Life Sciences 247 (2020) 117437

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

PD-L1/PD-1 axis as a potent therapeutic target in breast cancer

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ARTICLE INFO

Keywords:
PD-L1
PD-1
Breast cancer
Immunotherapy
Immune checkpoint

ABSTRACT

Although both the incidence and the mortality rate of breast cancer is rising, there is no potent and practical option for the treatment of these patients, particularly in advanced stages. One of the most critical challenges for treatment is the presence of complicated and extensive tumor escape mechanisms in the tumor microenvironment. Immune checkpoint molecules are of the main immunosuppressive mechanisms used by cancerous cells to block anti-cancer immune responses. Among these molecules, PD-1 (Programmed cell death) and PD-L1 (programmed cell death-ligand 1) have been considered as worthy therapeutic targets for breast cancer therapy. In this review, we intend to discuss the immunobiology and signaling of the PD-1/PD-L1 axis and highlight its importance as a worthy therapeutic target in breast cancer. We believe that the prognostic value of PD-L1 depends on the breast cancer subtype. Moreover, the combination of PD-1/PD-L1 targeting with immune-stimulating vaccines can be considered as an effective therapeutic strategy in breast cancer.

1. Introduction

As one of the most prevalent cancers, breast cancer is the leading reason for cancer-related fatality among females worldwide. According to literature, the global incidence of female breast cancer is estimated to be near 3.2 million annually by 2050. In terms of pathological features, breast cancer is comprised of various mixed carcinomas; for instance, some of them display a low growth rate with considerable prognostic capacity, whereas others show aggressive characteristics [1]. Currently, for reasons like categorization, prognosis, and treatment in breast cancer, some factors should be considered, such as the stage of the lymph node, histological grading of the tumor, and the stage of the tumor. Besides, some tumor markers need to be evaluated, which include human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and estrogen receptor (ER) [2–4]. Breast cancer occurrence has grown continuously over the past years; however, due to essential advances in therapeutic approaches, related deaths have decreased. Chemotherapy, targeted therapy, radiotherapy, and surgery, as well as hormone therapy, are well-established strategies for cancer treatment [5–7]. Since these approaches have not shown acceptable outcomes in sufferers, lately, the immune-based therapies have been reconsidered. In recent years, notable advances have been observed in cancer treatment via immunotherapy and authenticated immune agents encompass tumor vaccines, chimeric antigen receptor (CAR) T cells,

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https://doi.org/10.1016/j.lfs.2020.117437
Received 2 October 2019; Received in revised form 3 February 2020; Accepted 14 February 2020
Available online 16 February 2020

monoclonal antibodies, cytokines, and immune adjuvants [8,9]. Among them, accreditation and enhancement of immunomodulators (inhibitors of immune checkpoints) are of considerable importance in the clinic. Monoclonal antibodies (mAb) move toward inhibitors of immune checkpoints on immunological cells in order to trigger the reduction of other immune responses [10]. Programmed cell death 1 (PD-1) is a regulatory molecule, which selectively binds to programmed death-ligand 1 (PD-L1) and has been developed for utilization in tumor therapies in recent years. Two negative co-stimulatory moieties in PD-1 and its ligand can deactivate T cell functions and provide a safety mechanism for tumor cells against the immune system [11]. The expression of PD-L1 correlates with the prognostic aspect of various tumors, including non-small-cell lung cancer, gastric cancer, and breast cancer [12]. According to investigations, a negative correlation has been observed between the expression of PD-L1 and the survival rate of sufferers. The immune system likely has more influence on the progression of tumors due to the higher production of PD-L1 in patients. Therefore, immune-based approaches seem to have higher efficacy, particularly anti-PD-1/PD-L1 targeted approaches [13].

2. PD-L1/PD-1 axis: structure, expression, targets, and signaling pathways

2.1. PD-L1/PD-1 structure, expression, and targets

Ishida *et al.* carried out a study about cell apoptosis-related proteins in T cell hybridoma. Their study finally led to the discovery of PD-L1, a 40 kDa type 1 transmembrane protein, that significantly interlocks the PD-1 pathway and negatively regulates effector T-cell function [14]. The interaction between PD-L1 and PD-1 depends on their β-sheets V-domain [15]. Structurally, PD-1 belongs to CD28, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) co-receptor family with 25% of identicalness primarily functioning resemblance. Agonists of this family encompass B7-2 (CD86), B7-1 (CD80), PD-L2, and PD-L1 components; therefore, PD-L1 is also known as CD274 or B7-H1 [16]. It has been demonstrated that PD-1 and PD-L1 significantly contribute to adjust inflammation and mediate peripheral tolerance [17].

As mentioned before, the PD-1/PD-L1 axis mainly hinders T cell activation via T cell located PD-1 receptor, and its agonists (PD-L1/PD-L2) expressed on antigen-presenting cells (APCs). This pathway profoundly affects the generation of various cytokines by T cells [18]. T cell receptor (TCR) signaling in the presence of PD-1, shortened dwell time in T cell interactions with APCs, which leads to decreased T cell activation, T cell exhaustion, dysfunction, neutralization and may also favor the induction of regulatory T cells (Tregs) [19].

The expression of PD-1 is observed in activated cells, including B and T cells, monocytes, natural killer (NK) cells, dendritic cells (DCs), and natural killer T (NKT) cells. However, significant expression of PD-1 is observed in particular T cells including CD4 and CD8 T cells, T follicular helper cells (TFH), and exhausted CD8 cells [20,21]. The expression of PD-L1 is fundamentally observed in T cells, macrophages, B cells, and mesenchymal stem cells [22]. On the other hand, only activated macrophages, DCs, mast cells emerged from bone marrow, and more than half of peritoneal B1 cells express PD-L2 [22,23].

2.2. Signaling pathways inducing PD-L1 expression in tumor cells

There are two types of immune resistance due to PD-L1 expression. The PD-L1 expression is monitored either by the upregulation of PI3K-Akt kinases or secretion of IFN-γ. The former is known as innate immune resistance and the latter as adaptive immune resistance. The secretion of IFN-γ that represents the inflammatory responses contributes to adaptive immune resistance, but innate immune resistance or oncogenes is being continuously expressed and does not correlate with inflammatory responses. So, there is a correlation between PD-L1 expression and IFN-γ as inflammatory responses but only in adaptive immune resistance and not in innate immune resistance. In innate immune resistance, oncogenes can control PD-L1 generation in tumor cells, in which PD-L1 is being continuously expressed and does not correlate with inflammatory responses. According to findings, tumor cell attenuates PTEN (phosphatase and tensin homolog), which engenders the expression of PD-L1. PTEN carries out this function via upregulating STAT3 as well as lymphoma kinase (ALK) signaling resistance [24,25].

PD-L1 promoter also has numerous IFN-γ response elements that can induce PD-L1 expression. IFN-γ-mediated PD-L1 expression also implicates two signal transduction routes including JAK (Janus Kinase)/STAT3 and PI3K/AKT [26]. JAK/STAT1 axis is also involved in the stimulation of PD-L1 generation on cancerous cells in part through upregulating IRF-1 (Interferon regulatory factor 1) [27]. Probably, in the PI3K/Akt axis, Akt takes part in the regulation of PD-L1 after transcription. This protein induces NF-kB and mTOR, which directly act on the promotor of PD-L1 and enhance its transcription. mTOR/S6 as its subsequent transducer has been observed to mediate the PD-L1 expression stimulated by Akt [28].

Moreover, Toll-like receptors (TLRs)-involved signaling pathways, including TLRs/Myd88)/TRAF6/ IκB kinase (IKK)s are also supposed to participate in the induction of PD-L1 expression [29]. Newly, hypoxia-inducible factor 1-alpha (HIF-1α) has also been postulated to participate in the expression of PD-L1 [30]. HIF-1 and STAT3 cooperate for upregulating PD-L1 (Fig. 1) [31,32]. Similarly, it is demonstrated that OCT4 assists the metastasis and expansion of cervix tumor cells by accelerating the expression of PD-L1 using the miR-18a-dependent signaling network [33]. Tumors with mutated or deleted p53 tend to generate PD-L1 at high levels [34]. Other investigations have proven that in basal TNBC (triple-negative breast cancer) with EMT (epithelial-to-mesenchymal transition) attributes, MUC1-C (mucin 1 C-terminal) provokes the constitutive expression of PD-L1 (Fig. 2) [35]. In contrast, class I histone deacetylase HDAC8 negatively regulates expression of PD-L1 in melanoma cells in part through epigenetic regulation of HOXA5, STAT3 and HDAC6 localization in the promoter region of the PD-L1.

2.3. PD-1 signaling in T cells

The significant impact of PD-1 signaling in T cells is the prevention of TCR and critical signals in co-stimulation (e.g., CD28) [36]. Following the involvement of the appropriate agonist of PD-1, its signal transduction begins. Consequently, ligation of PD-1, tyrosine residues of intracellular immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasm are phosphorylated [37]. Remarkably, ITSM following phosphorylation employs SHP-1 (Src homology two domain phosphatase-1) besides SHP-2 [38]. SHP-2 performs dephosphorylation of ZAP70 (zeta chain of T cell receptor-associated protein kinase 70) besides PI3K (phosphoinositide 3-kinase) pathway triggered by CD28 as well as protein kinase θ (PKCθ) [39]. ZAP70 blockade decreases enhancer survival and proliferation pathways as Ras signals that inhibit extracellular receptor-activated kinase (Erk) activation. Suppression of PI3K prevents later phosphorylation of Akt (protein kinase B) [40]. Following inhibition of these pathways, PD-1 suppresses ubiquitin ligase SCF (Skp2), which influences the progression of cell cycle and T cell expansion. PD-1 equally enhances ATF-like (BATF) generation adequately in order to disrupt T cell expansion and cytokine secretion [41]. PD-1 initiated downstream signal transduction has many impacts comprising decline in cell survival gene, Bcl-XL and inflammation-related cytokines including TNF-α, IFN-γ, and IL-2. Additionally, this signal enhances energy in T cells (CD8+/CD4+). It also prevents amino acid metabolism and glycolysis, while it enhances the oxidation of fatty acids leading to the downregulation of T cell survival and proliferation [42].

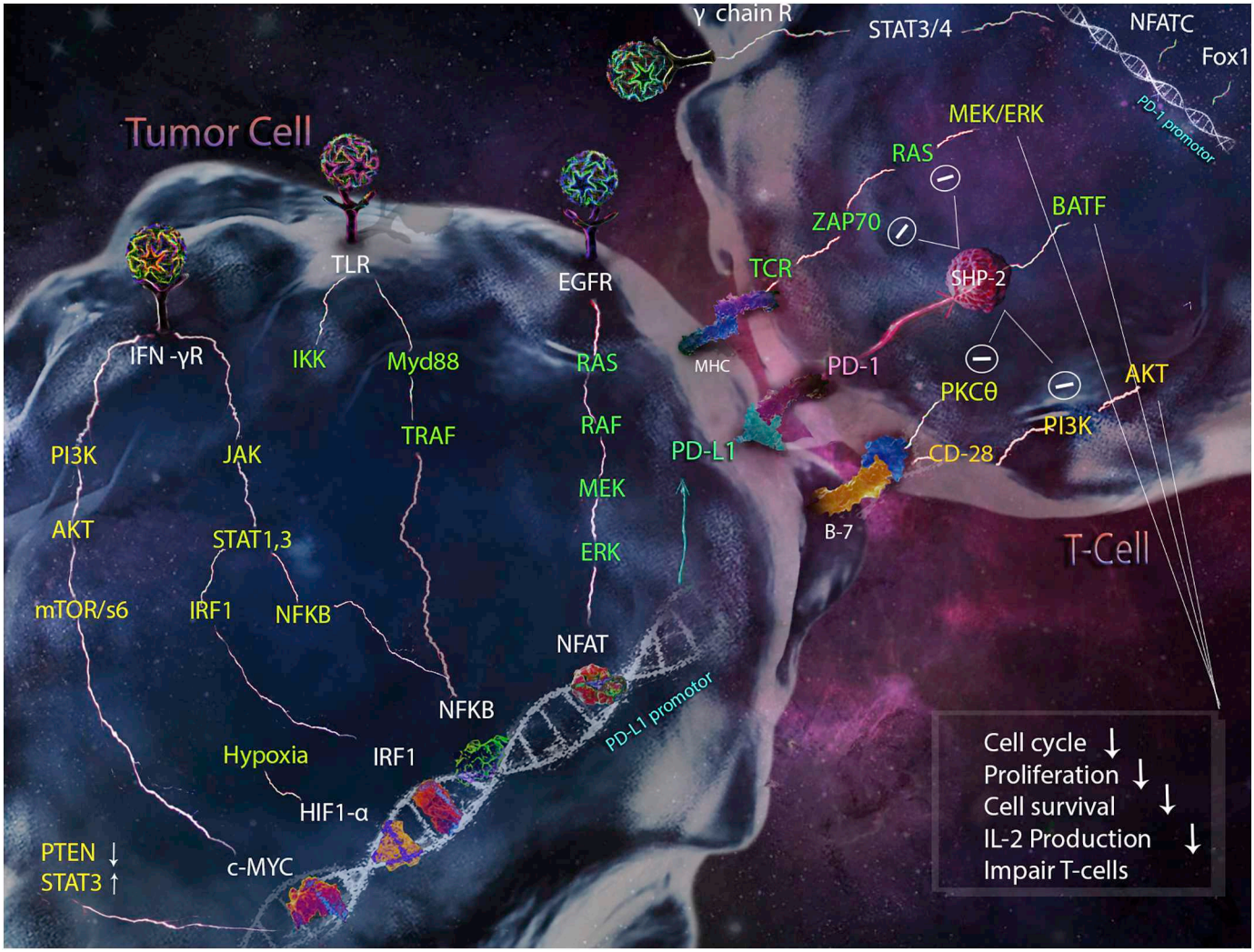

Fig. 1. PD-L1-inducing signaling pathways in cancer cells. IFN-γ can enhance PD-L1 expression in cancer cells through JAK/STAT/IRF1 pathway. PI3K/Akt/mTOR is another pathway by which IFN-γ induced PD-L1 expression. Akt induces NF-kB and mTOR, which directly act on the PD-L1 promotor and enhances its transcription. TLRs/Myd88/Traf6/IKKs pathway seems to be involved in the induction of PD-L1 expression. HIF-1α can also bind to HRE-2 and HRE-4 locations in upstream of the PD-L1 promoter and enhance the PD-L1 expression. The cooperative function of HIF-1 and STAT3 also promotes PD-L1 expression in cancer cells. PD-L1: Programmed death-ligand 1, JAK/STAT: Janus kinase/signal transducers and activators of transcription, IRF1: Interferon Regulatory Factor 1, IFNγ: Interferon-gamma, NF-κB: nuclear factor kappa light chain enhancer of activated B cells, Akt: Protein kinase B, PI3K: Phosphoinositide 3-kinases, mTOR: mechanistic target of rapamycin, TLR: Toll-like receptors, Myd88: Myeloid differentiation primary response 88, IKK: IκB kinase, Traf: TNF receptor-associated factor, HIF-1: Hypoxia-inducible factor-1, HRE: Hypoxia Responsive ERF genes.

2.4. Signaling pathways inducing PD-1 expression in T cells

Signals derived from cytokines are of great importance to adjust PD-1. Signal transduction via a shared γ chain seems to be pivotal. IL-2, IL-7, IL-15, and IL-21 are cytokines, which share γ chain and enhance PD-1 generation on T cells [43]. Besides, IL-6 via STAT3 and IL-12 via STAT4 can trigger PD-1 emergence in activated T cells via molecules that affect promoter of the PD-1 encoding gene. Among transcription factors, the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) can directly induce expression of PD-1. Distinctly, PR domain zinc finger protein 1 (Blimp-1) suppresses the expression of PD-1 in two ways; by NFATc1 repression and making inhibitory alterations to chromatin at the locus of PD-1 [44]. In macrophages, NF-kB/P65 regulates the PD-1 expression [45]. Moreover, IFN-α can stimulate overexpression of PD-1 in these cells through the involvement of IFN-sensitive responsive element (ISRE) in part via binding the transcription factor IRF9 to the STAT1 and STAT2 [46]. FoxO1 (Forkhead box O1) is recognized as another factor involved in transcription and play a role in expression maintenance and inhibition of PD-1 function in exhausted cytotoxic T lymphocytes (CTLs) (Fig. 3) [47].

2.5. PD-L1/PD-1 signaling in Treg differentiation

It is noteworthy that while exogenous TGF-β (transforming growth factor-beta) is absent, PD-L1 derived signals can stimulate Treg cells [48]. PD-L1-expressing DCs interact with T cells, thus trigger Treg production from naive T cells [49,50]. PD-L1/PD-1 axis decreases Th17 and Th1 and enhances Treg initiation by suppressing PI3K/AKT/mTOR and concurrently upregulating PTEN. Therefore, this axis can make a change to pathways involved in the differentiation of T cells [51].

2.6. Post-transcription regulation of PD-L1

It has been shown that various post-transcriptional regulations including ubiquitination and de-ubiquitination, phosphorylation, and glycosylation can affect the binding ability, affinity of ligation, stability, and function of PD-L1 in cancer cells. Accordingly, binding of some miRNAs to the 3′-UTR region of the PD-L1 transcripts destabilizes its mRNA [52]. This type of regulation can be done either directly through binding to mRNA binding or indirectly by influencing other factors

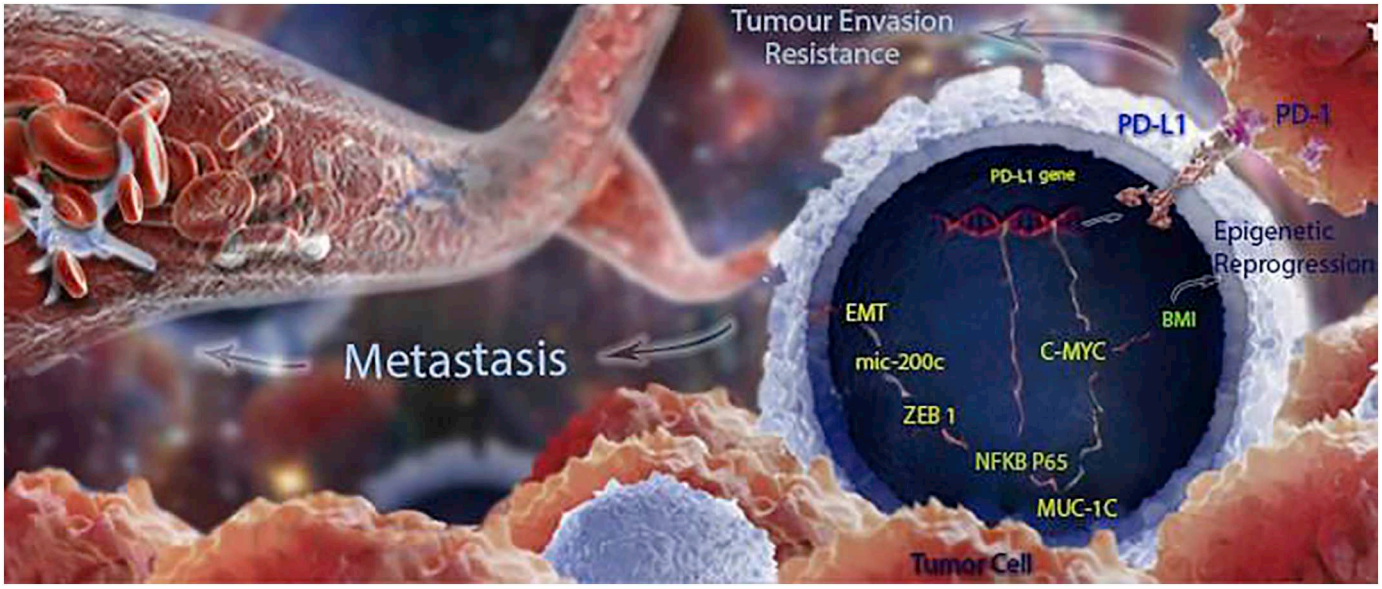

Fig. 2. PD-L1 triggers breast cancer metastasis. MUC1-C induces constitutive PD-L1 expression in breast cancer cells. It can enhance PD-L1 expression through MUC1-C MYC and MUC1-C NF-kB p65 pathways. MUC1-C can also induce epigenetic changes and MYC-associated expression of BMI1. NF-kB p65 induced by MUC1-C can interacts with the ZEB1 gene and form the ZEB1/miR-200c and induce the EMT process. The interrelation of these transcription factors culminates in PD-L1 promoter initiation. PD-L1: Programmed death-ligand 1, NF-κB: Nuclear Factor Kappa light chain enhancer of activated B cells, BMI-1, BMI1: B lymphoma Mo-MLV insertion region one homolog, ZEB1: Zinc Finger E-Box Binding Homeobox 1, MUC1-C: mucin 1 C-terminal, EMT: Epithelial-mesenchymal transition.

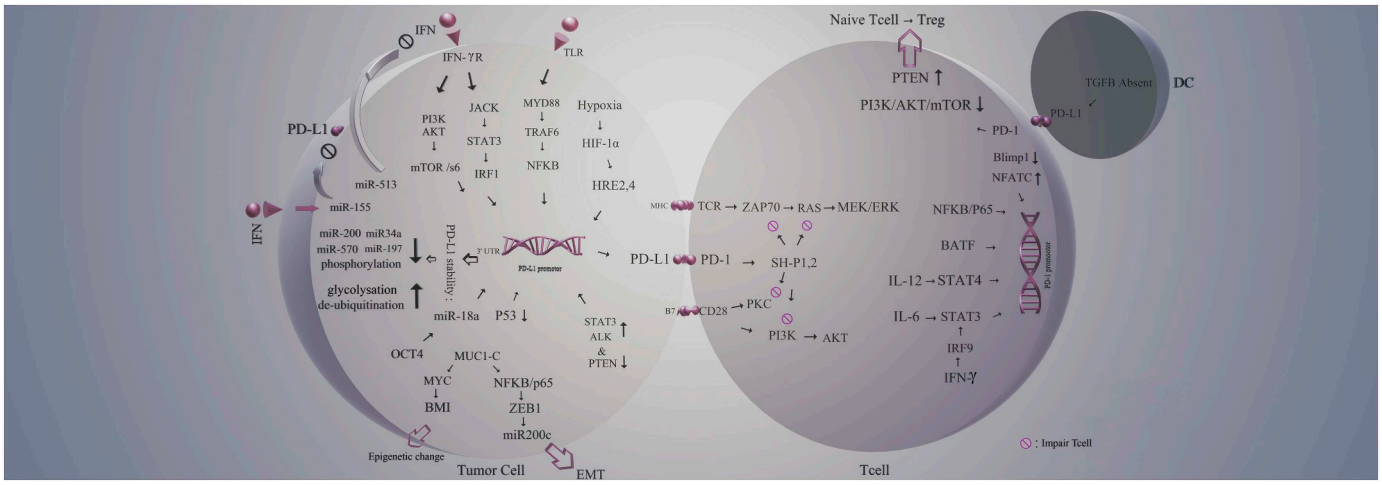

Fig. 3. Signaling pathways that modulate PD-L1/PD-1 expression. In the tumor cells, the expression of PD-L1 is increased by the two main (PTEN and IFN-γ) and some alternative pathways. Downregulation of PTEN unlike the upregulation of ALK and along with the IFN-γR signaling cascade including the JACK-STAT3 or PI3K/AKT pathway leads to the upregulation of PD-L1. Other pathways that modulate the expression of PD-L1 include the MYD88/TRAF6/NFKB, p53, OCT4, miR-18A, HIF-1, and HER2,4. Post-translational modifications of the PDL1 also regulate its stability in two different ways. Decreasing factors are miR-200/miR-34a/miR-570/miR-197, and phosphorylation of 3′UTR region. On the other hand, glycosylation and de-ubiquitination are considered as enhancer factors. miR-155 and miR-513 also regulate IFN-γ expression. Finally, the production of MUC1-C and subsequently induction of the NFKB/ZEB-1/miR200c pathway causes EMT occurrence and triggers epigenetic change by MYC/BMI pathway. Following PD-1/PD-L1 binding, SH-P1,2 is recruited in T-cells. This recruitment further inhibits TCR-induced ZAP70, RAS- and CD28-induced PKC and PI3K that ultimately impair T cells. PD-1 itself is increased by BATF, IL-12 (via STAT4), IL-6/IFN-γ (via STAT3, NFKB/P65) and NFATC factors. NFATC production is also regulated by Blimp-1 depletion. In the absence of TGF-β in DCs, PD-L1 is expressed. By further PD-1/PD-L1 interaction, PTEN expression is increased, whereas PI3K/AKT/mTOR decreases, resulting in Treg being generated from naive T cells.

[53]. Among the various miRNAs, it has been shown that miR-34a, miR-200, and miR-570 can suppress the expression of PD-L1 at the post-transcription stage. Another miRNA that can indirectly suppress PD-L1 expression is miR-197 which represses STAT3 leading to the down-regulation of PD-L1 [29]. The upregulation of miR-513 is also associated with the downregulation of IFN-γ-mediated PD-L1 expression. On the other hand, the induction of miR-155 by IFN-γ and TNF-α directly decreases the expression of PD-L1 [53]. Phosphorylation by various kinases is another post-transcription regulation of PD-L1 that can affect its expression and function. It is demonstrated that phosphorylation of PD-L1 by Glycogen synthase kinase-3 beta (GSK3β) facilitates its interaction with E3 ligase leading to proteasomal degradation of PD-L1 [54]. Moreover, it has been shown that N-glycosylation and de-ubiquitination can stabilize PD-L1 [55,56].

3. Expression of PD-1/PD-L1 in breast cancer tumor microenvironment and its prognostic value

So far, it has been shown that PD-L1 expression in breast tumors varies from 19% to 64% [57], however, in inflammatory carcinoma of

the breast, this percentage is more than other types of breast tumors [58]. Additionally, a large number of $HER2^{+}$ cells and especially TNBCs produce PD-L1. This protein ordinarily associates with poor prognostic characteristics including young age of patients, substantial size of tumor, high histological grade, significant index of proliferation, metastasis of lymph node, pervasiveness of tumor-infiltrating lymphocytes (TILs), features of aggressive human tumors (basal, TNBC, HER2-enriched) and ER, as well as status of PR-negativity [59]. Some studies also indicated that the expression of PD-L1 has a detrimental and disadvantageous prognostic value; however, this factor acts as a self-supporting parameter for triple-negative breast cancer prognostication. Mori and colleagues have shown that high expression of PD-L1 and low frequency of TILs in tumors of patients with primary TNBC (n = 248) is an independent poor prognosis factor for overall survival and recurrence-free survival [60].

Genomic instability in genes such as BRCA1- and BRCA2 can also affect breast cancer prognosis, which is associated with the expression of PD-L1 and PD-1 [61]. Interestingly, immunotherapy of breast cancer in cancers with a high mutation rate is usually more successful than cancers with a low mutation rate [62,63]. On the other hand, several studies have shown that low expression of PD-L1 is associated with low TIL frequency in metastatic breast cancer lesions in comparison with primary tumors, implying an immune-depleted nature of metastatic tumors that are resistant to immune stimulation and promote immune-editing phase of tumor progression [64]. Interestingly, mRNA and protein expression levels of PD-L1 have shown different prognostic values in patients with breast cancer. While high protein expression levels of PD-L1 are associated with poor prognosis, its high mRNA expression indicates a good prognosis. However, to have a definitive opinion on this, we need to conduct rigorous trials to find the prognostic value of PD-L1 mRNA and protein expression in patients with breast cancer [65].

In a study on 215 TNBC patients, it has been demonstrated that PD-L1 is upregulated and correlates with TIL numbers and indicates an excellent clinical outcome [66]. There is a similar report regarding the correlation between expression of PD-L1 in tumor cells and TILs, diabetic disease, and good prognosis in TNBC patients [67]. Alternatively, it has been shown that while the stromal PD-L1 expression was correlated with better disease-free survival (DFS) in 135 samples derived from TNBC patients, the expression of PD-1 was not correlated with disease prognosis [68]. Investigation of PD-L1 expression in 39 fresh tissues and 167 fixed tissues derived from HER2+ breast cancer patients exhibited a robust correlation of PD-L1 levels, with TIL frequency, and high histologic grade, negative hormone receptor expression, and better DFS. Therefore, it is suggested that PD-L1 expression levels can be considered as a favorable prognostic factor in patients with hormone receptor-negative HER2+ breast cancer [69]. Investigation of PD-L1 expression in tumor tissues derived from HER2+ breast cancer patients also showed a correlation of PD-L1 expression levels with high TIL levels and lower risk of tumor recurrence [70]. There is a similar report regarding the correlation of PD-L1 expression levels in 636 samples derived from primary breast carcinoma patients with high TIL levels and more prolonged recurrence-free survival (RFS) [71]. In contrast, it is demonstrated that the high frequency of CD8+ lymphocytes in primary tumor tissues is associated with low PD-L1 expression levels, high FoxP3-expressing TILs, and prolonged survival (only in hormone receptor-negative patients) [72]. Measurement of PD-L1 in tissue microarrays derived from 465 invasive breast carcinomas exhibited that high PD-L1 expression levels correlate with TIL frequency and better DFS and overall survival (OS) [73]. Regulatory B cells in patients with invasive breast carcinoma can also express high levels of PD-L1, which robustly associates with Tregs and inversely correlates with PD-1high effector T cells [74]. Correlation of PD-L1 levels with better patient survival has also been demonstrated in basal-like breast cancer [75]. Despite the PD-L1's association with poor pathologic and clinical characteristics, PD-L1 expression was correlated with better survival results, which implies its positive prognostic potential in breast cancer (stage I, II, and III) [59]. Evaluation of PD-L1 in 44 patients with breast cancer showed that half of the patients express intratumoral PD-L1, which is associated with PD-L1-expressing TILs and pathologic prognostic factors in infiltrating ductal carcinoma [76].

On the other hand, Tsang and colleagues showed that while the PD-L1 expression is associated with luminal cancers, it is an independent poor prognostic biomarker in patients with HER2+ breast cancer [77]. Another investigation also demonstrated that the expression of PD-L1 is highly correlated with the frequency of Tregs and poor prognosis, particularly in patients with basal-like carcinoma [78]. PD-1 expression was also correlated with poor prognosis in the basal-like, luminal B HER2-, and the luminal B HER2+ subtypes of breast cancer [79]. Similar results are also reported regarding the PD-L1 expression levels [80]. Association of PD-L1 expression levels with poor prognosis is also reported in 750 TNBC patients [81] and 64 invasive ductal carcinoma patients [82]. Li *et al.* also evaluated PD-L1 expression in synchronous axillary lymph node metastases and primary tumors of 101 TNBC patients. They showed that the expression of PD-L1 in cancer cells and lymphocytes of synchronous axillary lymph node metastases was higher than those which were present in primary tumors. However, both of them were associated with poor prognosis. They have suggested that the expression status of PD-L1 in metastases can be used for PD-1/PD-L1 targeted therapy in these patients [83]. Ács and colleagues have also shown that although the expression levels of PD-1 and PD-L1 are not different between early-onset non-pregnancy-related breast cancer and pregnancy-related breast cancer, expression levels of PD-L1 can be considered as a poor prognosis factor in early-onset breast cancer [84].

However, conflicting information has been reported about the universal and predictable value of PD-L1 in breast tumors. Immunohistochemical study on 1091 patients with breast cancer showed that PD-L1 expression was associated with luminal cancers and lower histologic grade. On the other hand, PD-L1 expression in HER2-positive breast cancers was an independent poor prognosis factor. High expression of PD-L1 in HER2-positive tumors was also associated with the low frequency of PD-1-expressing TILs. Moreover, patients with high PD-L1 and low PD-1-expressing TIL exhibited the worst survival implying the suppression of immune responses by PD-L1 [77]. These findings indicate that prognostic values of PD-L1 expression in tumor or PD-1 on TILs may vary based on breast cancer subtypes.

As discussed above, the majority of studies have evaluated the prognostic value of PD-L1 expression in breast cancer and little is known regarding the impact of PD-1 expression on disease prognosis. What is known is that PD-1 is expressed on infiltrated leukocytes to the tumor site and by binding to PD-L1 expressed on tumor cells inhibits anti-tumor leukocyte responses and enhances tumor growth and invasion. Therefore, it is expected that with increasing frequency of PD-1-expressing T cells, the growth and spread of breast cancer will increase. Studies to date on PD-1-expressing T cells in breast cancer have shown that the expression of this molecule promotes tumor progression by inhibiting T-cell response and increasing PD-L1 signaling in tumor cells. However, reaching these conclusions requires more detailed studies on large populations of patients with different subtypes of the disease.

The above-mentioned outcomes were derived from various distinctions in methodology such as tests on molecular subtypes, disparate populations, and diverse analytical levels (particularly considering levels of mRNA), as well as other emerging technologies. Regarding the distinctions above, there was some disagreement in results, which are summarized in Table 1.

It should be noted that cytotoxic therapeutics can also affect PD-L1 expression in breast cancer. For example, while paclitaxel could upregulate PD-L1, Epirubicin reduced its expression in MDA-MB-231 cells (the human breast cell line). Moreover, expression levels of PD-L1 have reduced in all evaluated cell lines following the tumor inoculation in mice [85]. Similarly, Soliman and colleagues have evaluated the expression of PD-L1 in various breast cancer cell lines including HCC38

Table 1
Studies related to the assessment of PD-1/PD-L1 expression levels in breast cancer.

Cancer	n	Method	PD-1/PD-L1 expression	Results	Ref.
TNBC	215	IHC	PD-L1: 32.6%	1. Upregulation of PD-L1 is associated with TIL frequency in TNBC. 2. PD-L1 + cancer patients had significantly longer DFS and OS. 3. Expression of PD-L1 and TIL frequency are independent prognostic factors.	[60]
TNBC	238	TMA	N/A	1. PD-L1 is an important prognostic marker. 2. PD-L1 expression positively correlates with (Ki-67), glycemia, and inversely with presence of lymph node metastasis and relapse. 3. PD-L1expression robustly associates with better DFS but not OS.	[61]
TNBC	136	H-score	PD-L1 51%	1. PD-L1 expresses in a high proportion of TNBCs. 2. Stromal expression levels of PD-L1 robustly correlated with better DFS. 3. Expression of PD-1 was not correlated with OS, DFS, and metastasis.	[62]
HER2-positive breast cancer	167	Allred scoring system	PD-L1: 48.5%	1. Expression PD-L1 was correlated with better DFS. 2. High expression level of PD-L1 was correlated with high TILs, high histologic grade, absence of lympho vascular invasion and negative hormone receptor expression.	[63]
HER2 + breast cancer	191	TMA	PD-L1: 21–59.5%	1. Higher expression of PD-L1 correlated with lower risk of tumor recurrence.	[64]
Primary breast carcinoma	636	Real-time PCR	PD-L1: 55%	1. PD-L1 expression and elevated TI Ls were associated with longer RFS.	[65]
Primary breast cancer tumor	256	IHC	94.9%	1. CD8 + TILs were increased in PD-L1^{low} tumors. 2. PD-L1 expression was more frequent in HR-positive BC	[66]
Invasive breast carcinomas	465	IHC	PD-L1 13.5%	1. Expression of PD-L1 was correlated with better DFS and OS, but is not an independent prognostic factor. 2. High PD-L1 expression was potently correlated with high histologic grade, negative lymph nodes, early pathologic stage, high TIL counts, negative ER and PR expression, HER2, EGFR and p53expression, and high Ki-67 proliferating index. 3. High expression levels of PD-L1 were correlated with the HER2 and TNBC type.	[67]
Invasive carcinoma of breast	98	Flow cytometry	14.02 ± 42.36	1. The PD-L1 expression on Bregs negatively correlates with PD-1 effector T cells and induces generation of Tregs.	[147]
Breast cancers	443	IHC	PD-L1 16.5%	1. The expression of PD-L1 was correlated with better patient survival in Basal-like TNBC patients. 2. PD-L1 associated with poor prognosis, high tumor grade, ER/PR negative status.	[69]
Breast cancer	1091	IHC	PD-1,TIL on 97	PD-L1 associates with luminal cancer, lower histologic grade, absence of necrosis, ER, PR, and AR expression. PD-L1 is an independent poor prognostic indicator.	[57]
Breast cancer	501	IHC	PD-L1: 46.1%	1. The expression of PD-L1 and Treg infiltrates correlated with poor prognosis, especially in the basal-like subtype. 2. Both markers are the independent predictors for reduced OS.	[71]
Breast cancer stages I, II, III	192	IHC, tissue microarray	PD-L1 56.6%	1. The expression of PD-L1 was a positive prognostic biomarker, whereas it was correlated with poor clinical and pathologic features in breast cancer.	[55]
Breast cancer	660	IHC tissue microarray	PD-1 15.8 %	The presence of PD-1 was correlated with a shorter OS in the luminal B HER2, luminal B HER2- and the basal-like subtypes.	[72]
Breast cancer	44	IHC	PD-L1 34%	1. PD-L1 is an important risk factor in breast cancer patients. 2. Tumoral expression of PD-L1 was significantly correlated with histologic grade III–negative, ER–negative, and PR–negative patients. 3. PD-L1 expressed in TIL was correlated with tumor size, histologic grade III, Her2/neu positivity, and high tumor lymphocyte infiltration	[70]
Breast cancer	650	IHC, tissue microarray	PD-L1: 23.4 %	1. PD-L1 expression is an independent poor prognostic factor in the HER2, luminal B HER2-, and the basal-like subtypes.	[73]
Metastasis Chinese breast cancer	870	IHC	PD-L1 21.7%	1. The expression of PD-L1 in TNBC was significantly higher than non-TNBC. 2. The expression of PD-L1 was associated with unfavorable prognosis in breast cancer patients.	[74]
Breast invasive ductal carcinoma	64	IHC and real time PCR	PD-L1 37.5%	PD-L1 may be a poor prognosis marker in invasive ductal carcinoma.	[75]
Primary lymph node metastases TNBC	101	IHC	PD-L1: 38.61%	1. The expression of PD-L1 was robustly higher in the tumor cells and lymphocytes of the LNMs. 2. The expression of PD-L1 was correlated with high grade and more TILs. 3. The DFS and OS between the PT /LNM patients exhibited worse. 4. PD-L1 status in LNMs can be used to predict whether PD-L1/PD-1-targeted therapy would be efficient for a node-positive TNBC patient.	[76]

(*continued on next page*)

Table 1 (*continued*)

Cancer	n	Method	PD-1/PD-L1 expression	Results	Ref.
PRBC & YWBC	42	IHC	PD-1: 9.56%– 16.87%, PD-L1: 1.07%–0.44%,	1. The optimal threshold of PD-L1 expression in cancer cells was at 10% for OS, and at 1% for DFS 2. Higher expression of PD-L1 was correlated with poor prognosis. 3. The expression of PD-L1 in early-onset breast cancer is an independent poor prognosis marker.	[77]
Different breast cancer cell lines	10 cell lines	Flow cytometry, IHC, Western blotting, Immunofluorescence	Different in esch cell line	1. High expression of PD-L1 was observed in MDA-MB-231 and JIMT-1 cells. 2. Epirubicin reduced and Paclitaxel induced expression of PD-L1 in MDA-MB-231 cells.	[58]
Basal breast cancer Pre-Epirubicin/Paclitaxel	Six cell lines	Flow cytometry, tissue microarray	Different in esch cell line	Basal type breast cancer cells (especially basal B) constitutively express high PD-L1 levels.	[59]

Abbreviations: PD-L1: Programmed death-ligand 1/PD-1: Programmed cell death protein 1/TIL: Tumor Infiltrating Lymphocyte/TNBC: Triple Negative Breast Cancer/HR: Hormone Receptor/ER: Estrogen Receptor/PR: Progesterone Receptor/AR: Androgen Receptor/IHC: Immunohistochemistry/TMA: Tissue Micro Arrays/DFS: disease-free survival/OS: overall survival/HER2: human epidermal growth factor receptor 2/EGFR: *Epidermal growth factor* receptor/N/A: not assigned/Bregs: regulatory B cells/Tregs: regulatory T cells/PT: primary tumors/LNM: lymph node metastases/PRBC: pregnancy-related breast cancer/YWBC: early onset non-PRBC

(the human breast/duct cell line) and MDA231 (basal B), MCF7 and AU565 (the human luminal breast cell line), HCC1143 (the human breast cell line), and BT20 (the human basal A breast cell line). Among them, basal B breast cancer cells exhibited the highest PD-L1 expression levels, which was associated with the upregulation of genes involved in proliferation, invasion, and chemo-resistance [86].

4. Targeting PD-1/PD-L1 in breast cancer

The PD-1/PD-L1 signaling cascade tends to suppress immune responses in solid tumors, such as breast cancer. Nonetheless, the impediment of immune checkpoint inhibitors has been demonstrated to trigger efficacious anticancer reactions, prevail over the obstacles, and improve the T cells activation and immunological trends and ultimately lessen breast tumor cells. Inhibitors of immune checkpoints such as antibodies aimed at PD-L1 or PD-1 have been translated in the clinics for various cancers [87].

What is known to date is that a group of breast cancer patients responded appropriately and durably to treatment with PD-L1 / PD-1 inhibitors. But what is really difficult is how to identify this group of patients who respond to treatment.

In order to predict correctly the outcome of treatment by inhibiting the PD-L1/PD-1 axis in breast cancer, the classification of patients based on PD-L1 expression pattern in tumor and PD-1 expression level in TILs may be helpful. Breast tumors can be categorized into four groups, namely PD-L1 expressing and TILs positive tumors, PD-L1, and TILs negative tumors, and PD-L1 expressing and TILs negative, finally PD-L1 negative and TILs infiltrating tumors. It is postulated that the first group has procured adaptive resistance against the immune system by generating PD-L1 to inactivate TILs. This group has the highest potential to undergo anti-PD-L1 treatment for immune stimulation. Tumors from the second group are supposed to be neglected by the immune system since they represent no tumor antigens. PD-L1 expression is perceived in third group of tumors because of the internal initiation of the expression. Either of the two last groups can be utilized for combination therapy. In the fourth group, it can be comprehended that tumor cells have boosted their tolerance to the immune system because of different immune blockers including suppressors of B and T lymphocytes and T cell immunoglobulin 3 (TIM3) [88,89]. Currently, evaluation of PD-L1 expression in tumor tissue by immunohistochemistry (IHC) seems to be the best approach for early detection of breast cancer patients' response to PD-L1 / PD-1 inhibitors [90,91].

Although the reported results are variable, it is demonstrated that PD-L1/PD-1 inhibitors can induce anti-tumor immune responses for up to three months. However, there are cases with a delayed response even several months after treatment, which may be in part related to delay in the initiation of the immune response.

Intriguingly, the appearance of fast disease progression following immune checkpoint immunotherapy has also been reported by some researchers who are also known as hyper progression phenomena in which the size of the tumor develops while the treatment is underway and may lead to unfavorable effects [92]. It has been found that in patients with breast cancer who have high expression of mouse double minute 2 homolog (MDM2), MDM4 or EGFR genes, a tumor hyperprogression process occurs [93–95]. This event has typically been associated with older-aged patients with breast cancer, a high tumor metastasis, and a history of radiotherapy. On the other hand, there is no relationship between this event and tumor burden, severe tumor growth before treatment, or the number of previous treatment sessions [96].

Multiple monoclonal antibodies (mAbs) have been explored to treat breast cancer encompassing transmembrane T cell-PD-1 targeting antibodies and breast cancer-PD-L1 targeting ones. Pembrolizumab and nivolumab aim at PD-1 and Durvalumab, Atezolizumab, as well as Avelumab, all of which target PD-L1 [97]. According to data derived

from phase I, it has been substantiated that using targeted antibodies for impeding the PD-1 immune regulators is hazardless and represents suitable tolerance with anticancer function in breast cancer. Initially, Pembrolizumab exhibited an 18.5% response in phase I clinical experiments in pretreated metastatic TNBC. Furthermore, an exploration of Atezolizumab in phase I has revealed that sufferers with positive TNBC PD-L1 show 19% total response according to criteria for evaluating response in solid tumors and have now entered phase III trials [98,99].

Pembrolizumab as humanized mAb (IgG4 isotype) has a considerable affinity and selectivity targets PD-1 molecule and inhibits its ligation to PD-L1/2. Although it is approved for the treatment of some cancers [100–102], there is no precise and comprehensive information on its efficacy in the treatment of breast cancer patients. Nivolumab was the first approved anti-PD-1 mAb for the treatment of some cancers [103–107], however, little is known regarding its efficacy in breast cancer. Avelumab, another anti-PD-L1 mAb (fully human, IgG1 isotype), is FDA-approved for treating metastatic Merkel cell carcinoma. Atezolizumab, which is humanized mAb (IgG1 isotype) against PD-L1, is FDA approved for some cancers [108]. It should be noted that, it is approved for the treatment of the PD-L1-positive TNBC. The objective response rate achieved by atezolizumab was 53% [109].

Several investigators have evaluated the impact of PD-1 or PD-L1 blockade on the anti-tumor immune responses and tumor growth both *in vitro* and *in vivo* in animal models and clinical trials related to breast cancer, which are summarized in Tables 2 and 3.

4.1. Animal studies

4.1.1. Monotherapy

Treatment of TNBC cell lines, including MDA-MB-231, IIB-BR-G (the human breast cell lines), and Hs578T (the human breast cell line) with anti-PD-L1 mAb, Avelumab, led to increased NK-cell mediated cytotoxicity, which was associated with expression levels of PD-L1 [110]. Consistently, administration of a humanized anti-PD-1 mAb, Pembrolizumab, into TNBC patient-derived xenograft tumor-bearing mice was associated with tumor regression and increased survival time [111,112]. Using transgenic mouse models (SV40 transgenic WAP-T/WAP-TNP), it is demonstrated that blockade of PD-L1 is more effective compared to PD-1 [113]. Interestingly, it is demonstrated that the downregulation of PD-L1 by shRNA results in EMT reversal in claudin^{low} MDA-MB-231breast cancer cells [114].

4.1.2. Combination with chemotherapy or cell-based immunotherapy

In *in vivo* study, anti-PD-1 therapy could potentially suppress tumor growth in the EMT-6/CDDP TNBC model. The addition of paclitaxel further prevented tumor growth [115].

Combining siRNA-mediated silencing of PD-L1 and chemotherapy was also an effective therapeutic strategy in the nude mouse model of MDA-MB-231 [116]. Moreover, combining anti-PD-L1 therapy with either paclitaxel or nintedanib (an anti-angiogenic VEGF (vascular endothelial growth factor) receptors) could effectively suppress tumor growth in EMT-6/CDDP and EMT-6 breast cancer models [117].

A combination of anti-PD-L1 therapy with cell-based immunotherapies was also associated with hopeful outcomes. Accordingly, blockade of PD-L1 significantly enhanced the anti-tumor potential of the DC vaccine in breast tumor-bearing hu-SCID mice [118]. Whole-cell vaccination could also increase the efficacy of anti-PD-L1 therapy in 4TO7 and D2F2 mouse breast cancer models [119].

4.1.3. Combination with other checkpoint inhibitors or cytokine inhibitors

Combined inhibition of PD-1 and other checkpoints is another promising strategy used to suppress tumor growth by various investigators. Efficacy of combined therapy based on anti-PD-1 and anti-

Table 2
Pre-clinical studies related to the blockage of PD-1/PD-L1 in breast cancer animal models.

Cancer	Therapy	Result	Ref.
Cell lines: Hs578T, IIB-BR-G, and MDA-MB-231	Avelumab	Avelumab-mediated ADCC is a valuable mechanism for elimination of tumor cells in TNBC.	[98]
TNBC PDX tumor models.	Anti-PD-1 antibody	Treatment significantly reduced tumor growth and increased survival.	[99]
Patient or cell line derived xenografts TNBC	Pembrolizumab	Treatment significantly reduced tumor growth.	[100]
4T1 mammary mouse model	A fluorescence-labeled PD-1 probe, PD-1-IRDye800CW	Immunotherapy blocked regrowth of tumor, inhibited metastasis and increased survival rate.	[117]
TNBC murine cancer cell line (EMT-6/CDDP)	Paclitaxel with or without anti-VEGF	Effective as a neoadjuvant therapy.	[103]
4T1-fLuc mouse breast cancer model	Anti-PD-1 antibody plus ZA	Combination therapy had efficient anti-tumor potential.	[53]
WT mice with 4T1 tumors	Anti–PD-1 mAbs	Partial growth inhibition with anti–IL-1β completely abrogated tumor progression.	[112]
Transgenic mouse model of breast carcinoma	Anti-PD-L1 with external beam radiotherapy	Optimize and monitor the efficacy of PD-L1 blocking.	[114]
TNBC AT3ova (TNBC) and MMTV-neu (HER2).	Anti-PD-1/PD-L1 and MEK inhibitors	Enhanced antitumor immune responses	[115]
4T1.2 breast cancer	Anti-CD73 mAb Anti-PD-1 mAb	Blockade of CD73 can increase therapeutic strategies targeting PD-1/PD-L1.	[109]
Xenotransplanted MDA-MB-231 in nude mice in the mammary breast cancer.	siRNA knock down of PD-L1 with chemotherapy	Anti-apoptotic role for PD-L1 in breast cancer cells.	[104]
Breast tumor, hu-SCID model.	Anti-PD-L1 during DC vaccination	Immunotherapy inhibited tumor growth and enhanced survival time.	[106]
SV40 transgenic WAP-T/WAP-TNP mouse models for mammary carcinomas	Anti-PD1 and anti-PD-L1 mAbs	Immunotherapy arrested tumor growth, with anti-PD-L1 treatment being more effective.	[101]
EMT-6, EMT-6/CDDP	Nintedanib, paclitaxel, PD-L1 antibody	Nintedanib or paclitaxel with the anti-PD-L1 mAb increased overall antitumor efficacy.	[105]
HER-2 positive mouse model of D2F2 and 4TO7 breast cancer cell line	Vaccination and PD-L1 blockade	Combination therapy showed high anti-tumor efficacy.	[107]
Claudin^{low} breast cancer cell line (MDA-MB-231)	PD-L1 shRNA	Therapy attenuated EMT.	[102]
Murine model of ER-negative breast cancer EO771 cells	Anti-IL-17A Ab and/or anti-PDL1	Combination therapy exhibited high anti-tumor efficacy.	[113]
Fo5 (MMTV–humanHER2) breast tumors	T-DM1 and anti-CTLA-4/PD-1	Combination therapy triggered innate and adaptive immunity.	[111]

Abbreviations: ADCC: Antibody-Dependent Cell-Mediated Cytotoxicity/ TNBC: Triple Negative Breast Cancer/ PDX: patient-derived xenograft/ PD-L1: Programmed death-ligand 1/ PD-1: Programmed cell death protein 1/ VEGF*: Vascular endothelial growth factor*/ ZA: Zoledronic *acid*/ WT: wild type/ MEK: Mitogen-activated protein kinase / SCID: severe combined immunodeficiency/ EMT: epithelial-mesenchymal transition/ shRNA: short hairpin ribonucleic acid

Table 3
Clinical trials related to the targeting PD-1/PD-L1 axis in patients with breast cancer.

Trial	Cancer	Therapy	n	Median PFS	Response	Median OS	ORR(%)	Adverse effect	Result	Ref.
KEYNOTE-012 phase Ib multicohort trial NCT01848834.	TNBC PD-L1 ≥1% positive	Single-agent pembrolizumab heavily pretreated median two previous therapy lines	27	1.9 months	17.9 weeks	11.2 months	18.5 1 CR and 4 PR	At least one grade 3 or 4 15.6%: grade 3 or greater, one treatment-related death	Acceptable safety profile and clinical efficacy	[118]
KEYNOTE-086 Cohort A of the Phase II NCT02447003	mTNBC 61.8% PD-L1 +	Pembrolizumab Monotherapy previously treated	170	2.0 months	6.3 months	9.0 months	5	12%: Grade 3 or 4 There was no death	Durable antitumor activity and manageable safety profile	[119]
KEYNOTE-086 Cohort B of the phase II NCT02447003	mTNBC positive score ≥1%.	Pembrolizumab as first-line therapy	84	2.1 months	8.4 months	18 months	23 4: CR	Grade 3 or 4 no patients dying or discontinuing	Manageable safety profile and durable antitumor activity	[147]
KEYNOTE-086 Cohort C phase II NCT02447003	mTNBC positive score 4.7%	Pembrolizumab prior metastatic treatment	170 positive score 4.7%	Months	6.3 months		One CR and seven PRs		An effective treatment	[148]
KEYNOTE-028 phase 1b NCT02054806	ER-positive and HER2-negative disease PD-L1 ≥1% in stroma 19% in tumors	Pembrolizumab heavily pretreated patients	24		7.3 months		12 Three patients: PR(last to 24 weeks)	16%: at least one grade 3 or 4 advers	Well tolerated with modest but durable overall response in certain patients	[121]
KEYNOTE-173 phase Ib study cohortA NCT02622074	TNBC	Pembrolizumab plus chemotherapy	10		pCR rate: 70% cohort A 90% cohort B		A:80 B: 100	Gr 3–4 treatment-related AEs (TRAEs) occurred in 8 pts in A and 10 pts in B; none were fatal	Manageable toxicity and promising antitumor activity	[122]
ENHANCE-1/KEYNOTE-150 phase Ib/II **NCT02513472**	mTNBC	Pembrolizumab eribulin combined received two or fewer lines of previous chemotherapy	107	4.2 months	8.3 months	17.7 months	26.4	19.5% of patients: grade 3 or 4	Improved ORR, with longer PFS, OS	[123]
PANACEA phase Ib/II KEYNOTE-014 NCT02129556.	Advanced HER2-positive breast cancer	Pembrolizumab With trastuzumab	58 trastuzumab-resistant patients	2.7/2.5 months	DCR 25%,	7 months	39 15	No dose-limiting toxicities to pembrolizumab were noted	Immunotherapy can work in patients whose tumors become resistant to first-line therapy.	[124]
A phase I/II study ECHO-202/KEYNOTE-037 NCT02178722.	TNBC	Pembrolizumab heavily pretreated combination with inhibitor of IDO: Epacadostat	39		DCR of 29%	N/A	10	grade ≥3 TRAEs occurred	well tolerated and showed antitumor activity consistent with previously reported P monotherapy	[125]
Phase I study NCT01375842	Metastatic TNBC	Atezolizumab therapy heavily pretreated	115	1.4/ 1.9 months	21 months	1-year OS rate: 41%	10 with long term response achievement	Grade 3–4 2%: grade 5	Acceptable safety and durable clinical benefit with stable disease in earlier lines of treatment	[127]
Phase Ib NCT01633970	mTNBC	Atezolizumab combination with nab-paclitaxel prior 3 lines of chemotherapy	33	5.5 months		14.7 months	39,4	73%: 3–5 grade	An efficient therapeutic strategy for metastatic TNBC patients with an acceptable safety profile	[129]
Phase III NCT02425891	mTNBC	Atezolizumab untreated combination with nab-paclitaxel or placebo plus nab-paclitaxel	902	7.2 months 5.5 months with placebo		15.5 months	N/A	99.3%: atezolizumab–nab paclitaxel group 15.9%: led to the discontinuation of any agent	Prolonged PFS With good safety profiles	[130]
		Durvalumab + tremelimumab	18	2.2 months	18–24%		17	No grade 4 or 5		[132]

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Table 3 (*continued*)

Trial	Cancer	Therapy	n	Median PFS	Response	Median OS	ORR(%)	Adverse effect	Result	Ref.
Phase II study NCT02536794.	HER2-negative MBC					Median OS was not reach in cohorts			Treatment might be beneficial in this subgroup of patients	
MEDIOLA phase I/II NCT02734004	BC Metastatic	Durvalumab + olaparib	25	7			67	Grade 3 or higher events	The combination was well tolerated.	[133]
JAVELIN Phase Ib study NCT01772004	MBC metastatic breast cancer & (TNBC) PD-L1 (81.0%)	Avelumab pre-treated three PD-L1 expression ≥10%	168 58: TNBC	5.9 weeks (5.7 to 6.9)	DCR: 28% 11.4 weeks	9.2 weeks	5.4 one CR eight PR	71% two treatment-related deaths	Acceptable safety profile and clinical activity	[128]
TONIC-trial phase II randomized study/ NCT02499367	TNBC	Nivolumab irradiation or chemotherapy	50	N/A	10.9 months		22 2 CRs 9 PRs	two patients had a SD (4%)	Promising response rate that appear higher than previous monotherapy studies	[126]

Abbreviations: PD-L1: Programmed death-ligand 1/PD-1: Programmed cell death protein 1/TIL: Tumor Infiltrating Lymphocyte/TNBC: Triple Negative Breast Cancer/MBC: Metastatic/IBC: Inflammatory Breast Cancer/EBC: early breast cancer/BC: breast cancer/CR: complete response/PR: partial response/HER2: human epidermal growth factor receptor 2/ER: Estrogen Receptor/PR: Progesterone Receptor/HR: Hormone Receptor/SRS: stereotactic radiosurgery/OS: overall survival/PFS: Progression Free Survival/ORR: Overall Response Rate/DCR: Disease control rate/AEs: adverse event/TRAEs: treatment emergent adverse events/

TIM-3 containing-mAbs was also higher than monotherapy in tumor models [120]. Blockade of CD73 has also been suggested as an effective combinatorial strategy besides the anti-PD-1 therapy for the treatment of metastatic 4T1.2 cancer model because it is demonstrated that activation A2AR promotes the expression of PD-1 [121,122]. Treatment of Fo5 (MMTV–humanHER2) mouse breast tumor models with a combination of anti-CTLA-4/PD-1 and T-DM1 also led to tumor regression [123].

Concomitant blockade of PD-1/PD-L1 axis and inflammatory cytokines was also efficient to attenuate cancer progression. Consistently, an anti-tumor synergistic effect was observed following the treatment of 4T1 breast cancer-bearing mice with anti-IL-1β and anti-PD-1 mAbs [124]. Neutralizing IL-17 could also enhance tumor regression in EO771 (mouse breast tumor model) treated by anti-PD-L1 mAbs [125].

4.1.4. Other combinations

A combination of anti-PD-L1 with radiotherapy was also an effective anti-cancer strategy in the breast tumor model [126]. A combination of anti-PD-1 and MEK inhibitor, selumetinib, was also effective for inducing potent anti-tumor immune responses in MMTV-neu (murine model of HER2 breast cancer) and TNBC tumor models [127]. Similarly, blockade of PD-1 in combination with zoledronic acid had higher tumor-suppressive effects compared to monotherapy in 4T1 tumor-bearing mice [128]. Interestingly, anti-PD-1 immunotherapy in combination with image-guided tumor resection using a fluorescence-labeled PD-1 probe was associated with suppressed regrowth of tumor and metastasis in 4T1 tumor-bearing mice [129].

4.2. Human studies

In addition to *in vivo* mouse breast tumor models, several clinical trials have tried to evaluate the efficacy of anti-PD-L1/PD-1 therapy in breast cancer patients.

4.2.1. PD-1 inhibitors

4.2.1.1. Monotherapy. In phase 1b clinical trial (KEYNOTE-012 study; NCT01848834) in 111 patients with TNBC, Nanda, and colleagues evaluated the anti-tumor potential and safety of Pembrolizumab. They showed that 58.6% of patients had PD-L1-expressing tumors. Intravenous administration of Pembrolizumab (10 mg/kg; median five doses every 2 weeks) into PD-L1 + patients was associated with signs of clinical efficiency and a conceivable safety profile of treatment in advanced TNBC patients [130].

In another phase II clinical trial (KEYNOTE-086 study; NCT02447003) for treatment of previously treated TNBC patients, Adams and coworkers treated 170 patients with intravenous administration of Pembrolizumab (200 mg; every 3 weeks for up to 2 years). 61.8% of patients expressed PD-L1 in tumor cells. Similar to the previous studies, monotherapy of metastatic TNBC patients with Pembrolizumab led to good clinical outcomes in association with safety profile [131]. In another phase II clinical trial (KEYNOTE-086 study; NCT02447003), the same research group investigated the safety and anti-tumor potential efficacy of Pembrolizumab in PD-L1-expressing metastatic TNBC (n = 84)). Monotherapy of patients by Pembrolizumab (200 mg; intravenously every 3 weeks, up to 2 years) induced durable antitumor response in association with manageable safety profile [131]. In cohort C, which was performed in PD-L1-expressing breast cancer patients, 4.7% of overall response rate (ORR), one CR (complete response), and seven PRs (partial response) were detected. The median progression-free survival (PFS) and duration of response (DOR) were 2 and 6.3 months, respectively [132].

Rugo and coworkers also evaluated the safety and potential anti-tumor activity of Pembrolizumab (10 mg/kg, every 2 weeks, up to 2 years) in 248 ER + /HER2-advanced breast cancer patients (luminal subtype), among them, 48 patients were PD-L1-positive (multi-cohort, phase Ib, open-label, KEYNOTE-028; NCT02054806). Their results

showed that treatment had a good safety profile, which was associated with a modest but durable overall response [133].

4.2.1.2. Combination with chemotherapy. In addition to the above-discussed studies, some investigators have tried to elucidate the effect of combination therapy using Pembrolizumab. Accordingly, the impact of combination therapy of TNBC patients (n = 10) using Pembrolizumab and chemotherapeutics also had surprising preliminary results demonstrating ORR of 80% to 100% (KEYNOTE-173 study) [134]. Another clinical combination strategy was evaluated by Tolaney and colleagues based on Pembrolizumab (200 mg, on day 1 of a 21-day cycle) and eribulin (1.4 mg/m^2, intravenously, on day 1 and 8) in 39 patients (7 patients in phase 1b and 32 patients in phase 2) with metastatic TNBC. They suggested that this combination approach can be further considered as a novel promising therapeutic strategy. However, combination therapy showed comparable side effects when compared to monotherapy [135].

4.2.1.3. Combination with other inhibitors. In the combinatorial strategy in a phase 1b/2 clinical trial (NCT02129556; KEYNOTE-014) in 11 centers located in five countries, Loi *et al.* tried to assess safety and anti-cancer capability of Pembrolizumab (2 mg/kg and 10 mg/kg, 3 doses, every 3 weeks) in combination with Trastuzumab (6 mg/kg; 3 intravenous doses) in metastatic HER2+, Trastuzumab-resistant breast cancer patients. They showed that this combination therapeutic approach was safe accompanied by conceivable anti-tumor activity and durable clinical outcomes [136].

A phase I/II trial in heavily pretreated TNBC patients evaluated the efficacy of Pembrolizumab in combination with Epacadostat (inhibitor of indoleamine 2,3-dioxygenase 1) and demonstrated a DCR of 36% and ORR of 10% [137]. Moreover, an initial report regarding a phase II clinical trial (TONIC study, NCT02499367) in 50 metastatic TNBC patients treated with nivolumab in combination with radiation therapy or low-dose chemotherapy implied clinical benefit [138].

4.2.2. PD-L1 inhibitors

4.2.2.1. Monotherapy. In literature, the impact of PD-L1 targeting in breast cancer progression has already been reported. Schmid and coworkers showed that intravenous administration of Atezolizumab (15 or 20 mg/kg or flat dose 1200 mg; every 3 weeks) into metastatic TNBC patients (n = 115) was associated with good tolerability and clinical outcome [139].

In another phase I clinical trial (JAVELIN study; NCT01772004), Dirix and coworkers evaluated the safety and activity of Avelumab (10 mg/kg, intravenously, every 2 weeks) in metastatic breast cancer patients (n = 168), who were resistant to standard therapies. They showed that treatment exhibits conceivable safety and excellent clinical activity. Interestingly, better clinical activity was correlated with PD-L1 expression in tumor-infiltrating immune cells [140].

4.2.2.2. Combination with chemotherapy. Similar to studies targeting PD-1, blockage of PD-L1 is also investigated alone or in combination with other anti-cancer therapeutics. Accordingly, Adams and coworkers have assessed the efficacy and safety of Atezolizumab in combination with nab-paclitaxel in pre-treated metastatic TNBC patients (n = 33, phase Ib trial; NCT01633970). Results indicated the excellent safety profile and marked clinical benefit following the application of this combination approach [141]. The efficacy of this therapeutic strategy in TNBC patients (n = 902) was further substantiated in the phase III study Impassion 130 (NCT02425891) by Schmid and coworkers. Results demonstrated that combination therapy with nab-paclitaxel and Atezolizumab markedly increased OS and PFS in treated patients compared to the placebo group [142]. Treatment of TNBC patients (n = 53) by Durvalumab in combination with chemotherapeutics such as anthracycline, taxane, epirubicin and cyclophosphamide in the phase 2 trial (GeparNuevo study; NCT02685059) was also associated with acceptable safety profile [143].

4.2.2.3. Combination with other inhibitors. A combination blockade of PD-L1 and CTLA-4 (by Durvalumab and Tremelimumab, respectively) in the metastatic ER-positive (n = 11) or TNBC (n = 7) patients had no remarkable clinical benefit implying a low response rate in unselected patients [144]. On the other hand, combination therapy of ER-positive (n = 12) or TNBC (n = 13) patients by Durvalumab (1.5 g IV every 4 weeks) and PARP (poly ADP ribose polymerase) -inhibitor, Olaparib, (300 mg OD for 4 weeks) in a phase I/II trial (MEDIOLA study) had acceptable safety profile, which was associated with marked clinical outcome [145].

4.3. Underway clinical trials

There are also several ongoing clinical trials investigating the safety and clinical activity of pembrolizumab, nivolumab, Avelumab, Durvalumab, Atezolizumab, and other agents as a possible treatment for breast cancer as monotherapy or in combination with chemotherapy regimens, Nab-paclitaxel, Paclitaxel, Tremelimumab, Trastuzumab, Pertuzumab, Ipilimumab, radiation therapy, and hormonal therapy, which are summarized in the Supplementary Table 1.

5. Discussion

The blockade of immune checkpoint molecules has attracted extensive attention during the last two decades. The importance of this issue led to the achievement of the 2018 Nobel Prize by Tasuku Honjo and James P. Allison for their discoveries in the blockade of immune checkpoint molecules in cancer therapy. Although PD-L1/PD-1 blockade is FDA approved for the treatment of some cancers, its efficacy in breast cancer is yet a matter of debate. Several investigations have shown upregulation of PD-L1 in breast cancer, which was correlated with increased frequency of PD-1-expressing TILs. However, there is controversy as to whether it is a positive or a negative prognostic factor. Consequently, Stovgaard and colleagues have recently analyzed PD-L1 expression and its prognostic value in breast cancer in a comprehensive systematic review and proposed that further studies are needed in this issue because there is little consensus on the methods used for evaluating the expression of PD-L1 [146]. Therefore, it seems that further investigations are required to precisely characterize the prognostic value of PD-L1/PD-1 expression in breast cancer. Moreover, several studies are trying to combine PD-L1/PD-1 target therapy with other anti-cancer therapeutics, which can increase the chance of tumor eradication. It is suggested that blockade of checkpoint inhibitors can not only prevent T cell inhibition but can also improve the efficacy of other immunotherapeutic approaches including DC vaccines through enhancing the maturation of DCs and response of anti-tumor primed T cells [147–150].

The main mechanism that promotes breast cancer by the PD-1 molecule is another important issue that needs to be further focused on in future studies. We believe that the expression of this molecule on TILs could increase the growth of breast cancer by inhibiting the anticancer activity of leukocytes. Moreover, even in patients who do not express the PD-L1 (PD-L1-negative tumor cells), expression of the PD-1 molecule can accelerate the growth of breast cancer. This increase can be mediated by the binding of PD-1 molecule to PD-L1 molecules expressed on APCs, leading to inhibition of cells expressing both molecules and ultimately breast cancer progression. Thus, PD-1 expression on TILs may be more important than PD-L1 expression on cancer cells, and therefore, PD-1 may be a better target than PD-L1 for the treatment of breast cancer. However, the efficacy of breast cancer treatment using PD-1 and PD-L1 inhibitors must be evaluated and compared in several studies. Unfortunately, there are very few studies comparing the efficacy of inhibitors of these molecules. In a few studies comparing this, it has been shown that PD-L1 inhibition is more effective than PD-1 inhibition in the animal model of breast cancer [113].

Moreover, there are several unknown issues, which require further

investigation. The adverse effect is a critical issue in the design and development of immunotherapies. It is observed that inhibitors of PD-1/PD-L1 exert modest immune toxicities which may appear several months after treatment and are usually ameliorated using forbidding and immune-suppressive drugs [151]. Skin-related adverse effects (usually with grade 3–4 events) such as dermatitis, pruritus, erythema, rash, photosensitivity reaction, palmoplantar erythrodysesthesia, urticaria, vitiligo, and toxic epidermal necrolysis are of the most common events following administration of PD-1 blockers such as Pembrolizumab and Nivolumab. A maculopapular rash is the most common adverse event following treatment with anti-PD-1/PD-L1 mAbs, which can be managed with topical or oral corticosteroids, depending on severity, along with oral antipruritic agents. Permanent discontinuation of therapy due to dermatologic toxicity has been reported in < 5% of patients in clinical studies [152,153]. Other side effects including gastrointestinal disorders, abdominal pain, diarrhea, anal pain, fever, vomiting, rectal bleeding, and nausea can also appear in some patients treated with PD-1/PD-L1 inhibitors which can be managed with ameliorative medications such as antidiarrheal medications in grade 1 or 2 cases, and immunosuppressive agents in association with life-threatening immunotherapy in grade 3 and 4 events. American Dietary Association's colitis diet and anti-diarrheal medications including atropine and oral diphenoxylate hydrochloride can alleviate the mild or grade 1 colitis [153,154]. Endocrine-related diseases such as hypothyroidism, hyperthyroidism and to a lesser extent acute thyroiditis are other adverse effects of treatment of patients with PD-1 inhibitors which are observed in about 10% of patients and can be managed by anti-thyroid peroxidase and anti-thyroglobulin Abs. Comparing adverse events that emerged following treatment with Pembrolizumab or Ipilimumab showed that hyperthyroidism and hypothyroidism were higher in the Pembrolizumab treated patients, whereas hypophysitis was higher in the Ipilimumab treated patients. It should be noted that immune-related adverse events may appear during the highly variable period. Therefore it is crucial to monitor safety issues for a long time after treatment [153,155].

The current data regarding the efficacy of using PD-1/PD-L1 inhibitors for the treatment of breast cancer points to a hopeful future in this issue. One crucial point is the evaluation of PD-L1/PD-1 expression levels in patients before treatment. It is evident that we expect to have a better outcome in patients with positive expression of these checkpoints; however, it is observed that ameliorative effects are also detectable in negative PD-L1 tumors, which seems to be related to the expression of this molecule on non-tumor cells. It is recommended that for having an optimum outcome, it is necessary to combine anti-PD-1/PD-L1 therapies with novel immunotherapeutics, targeted therapy, radiotherapy, and chemotherapy [90]. In brief, it is expected to have a better ameliorative effect in patients who have a low frequency of TILs and low expression of PD-L1 and thereby low immune response, when anti-PD-1/PD-L1 therapies are combined with conventional chemotherapies [156]. Accordingly, as shown in Supplementary Table 1, several trials are now underway to investigate the efficacy of various combination therapies based on the blockage of the PD-L1/PD-1 axis.

Management of immunosuppressive status generated following treatment of patients is another issue, which should be considered before the use of anti-PD-1/PD-L1 medications. It is suggested that the use of paclitaxel and low-dose cyclophosphamide, which can deplete Tregs or docetaxel and gemcitabine which can suppress MDSCs may control the immunosuppression induced by increased TILs. On the other hand, radiotherapy may increase immune responses in less immunogenic tumors [157,158].

Although overall response rates in trials on the evaluation of the efficacy of anti-PD-1/PD-L1 medications was relatively low, the durable responses in association with safety issues are hopeful for performing future studies. There are some issues, which should be addressed in future investigations. For instance, the exploration of biomarkers, which can anticipate the clinical benefit and response to anti-PD-1/PD-L1 medications is a critical issue for the design of future studies. Moreover, evaluating the efficacy of anti-PD-1/PD-L1 medication combined with various checkpoint inhibitors, radiotherapy, chemotherapy targeted therapy, or novel immunotherapeutics is an essential subject for future investigations.

There is some controversy regarding the prognostic value of PD-L1 expression in breast cancer, which should be clarified in future studies. Although expression levels of PD-L1 were correlated with high-grade histologic characteristics and large tumor size, survival results were correlated with high expression of PD-L1. We believe that longer survival outcomes may be in part related to the induction of robust anticancer immunity, which leads to tumor excitation for expression of evasion mechanisms such as expression of PD-L1. However, it needs further investigations for precise clarification.

The standard for selection of breast tumor sufferers for inhibitors of immune checkpoint inhibitors relies upon the diversity of primary cancer and subtypes concerning PD-L1 expression and the particular outline of TILs. Regarding the subtypes of breast tumors, diversity in PD-L1 expression alters the existence of TILs [77]. Membrane, cytoplasm, and stromal of tumor tissue are three places that contain PD-L1 as much as 64%, 80%, and 93%, respectively, which is demonstrated by immunohistochemical staining and is accompanied with different results [66,159]. It is substantiated that according to a subset of various histological breast tumor subtypes, to some extent PD-L1 generation in tumors and immune tissues can be distinguished. The PD-L1 expression in cancerous cells and TILs is locationally changeable through various site cores in an identical tumor, which suggests that individual biopsies do not exhibit overall PD-L1 condition. Extensive investigations are of paramount importance for finding out the association between PD-L1 expression levels in tumors and stromal immune cells with medical treatment reactions to PD-L1 suppression.

It is worthy to note that elevated PD-L1 expression cannot confirm the response and the absence of its expression on tumor cells cannot eliminate the response occurrence. We believe that this is due to PD-L1 expression on non-tumor cells in the tumor microenvironment. Although tumor cells may be detrimental for PD-L1, other tumor-resident non-tumor cells such as APCs can express this molecule and inhibit tumor-infiltrating T cells by interacting with their PD-1. Therefore, elevated expression of PD-L1 is a factor from the pile of parameters that influence the way that patients respond to PD-1 impediment.

Another central concern is to identify tumor markers, which necessitates having the potential to anticipate the merits of PD-L1/PD-1 blockers in clinical implementations. Moreover, it paves the way for personalized therapeutic strategies. Diverse biomarker classes have been applied and are being upgraded [94], however, the optimal biomarkers have not yet been distinguished.

Till now, the investigation of PD-L1 and other biomarkers has led to variable and sometimes different outcomes [160]. PD-L1 biomarker currently has no suitable test, which is the challenge of this approach. Probably, this is obtained from various assessment strategies such as DNA microarray, IHC, and in situ hybridization. Besides, diverse IHC-antibodies that are used for histological assessment with changeable calculating systems which utilize distinct cutoff scores for various purposes. These purposes include analyzing positivity, and mRNA or protein evaluations. Other strategies include diverse methods for score presumption of mRNA analysis and various examinations concerning expressing cell types, cancer cells, immunological cells capable of penetrating cancer cells, or any of them.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.117437.

Declaration of competing interest

There is no conflict of interest.

Acknowledgment

None.

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