patients with breast cancer in different stages of the disease, for treatments with indoximod, an IDO inhibitor (IDO is expressed by a number of tumor types and correlates with poor prognosis), an anti-OX40 antibody (OX40 is a costimulatory molecule expressed after T cell activation that enhances T cell survival and anti-cancer effector function) and an anti-PD-L1 checkpoint inhibitor [52].

f) Cytokines are messenger molecules that are able to help

controlling the growth and activity of immune system cells. Monoclonal antibodies are molecules generated in the laboratory that can target specific antigens on tumors. Combining them seems to be a good immunologic treatment. There is a fusion of the cytokine Interleukin-2 (IL-2) and an antibody that recognizes peptides on the surface of the tumor cells that was studied in clinical trials. Treatment with IL-2 can enhance the activity of the immune system against tumors and, by linking IL-2 to the antibody, ALT-801 can target IL-2 to cancer cells [68]. IL-2 cytokine used in solid tumors like melanoma were discovered [52].

g) Monoclonal Antibodies (mAbs) are generated in the lab and target specific antigens on tumors. Many antibodies are currently used in cancer treatment. New antibodies are tested in breast cancer: glembatumumab vedotin - an antibody-drug conjugate used in patients with advanced triple-negative breast cancer with cells that produce a protein called glycoprotein NMB, margetuximab, an anti-HER2 antibody used in patients with relapsed or refractory advanced breast cancer.

### Treatment of Breast Cancer

Selection of breast cancer treatment is based on the stage and type of the tumor. Early stages BC can be treated by surgical removal called "mastectomy". There are two types of surgery, the first one called "modified radical mastectomy" which means complete removal of the whole breast and many of lymph nodes while the second type is called "breast conserving surgery" at which resection of tumor takes place and some normal tissue around it. Even after surgical procedures, recurrence may occur, so "adjuvant therapy" given after surgery such as chemotherapy or radiation therapy to reduce the risk of relapse. Moreover, chemotherapy may be given before surgery because it will shrink the tumor and reduce the amount of tissue that needs to be removed, such treatment is called "neoadjuvant or preoperative therapy" [69].

### Treatment of Estrogen receptor-positive breast cancer

Estrogen Receptor (ER) expression is the main indicator of potential responses to Endocrine Therapy (ET), and approximately 70% of human Breast Cancers (BCs) are hormone-dependent and ER-positive [70]. In patients with BC, the introduction of adjuvant systemic therapy led to a significant improvement in post-surgical survival and a reduction in disease relapse. ET may be received alone or in combination with cytotoxic therapy [71,72].

Adjuvant ET currently consists of (i) ovarian suppression, (ii) Selective Estrogen Receptor Modulators (SERMs), and (iii) Aromatase Inhibitors (AIs).

(i) **Ovarian suppression:** In patients with ER+ tumors ovarian suppression with gonadotropin-releasing hormone agonists, such as goserelin and triptorelin in combination with standard adjuvant therapy is generally more effective than adjuvant chemotherapy alone [73,74]. The continuous administration of GnRH agonists lead to decreased gonadotropin and estrogen levels but the normal hypothalamic-pituitary axis usually recovers after the treatment is stopped [75]. It has been shown that in premenopausal women with BC who did not receive chemotherapy, OS may reduce the recurrence and mortality rate by 25% [76].

(ii) **SERMs:** ERs are nuclear proteins that act as transcription factors regulating the expression of estrogen-responsive genes

and SERMs are drugs that block signaling at the level of ERs by binding to them and inhibit the DNA synthesis such as tamoxifen, raloxifene and toremifene [77]. Tamoxifen is the prototype of SERMs and it acts as ER antagonist (competitive inhibitor) on breast but acts as a partial agonist on uterus, liver and other tissues. In patients with ER+ tumors, 5 years of tamoxifen reduces the annual BC death rate by 31% [78]. Usually patients do not benefit from treatment longer than 5 years, but more recent studies suggest stopping tamoxifen administration after 7 or 10 years [79]. Toremifene is similar to tamoxifen in estrogenic effects but induces less positive effects on the bone. Moreover, it is completely cross resistant with tamoxifen. Raloxifene is another SERM approved by the FDA that causes less toxicity, including reduced thromboembolic events and endometrial cancer risk [80]. Several studies confirmed the efficacy of raloxifene in preventing invasive BC in postmenopausal women by reducing the risk by 38% but showed that it has similar efficacy to tamoxifen as adjuvant therapy [81]. Additionally, Selective Estrogen-Receptor-Downregulators (SERDs) are distinguishable from SERMs both pharmacologically and in terms of their molecular activity [82]). They have more antagonistic profile than SERMs and are capable of down-regulating ER and inhibiting the growth of tamoxifen-resistant BC cells [83]. SERDs are selective ER antagonists and have a pure anti-estrogenic effect by binding to intracellular ERs and accelerate their degradation [75]. Fulvestrant is currently the only SERD approved for the treatment of BC [84]. Endocrine resistance is a serious challenge because 30% of ER+ tumors do not respond to tamoxifen.

(iii) **Aromatase Inhibitors (AIs):** They act by blocking aromatase enzyme activity and thus reducing the circulating levels of estrogen. They represent an effective alternative therapy to SERMs [85]. A recent study found that following combination of Ovarian Suppression (OS) with AI (anastrozole), complete or partial response was obtained in 70.4% of patients compared with 50.5% of those treated with OS and tamoxifen [86].

### Treatment of HER2+ breast cancer

The Human Epidermal growth factor Receptor 2 gene (HER2) is overexpressed in approximately 15% of BC patients. It is of great scientific research interest as HER2 overexpression is associated with worse prognosis in the absence of therapy [87]. Four separate HER2-targeted agents (trastuzumab, pertuzumab, ado-trastuzumab and lapatinib) have been approved for the treatment of HER2-positive BC patients. First line immunotherapeutic agent is "trastuzumab" which is a humanized monoclonal antibody against HER2 has been approved to be combined with chemotherapy. There was a significant improvement in survival and response rate [88]. Administration of trastuzumab along with anthracyclines results in high risk of cardiotoxicity. Pertuzumab was approved by the FDA in 2012, it targets HER2 dimerization. Combination of pertuzumab along with trastuzumab and docetaxel considered as a first line therapy in metastatic breast cancer patients. Additionally, lapatinib is a dual Tyrosine Kinase Inhibitor (TKI), blocking HER2 tyrosine kinase activity by binding to the ATP-binding site of the receptor's intracellular domain so this results in the inhibition of tumor cell growth. In patients, lapatinib is well tolerated with low grade adverse effects. In 2007, lapatinib has been approved in combination with capecitabine in patients with advanced HER2-positive breast cancer. In 2010, the approval was extended to the treatment of post-menopausal women with advanced HER2-positive BC [89].

### Treatment of basal-like breast cancer

This type is also known as Triple Negative Breast Cancer (TNBC). It is defined by negative immunohistochemical staining for ER and PR and lack of HER2 overexpression. TNBC is associated with aggressive pathologic features and poor clinical outcomes [90]. Patients with TNBC do not benefit from hormonal or trastuzumab-based therapy due to the loss of target receptors. Surgery and chemotherapy, individually or in combination, appear to be the only available options. Traditionally radiotherapy is given in TNBC following mastectomy but there is still controversy on this issue as TNBC are rapidly growing and locally aggressive cancers [91]. Targeted therapies for TNBC remain elusive and chemotherapy remains the only systemic treatment option. Potential targets include Poly ADP-Ribose Polymerase (PARP1) inhibitors, Epidermal Growth Factor Receptor (EGFR) inhibition, angiogenesis inhibitors as Vascular Endothelial Growth Factor (VEGF) inhibitor, PI3K/mTOR pathway inhibitors, apoptotic pathways inhibitors, Heat Shock Protein pathway 90 (HSP90) and immune checkpoint inhibitors [92-94]. This sheds the light on PD-1/PD-L1 checkpoint in treatment of breast cancer.

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