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REVIEW ARTICLE

Targeting PD-1/PD-L1 in cancer immunotherapy: An effective strategy for treatment of triple-negative breast cancer (TNBC) patients

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Abstract Maintaining the balance between eliciting immune responses against foreign proteins and tolerating self-proteins is crucial for maintenance of homeostasis. The functions of programmed death protein 1 (PD-1) and its ligand programmed death ligand 1 (PD-L1) are to inhibit immune responses so that over-reacting immune cells does not cause any damage to its own body cells. However, cancer cells hijack this mechanism to attenuate immune cells functions and create an immunosuppressive environment that fuel their continuous growth and proliferation. Over the past few years' rapid development in cancer immunotherapy has opened a new avenue in cancer treatment. Blockade of PD-1 and PD-L1 has become a potential strategy that rescue the functions of immune cells to fight against cancer with high efficacy. Initially, immune checkpoint monotherapies were not very successful, making breast cancer less immunogenic. Although, recent reports support the presence of tumor infiltrating lymphocytes (TILs) in breast cancer that make it favorable for PD-1/PD-L1 mediated immunotherapy,

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which is effective in PD-L1 positive patients. Recently, anti-PD-1 (pembrolizumab) and anti-PD-L1 (atezolizumab) gets FDA approval for breast cancer treatment and make PD-1/PD-L1 immunotherapy is meaningful for further research. Likewise, this article gathered understanding of PD-1 and PD-L1 in recent years, their signaling networks, interaction with other molecules, regulations of their expressions and functions in both normal and tumor tissue microenvironments are crucial to find and design therapeutic agents that block this pathway and improve the treatment efficacy. Additionally, authors collected and highlighted most of the important clinical trial reports on monotherapy and combination therapy.

Introduction

The unique discrimination ability of our immune system to recognize self from non-self is required both the instruction of innate immunity and guidance of acquired or adaptive immunity. During the transition from unicellularity, multicellular organisms evolve to tightly regulate their structural and functional integrity through intercellular communication between individual cells. But when they fail to regulate their normal developmental programs that appeared during early metazoan life, cancer develops, and its widespread occurrence was observed across the tree of multicellular life[1]. In Immune Surveillance Theory, Burnet hypothesized that, in our body, sentinel lymphocytes are capable to identify and eliminate transformed cancerous cells.[2] T lymphocytes are trained under the supervision of various cell surface molecules where they are educated to recognize the ID card or MHC molecules of self/own cells.

The first mention of any kind of cancer was Breast Cancer (BCa), which was demonstrated in the ancient Egyptian textbook 'The Edwin Smith Papyrus' around 1600 BC. In 1891, Willium B Coley, also known as 'Father of Immunotherapy', first attempted to develop infection by injecting mixtures of live and attenuated bacteria into patient's tumor and achieve complete remission in several types of malignancies. In 1975, Lafferty and Cunningham in their modified 'Two Signal Model' stated that, co-stimulatory second signal is essential for the activation of T cells beside antigenic stimulation.[3] In 1990s, co-inhibitory molecules CTLA-4 and PD-1 were discovered as 'brakes' on immune cells and revolutionized the area of immunotherapy via regulation of the control point of T cells and permit them to eradicate cancer cells.[4] BCa is a heterogeneous disease impacting 2.1 million women each year and causes the greatest number of cancer related deaths among women.[5] Treatment options for aggressive subtypes of BCa such as TNBC is limited. Though BCa was not originally considered as immunogenic, however in case of TNBC and HER2 positive BCa, they respond well to immune checkpoint blockade when applied as monotherapy or in combination with other conventional treatments. In this review, we provide an overview of PD-1/PD-L1 axis and therapeutic application of PD-1/PD-L1 targeting immunotherapy in TNBC.

PD-1 and PD-Ls — linkages with their co-signaling partners

Over the past few decades, the B7-CD28 family has been intensively studied for their crucial roles in various immune regulatory pathways. CD80/CD86/CD28 and ICOS-ICOSL signaling pathways send positive signals that promote T-cell responses whereas, CTLA-4/CD80/CD86 and B7—H1/B7-DC/PD-1 pathways deliver negative signals that limit T-cell responses. BLAST searching identifies B7 and CD28 family like proteins in mammals, birds and probably also in fish dictate the importance of this receptor—ligand interaction. The close proximal presence of CD28, CTLA-4 and ICOS gene cluster on chromosome 2q33 indicate their common evolutionary origin by gene duplication. Whereas PD-1gene is located separately on chromosome 2q37. The B7.1 and B7.2 as well as PD-L1 and PD-L2 are closely linked but the genes for ICOS-L, B7—H3 and B7—H4 are not linked.[6,7]

Programmed death-1 (PD-1; also called CD279) is a 55-kDa monomeric type 1 surface transmembrane glycoprotein consists of 288 amino acid (AA) residues with an immunoglobulin superfamily domain, a ~20 AA stalk, a transmembrane domain and an intracellular domain of ~95 AA residues containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM) (Fig. 1). Engagement of PD-1 with its specific ligands B7—H1 (PD-L1, also known as CD274) or B7-DC (PD-L2, known as CD273) leads to phosphorylation of both the motifs and recruits Src homology region 2 domain containing phosphatases (SHP-1 and SHP-2). PD-1 has a characteristic disulphide bridge between Cys54 and Cys123. However, it lacks the second disulphide linker segment which connects the IgV and transmembrane domains. Additionally, the proline-rich ligand recognition loop was also absent, which makes PD-1 as a unique member of the CD28/B7 family. PD-1 inhibitory function can be lost if ITSM is mutated which indicates its important role. PD-1 ligation occurs only if it is close to antigen receptor engagement by mostly recruiting SHP2 to the phosphorylated Tyr residue of ITSM. PD-1 was identified as an apoptosis associated molecule and shares 21—33% sequence similarity with CTLA4, CD28, and ICOS. Both PD-L1 and PD-L2 are type 1 transmembrane glycoproteins, having IgV and IgC domains. These two ligands share 40% sequence

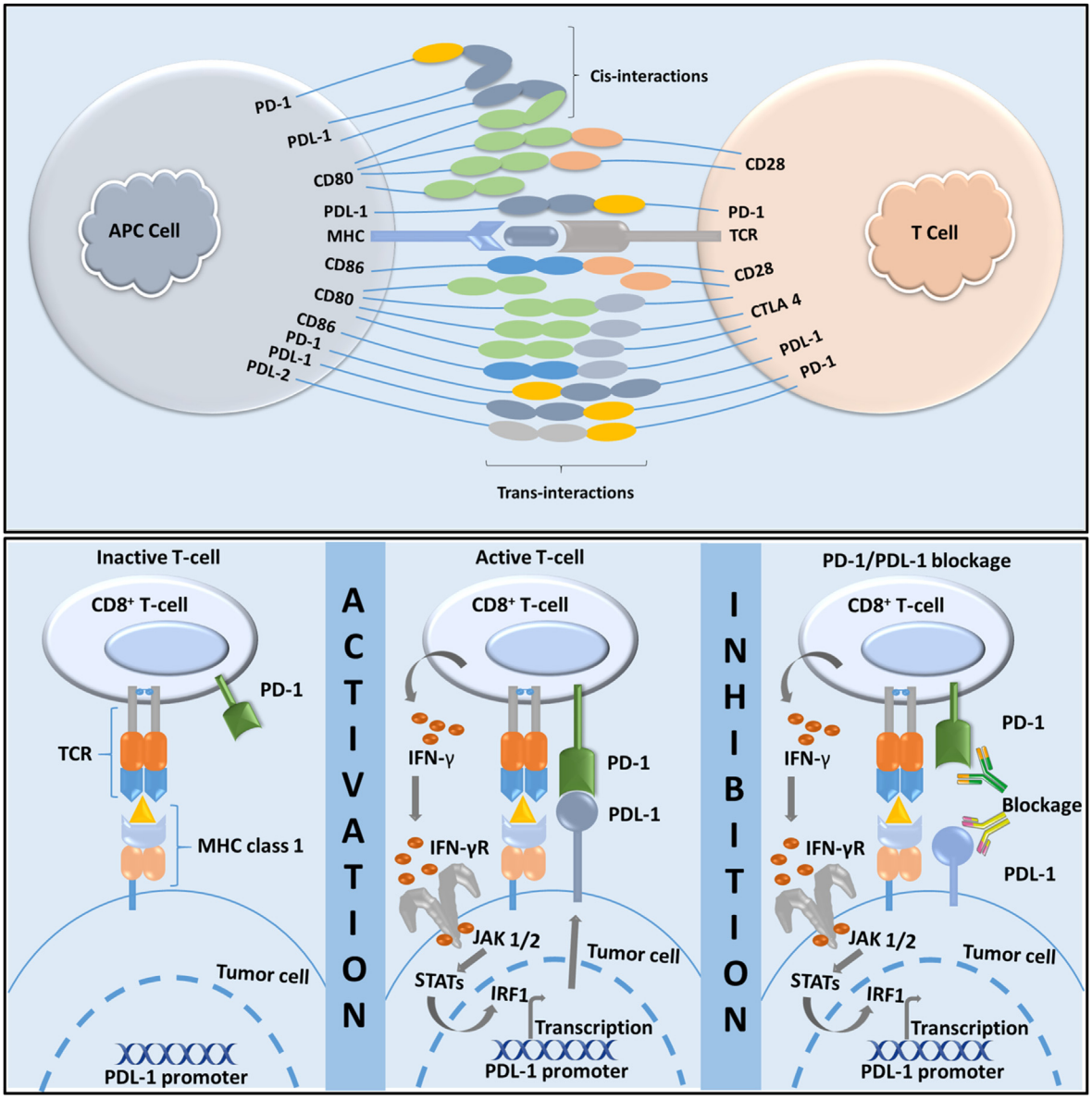

Figure 1 Co-signaling molecules, their linkages and mechanism of PD-1/PD-L1 blockade. Upper panel shows the types of interaction (cis/trans) between APC and T-cell surface. And lower panel shows the mechanism of anti-PD-1/PD-L1 immunotherapy.

similarity with each other and 20% similarity between PD-Ls and B7s. PD-1 is expressed on activated T cells, B cells, natural killer (NK) cells, and monocytes. The 40-kDa PD-L1 and the 25-kDa PD-L2 are encoded by the adjacent genes CD274 and Pdcd1lg2 on chromosome 9 in human. PD-L1 was found during homology searching with B7-1 and B7-2 whereas presence of PD-L2 was identified when T cell response mediated inhibitory function of dendritic cell was recognized. Engagement of PD-L2 to PD-1 exhibits 2—6 folds higher affinity than PD-L1 binding with PD-1. PD-L1 is widely expressed on T cells, B cells, Tregs, macrophages, resting dendritic cells, non-hematopoietic cells and also on some parenchymal cells whereas PD-L2 expression is restricted on macrophages and dendritic cells. Recently, it was found that PD-L1 was also expressed on several cancer cells including BCa.[8,9]

PI3K-AKT-PKCθ signaling axis in T cell regulation

T cell priming initiates when CD3 associated TCR can recognize CD4 or CD8 clustered antigenic peptide-MHC and its intracellular domains are phosphorylated by PTKs (Lck and Fyn). PTKs also phosphorylate ZAP70 kinase and recruit it to the CD3ζ. Phosphorylated ZAP70 kinase then activates LAT, GRB2 and PLCγ1 through which eventually activates MAPKs (ERK1/2) and JNK pathways which are essential for proliferation, differentiation, motility, stress response, apoptosis, survival of T cells and the transcription of IL2. LCK phosphorylation also recruits PI3K complex at the intracellular domain of CD28 and generates PIP3. Next, PIP3 activates PKCθ that produce IL2 and PDK1, which activates PKB/AKT. PKB then further activates its downstream molecules viz., BAD, caspase-9, transcription factors CREB1,

mTOR and GSK3α and GSK3β for regulation of protein synthesis, cellular metabolism and cell survival. In the absence of PD-1 ligation, SCF^{skp2} ubiquitin ligase tags CDK2 inhibitor $p27^{Kip1}$ to the proteasomal degradation machinery and allow CDK2-dependent cell division. But after engagement with its ligand, PD-1 recruits SHP1 and SHP2 phosphatases to the ITIM and ITSM and terminates ZAP70 and PI3K phosphorylation results in inhibition of downstream members viz., AKT, ERK and PKCΘ and transcription of SKP2 (part of SCF^{skp2} ubiquitin ligase). On contrary, accumulation of $p27^{Kip1}$ inhibits CDK2 and eliminates the inhibiting phosphorylation of SMAD3. Activated SMAD3 then expresses the p15-INK4B gene and represses transcription of tyrosine phosphatase CDC25A and removes inhibitory tyrosine phosphorylation of CDK4, CDK6 and CDK2 which results in T cell arrest at the G_0-G_1 phase. Normally for the proceeding of signal transduction, TCR increases the expression of CK2 and inactivates PTEN by phosphorylation that leads to PIP3 accumulation and increase in its activity. But PD-1 engagement reduces the expression and activity of CK2 and activates PTEN which terminates the PI3K-AKT-PKCθ pathway and inhibits the growth and survival of T cell.[10–12]

PD-L1's cryptic expression dictates immunosuppression

Several studies were demonstrated that, PD-L1 binds with PD-1 in trans whereas, it can also bind with CD80 in cis position. This interaction represses CD80/CTLA-4 signaling due to overlapping interface but leave CD80:CD28 pathway unaffected. This indicates that there is another layer of crosstalk present among PD-L1, PD-1, CD-80 and CTLA-4 molecules.[13] Several hours delayed expression of PD-1 in T cell after antigenic stimulation seems not to be the core mechanism. This is answered by some studies indicating that besides co-stimulation which is essential for the activation of T cells, CD-80 also attenuate PD-1 mediated co-inhibitory effect via interaction with PD-L1 and maintain the optimum immune response by restricting PD-1 expression. At the same time this signaling also protect CD-80 from CTLA-4 mediated trans-endocytosis.[14] Another study suggested that TILs, which express both PD-1 and PD-L1, where PD-L1 may act as both ligand and receptor. PD-L1 acts as a receptor and inhibit the differentiation of $CD4^+$ T cells into T helper cells and also prevent the effector function of $CD8^+$ T cells through binding with PD-1 or CD-80. However, as a ligand in forward signaling, PD-L1 binds with PD-1 on another T cell or macrophages. In both the cases PD-L1 can induce immune suppression.[15] By analyzing evolutionary data, it was found that, in the flexible stalk domain of PD-L1 it has many positively selected sites which allow it to bend and form cis interaction with CD-80 molecule on the surface of same cell. The result of the competition between PD-1 and CD-80 to bind with PD-L1 are based on the number of available CD-80 or PD-1 molecules on the local cell surface.[16] Recently a group of studies reported that PD-L1 expressing tumor cells also overexpressed CD-80. Based on this they proposed that soluble CD-80 protein (CD80-Ig) can be used to block the inhibitory signaling that mediated by PD-L1/CD-80 as well as PD-L1/PD-1.[17] Beside this, it was also reported that PD-L1 is essential for the survival of $CD8^+$ T cells. These cells may survive and involve in killing of other CTLs by upregulating PD-L1 which maintain the expression of anti-apoptotic molecule Bcl-xL.[18] Another study found that on tumor cells PD-L1 may act as receptor and upon binding with its ligand PD-1 on T cells, PD-L1 transmit anti-apoptotic signal to the cancer cells and make them more resistant to cytolysis and drug mediated apoptosis.[19]

Role of PD-1 and PD-L1 in tumor microenvironment

A tumor is composed of malignant cells together with other micro environmental factors such as stromal fibroblasts, hematopoietic cells, adipose cells, vascular endothelial cells, immune cells, ECM components as well as several signaling molecules, together they form a dynamic, complex network which is crucial for the growth and survival of tumor. In many ways cancer cells mimic several features of wound healing and embryogenesis to escape immune surveillance. It is likely that, understanding of these common pathways and micro environmental factors may provide advancement of our knowledge to improve the therapeutics of cancer pathophysiology.[20]

In 1863, Virchow first showed a common link between inflammation and cancer. In the first phase of wound healing process, neutrophils secrete various pro-inflammatory cytokines and chemokines to kill microbes and recruit macrophages, NK cells, and T cells which amplify Th1 mediated pro-inflammatory responses. In proliferation phase, neutrophils, macrophages convert from Th1 type to Th2 like phenotype and release anti-inflammatory cytokines and chemokines which in turn suppress immune cells to stop further tissue damage. Macrophages also encourage the proliferation and migration of fibroblasts to start re-epithelialization process and secretes VEGF, TGF-β and IL-18 to promote new vascularization. During ECM remodeling, keratinocytes hyper-proliferate and migrate to the wound bed under the guidance of cytoskeletal proteins as well as cell–cell and cell–matrix interactions with the help of MMPs in a tightly regulated manner. In contrast cancer cells remain in the proliferative and resolution phase and lead to uncontrolled cell proliferation, angiogenesis, abundant expression of MMPs for metastasis and maintain a vascular enriched stroma with immunosuppressive environments. Hence, Dvorak describe tumor as a wound that does not heal.[21]

Common pathways shared by cancer and placenta include hyper-proliferation of trophoblast cells by IGF/MAPK, promotion of angiogenesis by VEGF, FGF as well as HIFs, also cell migration via EMT and activation of WNT pathways which induces loss of cell–cell adhesion. To protect fetus from maternal immune response trophoblast cells downregulate the expression of MHC class I molecules and induce the expression of PD-L1, Tregs, MDSC, macrophages and NK cells. All these molecules and cells suppress maternal immune response by binding their receptors on NK cells, CTLs, B cells, neutrophils and DCs. It seems that cancer cells also achieve immune tolerance by expressing these molecules on their cell surface.[22] HIF play a major role by inducing expression of HLA-G, PD-L1 as well as

promoting angiogenesis, EMT, alteration of cell—cell and cell—matrix adhesion by expressing MMPs in both placenta and tumor hypoxic microenvironment.[23] Pregnancy is a Th2 type phenomenon where maternal cytokines regulate placental development and placental cytokines determine the expression pattern of maternal cytokines, which is very much similar to cancer cells mediated cytokine environment alteration.[24] In tumor microenvironment, CSC is responsible for tumor relapse and metastasis where the reactivation of several crucial embryonic developmental pathways such as Notch, Wnt, Hedgehog, JAK/STAT, PI3K/AKT and NF-κB have been reported.[25] Surprisingly, Feto-maternal tolerance and invasive placenta, which is the unique feature of mammals, may also favor the progression of cancer as it occurs in mammals in higher rates.[26]

Due to their rapid proliferation and lack of comparable vascular support cancer cells quickly consume all the available nutrients and oxygen from surroundings and create a competitive hypoxic microenvironment for other cells. To survive in this condition, cancer cells express HIFs which promote angiogenesis and induce the expression of glycolytic enzymes and several glucose transporters and suppress TCA cycle enzymes to make glycolysis as a central mechanism of metabolism. Cancer induced mutation can activate PI3K-AKT pathway may also enhance the expression of HIFs to induce glycolysis in cancer cells and allow rapid generation of several essential metabolic intermediates to support their ever-increasing cellular needs. Not only cancer cells, but various immune cells also depend upon glycolysis for their transition phase from quiescent stage to effector phase after antigenic stimulation.[23,27]

HIFs can induce the expression of several transporters which helps to release glycolysis derived lactate from cancer cells to stabilize their intracellular pH and acceptance of this lactate as a metabolic fuel by other cancer cells which are present near the blood cells so that they spare glucose utilization for hypoxic tumor cells. Cancer cells also enhance glutaminolysis, and fatty acid synthesis to create a toxic, hypoxic, nutrients depleted microenvironment which severely hamper the differentiation and function of immune cells. Also, HIF1-α induced PD-L1, TAM, MDSC and Treg together make an immune-suppressive environment where immune cells will be able to identify cancer cells but unable to destroy them.[28]

Tumor stromal cells secrete GM-CSF, VEGF and T-helper cells and release various cytokines such as IL-10, IL-40, TNF-α which induces the expression of PD-L1 in cancer cells. Although activation of several oncogenic pathways such as INF-γ/JAK2/IFN, PI3K, AKT/STAT3, MEK/ERK and mutation in tumor suppressor genes PTEN and SOCS1 also upregulate PD-L1 expression via JAK/STAT pathway.[29]

Although PD-1 is not expressed on naïve T cells, after antigenic stimulation multiple transcription factors such as NFATc1 and AP1 can induce PD-1 expression. In response to multiple cytokines stimulation, NFAT/STAT interact with Pdcd1 promoter and induce PD-1 expression. Also, ADAP-SkAP55 induces PD-1 expression through Fyn, Ca^{2+} and NFATc1 control T cell adhesion as well as activation in the synapse between $CD8^+$ CTLs and tumor cells. Persistence of antigens such as TAAs, enhance PD-1 expression on $CD8^+$ T cells results different PD-1 induced exhausted T cells with different recovery potential like intermediate PD-1 expression to high PD-1 expression which may shed light on reversion of exhaustion and restore $CD8^+$ T cell's antitumor function. PD-1 mediated T cell exhaustion are regulated by FoxO1, NFATc1, NF-κB and NR-A. IL-6, IL-12, VEGF-A, TGF-β can suppress T-bet and Blimp1 by directly binding to their promoters. PD-1 ligation induces hypo-responsiveness of T cells through enhancing their metabolic profile from glycolysis to lipolysis by inhibiting several key metabolic pathways such as mTOR, AMPK, PKC1-α as well as down-regulating anti-apoptotic Bcl-xL gene and increasing ROS production which make T cells susceptible to F0-ATP synthase inhibitors.[30,31] Some studies showed that ubiquitous presence of glucocorticoids along with cytokines induce the expression of PD-1 by directly binding to GRE in the PD-1 promoter region on CD56-bright NK cells which are very abundant subtype of tumor infiltrating NK cells. It was also reported that deletion of glucocorticoid receptor (GR) from Treg also reduces PD-1 expression in spleen.[32] GR is also reported to activate the expression of PD-L1 and further transcriptionally repress the expression of MHC class I in pancreatic ductal adenocarcinoma (PDAC) cells. In contrast, the opposite results have also been found upon GR inhibition or downregulation in mouse model of PDAC which results in T cell infiltration and activation. Thus, this study concludes that the anti-tumor immunity and resistance against immune checkpoint blockade (ICB) can be minimized by GR inhibition or downregulation.[33] Additionally, PD-1 can suppress the conversion of functional $CD8^+$ T effector memory cells into $CD8^+$ T memory cells and also regulate the number of memory phenotypes of $CD4^+$ T cells as well as T effector cells by promoting their apoptosis. Thus, it may reduce the long-term memory against cancer cells.[34] Treatment with PD-1 inhibitors showed greater efficacy and several PD-1/PD-L1 mAbs also get FDA approval for the treatment of various types of cancers.[30]

Milestones of cancer immunotherapy

Immunotherapy involves regulation of body's immune system for treatment of various diseases, and this domain is primarily associated with immuno-oncology. Immuno-oncology in cancer therapy involves modulation of immune system for cancer suppression. One of such approaches is inhibition of Programmed Cell Death Protein 1 (PD-1)/Programmed Cell Death Ligand 1 (PD-L1) pathway. Studies have shown that PD-1 inhibition leads to an increase in cancer cell specific immune response. Furthermore, the suppression of PD-1 signaling has been widely associated with the effective penetration of T-cells inside the tumor. Similar response was also reported upon PD-L1 inhibition.[10]

In the late nineteenth century, William B. Coley injected Coley's toxin, a mixture of *Serratia marcescens* and *Streptococcus pyogenes* (live and inactivated bacteria) in more than one thousand patients for induction of sepsis by triggering a strong immunogenicity followed by antitumor response. A stable reduction of malignancies viz., testicular carcinoma, lymphoma and sarcoma was achieved but the limited knowledge about the involved mechanisms and high risk factors make it difficult for oncologists to choose invasion therapies like radiotherapy and surgery as standard treatment.[35] Similar concept resurfaced in 1909 as *Cancer*

immunosurveillance hypothesis which presents the idea of immune system dependent repression of "overwhelming frequency" in carcinoma. Paul Ehrlich hypothesized that during fetal/post-fetal development, occurrence of aberrant cells are normal and it remains latent due to the natural defense system of the body.[36] Almost sixty years later, major neoplasia controlling immune system molecules viz., suppressor T cells (now known as regulatory T-cells or Treg), T-cell receptors (TCRs) and cytotoxic lymphocyte antigen-4 (CTLA-4) were identified in late 70's and 80's. $CD4^+$ and $CD25^+$ suppressor T cells showed suppression of antigen mediated response of other thymocytes sub-population without any involvement of B cells or antibodies.[37] TCRs are presented on thymocytes surface and are responsible for the recognition of antigen-MHC complex[38] whereas CTLA-4 was identified as receptor for B7, a co-stimulatory ligand of T cell, which is associated with decreased immune response.[39]

Discovery of CD28 and T cell activation mechanism, inhibitory response of CTLA4, co-stimulatory response of CD28 on T cells[40] and tumor rejection upon administration of anti-CTLA4 antibodies are the major milestones acquired between 1994 and 1998. CD28 is expressed on activated as well as resting cells and stimulates T cells proliferation via bound anti-TCR antibodies.[41] T cell activation was found to be dependent on interaction of TCR with antigen-loaded MHC, binding of CD28 to B7 and cytokine activation.[42] Moreover, it was reported that T-cell proliferation was partially inhibited in presence of B7.2 due to CTLA-4 interaction.[40] In the preclinical studies it was shown that administration of anti-CTLA4 antibodies in mouse leads to the rejection of stabilized/new tumors and also induced immune memory upon secondary exposure.[4] From 1997 to 2007, development of ipilimumab, human monoclonal antibody against CTLA-4 and recognition of PD-1 and PD-L1 were the prime discoveries.[43] Ipilimumab is an IgG1—kappa immunoglobulin which overexpressed in CHO cells and available as single dose IV infusion of 50 mg per 10 ml or 200 mg per 40 ml sterile solution.[44] PD-1 (288 amino acids), an activated T cells surface protein, was reported as immune system down regulator when PD-1-deficient mice developed autoimmune features with late onset, organ-targeted effects and hampered penetrance.[45] PD-L1 was identified as B7—H1, a B7-1/B7-2 homologous molecule which is able to co-stimulate and activate T cells through a non CTLA4/CD28/ICOS receptor leading to enhanced IL-10 production.[46] Cancer immunotherapy trials were established in 2010 and inhibition of PD-1 checkpoint was one of the major approaches (Fig. 2).

In 2011, hallmarks of cancer were elaborated in detailed. Ipilimumab (anti-CTLA4 antibody) was approved by FDA after testing of clinical safety marketed under Yervoy brand name. This approval was for melanoma treatment upon 10 months overall survival.[44] In 2014 and 2016, nivolumab (OPDIVO) and atezolizumab (Tecentriq) were developed as FDA approved antibodies specific to PD-1 and PD-L1 respectively. In comparison to docetaxel, risk of dying decreased by 27% in OPDIVO recipients and Tecentriq had 12.3 months overall survival.[47] In 2017, axicabtagene ciloleucel (Yescarta) was approved for chimeric antigen receptor (CAR) T-cells therapy having ORR of 72%, 51% Complete Remission (CR) rate and 21% Partial Remission (PR) rate respectively. During 2017—2018, PD-1 blockers: pembrolizumab (Keytruda) and cemiplimab (Libtayo); PD-L1 blockers: durvalumab (Imfinzi) and avelumab (Bavencio) were approved by FDA. Keytruda and Libtayo were reported with 68% and 41% ORR respectively.[48,49] Imfinzi showed 47.5 months median OS upon 5 years

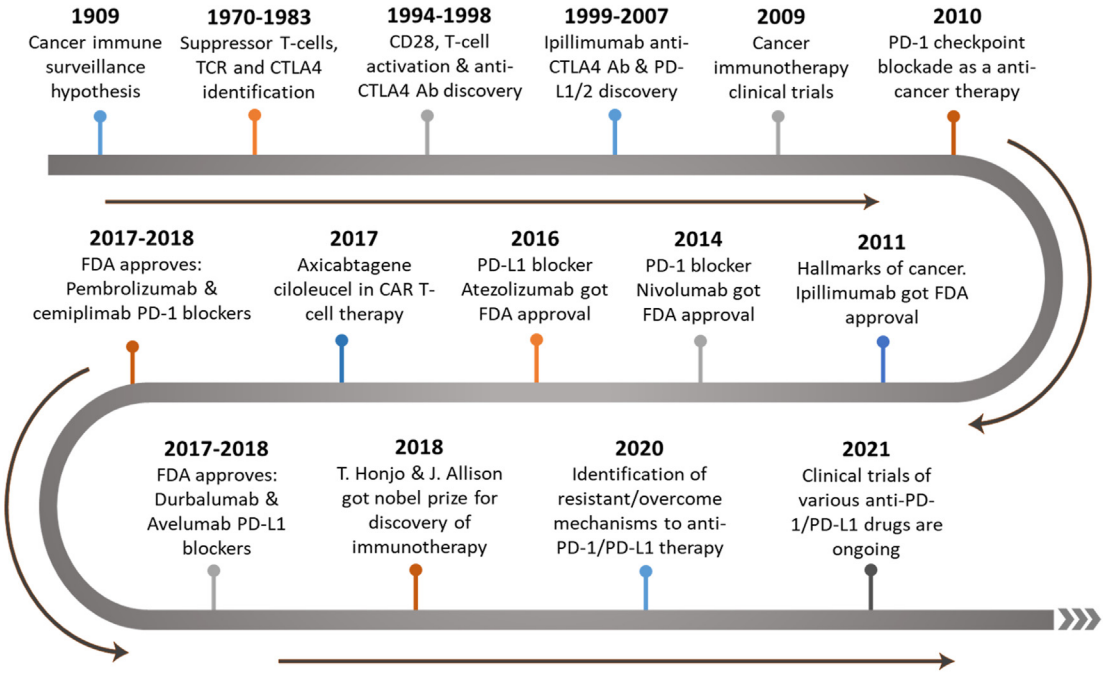

Figure 2 Milestones of PD-1 and PD-L1 immunotherapy. The figure depicts the timeline of all the major milestones in immune-oncology research and the chief modulators of PD-1 and PD-L1 signaling pathways.

analysis whereas Bavencio showed 31% reduction of death risk and improvement in OS by 7.1 months.[50] Furthermore, T. Honjo and J. Allison received Nobel Prize in 2018 for the discovery of cancer immunotherapy by preventing the negative immune regulation. This study explored negative immunomodulation for therapeutic suppression of cancer by inhibiting immune checkpoint for effective elimination of cancer cells.[51] In 2020, primary as well as acquired resistant mechanisms of PD-1/PD-L1 targeted immunotherapy were reported. Simultaneously, the various combinatorial therapeutic strategies were developed to overcome these resistance mechanisms. Some of these include enhanced immunogenicity by modulating tumor microenvironment, epigenetic regulations, exhaustion of T cell and increasing T-cell infiltration in tumor site as discussed in the later part of this review.[52]

At present, number of anti-PD-1/PD-L1 antibodies are in clinical trials (Table 1). To better understand the therapeutic perspectives of PD-1/PD-L1 axis, we should know about the types of resistance developed during immunotherapy. In the following section of this review, the authors are interested to discuss the resistant mechanisms acquired and strategies to overcome them.

Resistance mechanisms in cancer immunotherapy

Anti-PD-1/PD-L1 immunotherapy is a promising therapeutic strategy against multiple cancers including BCa. But reduction in effectiveness is also noticed due to their primary or acquired resistance developed with progression of therapy. Patients group who never respond to the first time use of anti-PD-1/PD-L1 immunotherapy is termed as a "primary resistance".[53] Several studies have explored the phenomenon of primary resistance in which either $CD8^+$ T-cells are not able to recognize cancer cells or rendered ineffective. This resistance mechanism also depends upon the cell types which exerts local immunosuppressive effects within tumor microenvironment or inherited resistance of tumor cells from the T-cells[54] (Fig. 3). On the other half group of patients who respond initially but adopt resistance after their frequent/continuing use is termed as "acquired resistance". Mechanisms of resistance are too much complex because it depends upon multiple genetic factors and tumor microenvironment.[55]

Primary resistance

Poor T-cell infiltration — Host $CD8^+$ T-cells are failed to recognize and localize the tumor, due to the absence or insufficiency of antigens over the tumor cells.[56] Additionally, occurrence of mutational burden in the tumor cells contributes to the limited expression of tumorigenic antigens which may leads to limited or no recognition by host $CD8^+$ T-cells. That is why some cancer patients with low mutational burden are non-responders to the anti-PD-1/PD-L1 immunotherapy. Cancer patients with high mutational burden (viz., head and neck, bladder, NSCLC, melanoma, and microsatellite unstable cancers) are mostly respond to the anti-PD-1/PD-L1 immunotherapy as the neo-antigens has capability to activate the $CD8^+$ T-cells which leads to PD-1/PD-L1 interaction between T-cells and tumor cells. So anti-PD-1/PD-L1 immunotherapy will give rise to anti-tumor activity on tumor cells with high mutational burden.[57] Exceptionally, the renal cell carcinoma (RCC) is a tumor with low mutational burden hence non-responder[58] but the prostate and pancreatic cancer patients with high somatic mutations are non-responders to the anti-PD-1/PD-L1 immunotherapy.[59,60]

To encounter this problem, supply of T-cells specific antigens to the patients may be an effective strategy. Adoptive cell therapy (MART-1 and NY-ESO-1 in melanoma and sarcoma respectively) is a strategy that involves the induction of huge amount of $CD8^+$ T cells those are specific to tumor antigens.[61,62] Induction of tumor antigen specific $CD8^+$ T cells can be done by *ex-vivo* expansion of endogenous $CD8^+$ T cells or *ex-vivo* genetic modification of peripheral mononuclear blood cells, and then re-infused into patient.[63] Alternatively, tumor vaccines may be an effective strategy which is capable to stimulate the patient's dendritic cells that produces T-cells activity specific to tumor antigens.[64] Recently, for the treatment of melanoma the modified human herpes simplex virus (HSV) in

Table 1 FDA approved anti-PD-1/PD-L1 antibodies against different cancers.

Target	Agent	Brand	Class	Cancer type
PD-1	Pembrolizumab	Keytruda (Merck)	IgG4K	BCa, NSCLC, SCLC, HL, UC, GEJ, HNSCC, HCC, RCC, MCC, Melanoma, Cervical cancer, Primary mediastinal large BCL, Adenocarcinoma, Endometrial carcinoma
	Nivolumab	Squibbi Opdivo (Bristol-Myers)	IgG4	HNSCC, ESCC, UC, CRC, HCC, RCC, NSCLC, Melanoma, Classical HL
	Cemiplimab	Libtayo (Sanofi and Regeneron)	Combination of Ab and drug	Cutaneous squamous cell carcinoma
PD-L1	Atezolizumab	Tecentriq (Genentech/Roche)	IgG1K	BCa, HCC, NSCLC, SCLC, UC
	Avelumab	Bavencio (Pfizer)	IgG1	MCC, UC, RCC
	Durvalumab	Imfinzi (Astrazeneca)	IgG1K	UC, NSCLC, SCLC

Abbreviations: BCa: Breast Cancer; NSCLC: Non-small cell lung cancer; SCLC: Small-cell lung cancer; HNSCC: Head and neck squamous cell carcinomas; GEJ: Esophagogastric junction; HCC: Hepatocellular carcinoma; RCC: Renal cell carcinoma; ESCC: Esophageal squamous cell carcinoma; MCC: Markel cell carcinoma; HL: Hodgkin's lymphoma; BCL: B cell lymphoma; UC: Urothelial carcinoma, CRC: Colorectal cancer and Ab: Antibody.

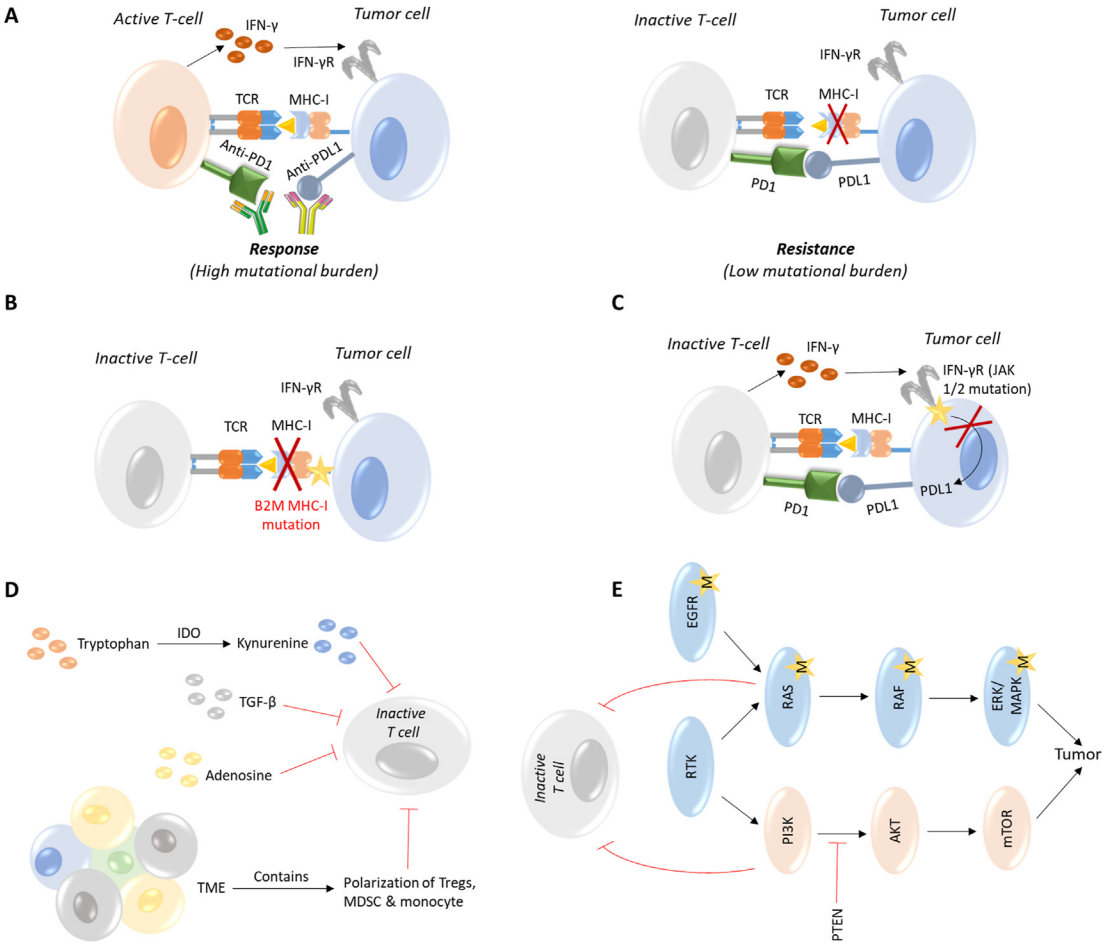

Figure 3 Primary resistance mechanisms. The figure depicts primary resistance mechanisms of anti-PD-1/PD-L1 immunotherapy occurs due to **(A)** poor T-cell infiltration, **(B)** abnormality in MHC's, **(C)** mutations in Interferon- γ (IFN- γ) signaling, **(D)** immunosuppressive TME, and **(E)** oncogenic mutations.

combination with anti-PD-1 agents is studied in phase 1b clinical trial.[65] Thus, the combination of adoptive cell therapy or tumor vaccines with anti-PD-1/PD-L1 agents may be an ideal strategy to fight against cancer in future.

Irreversible T-cell exclusion — T-cell exclusion is a dysfunctional state which may occur due to the extensive expression of tumor antigens, primary mutations in tumor and immunosuppressive tumor microenvironment (TME). In this state, T-cell tracking system is not able to trace the tumor antigen expressed in TME. Recently, activation of Wnt/β-catenin signaling is reported to result into T-cell exclusion. Inverse correlations were reported between $CD8^+$ T cells infiltration and activation of Wnt signaling in the mouse model of melanoma.[66] They also found activation of this pathway in melanoma model responded poorly to anti-PD-1 immunotherapy, while decreased expression of Wnt/β-catenin gives better response. Multiple drugs inhibiting Wnt signaling are under clinical trials. To overcome this primary resistance, anti-PD-1/PD-L1 therapy can be used in combination with Wnt signaling inhibitors.[67]

Mutation in the members of mitogen activated protein kinase (MAPK) signaling cascade is also associated with T-cell exclusion. These mutations will cause generation of immunosuppressive cytokines viz., VEGF, IL-8; results into inhibition and loss of functionality of T-cells that make cells resistant to anti-PD-1/PD-L1 immunotherapy.[68] Inhibition of this signaling cascade in combination with anti-PD-1/PD-L1 immunotherapy may overcome this primary resistance.[69] Similarly, loss of function of PTEN also contributes resistance to the anti-PD-1/PD-L1 immunotherapy, due to the overproduction of VEGF and reduced $CD8^+$ T cells infiltration in tumor cells. Addition of PTEN/PI3K inhibitors to the anti-PD-1/PD-L1 immunotherapy will improve such kind of primary resistance mechanism.[70]

Interferon-γ (IFN-γ) signaling — Upon antigenic recognition by TCR, T-cells produces the IFN-γ which activates IFN-γ receptors and subsequently activates JAK1/2, and subsequent phosphorylation of STAT1/3. By this signaling cascade, most of the activated STAT1/3 target genes contribute to the anti-tumor immunity; but activation of CD274 (PD-L1 encoding gene) leads to inactivation of tumor specific T-cells. Mutation in JAK1/2 disrupts the IFN-γ signaling and limits the expression of PD-L1. Similarly, in some kind of tumors researchers have noticed high mutational burden and is also resistant to anti-PD-1/PD-L1 therapy viz., colon cancer and melanoma. JAK1/2

mutations are also mostly found in the tumor with high mutational burden. Hence, mutations in IFN-γ signaling is a major contributor of resistance to anti-PD-1/PD-L1 immunotherapy.[71,72]

Abnormality in MHC's — MHC class-I is mostly involved in tumorigenic antigen presentation in the TME and then T cells are able to recognize and destroy these cells. Inactivation of MHC class I complex may contribute to the failure of tumor rejection. Mostly, B2M mutation in HLA class I complex causes abnormality in antigen presentation and results in failure of T cells to reject tumor.[73] B2M mutations are more frequently occur in the colorectal cancer tissues. One more recent study observed that B2M mutation occurs three times higher in the non-responders as compared to the responders to anti-PD-1 immunotherapy in melanoma. Altogether, it concludes that mutational abnormality in MHC class—I complex may contribute and a major cause of resistance to anti-PD-1/PD-L1 immunotherapy.[74]

Beside B2M mutations, autophagy or lysosome mediated degradation of MHC-1 proteins with the involvement of cargo receptor NBR1 was also reported as a resistance mechanism against anti-PD-1/PD-L1 immunotherapy. Here, authors have found the reduced expression level of MHC-I protein at cell surface and enhanced expression in autophagosome and lysosome. Additionally, they suggested a novel approach to overcome this resistance by inhibition of autophagy either genetically or using small molecule (viz., chloroquine) which leads to the restoration of surface MHC-1 protein expression results in improvement of antigen recognition and promotion of anti-tumor T cell response. Furthermore, they also demonstrated the synergistic role of autophagy inhibition in combination with other ICB therapies using anti-PD1 and anti-CTLA4 antibodies to overcome this resistance and enhance the anti-tumor immune responses.[75]

Immunosuppressive TME — Tumor cells instruct immunosuppressive cells, cytokines and tumor metabolites to defeat the antitumor immunity and promote their differentiation, proliferation, elongation, and invasion.[76] Regulatory T (Treg) cells act as a negative regulator of $CD8^+$ T cells in TME through the generation of various immunosuppressive molecules viz., TGF-β, IL-10 and extracellular adenosine. However, the reduction of IL-2 in TME is also a major contributor of Treg cells mediated negative regulation.[77—80] Furthermore, the *in-vivo* studies conclude that Treg cells promote expression of PD-1 in $CD8^+$ T cells and causes primary resistance to anti-PD-1 immunotherapy.[81]

Secondly, myeloid-derived suppressive cells (MDSCs) a component of TME is also responsible for the production of various immunosuppressive factors (viz., NO, ROS and IL-10), that can suppress both specific as well as non-specific immune responses of $CD8^+$ T cells and promotes cellular invasion and angiogenesis. Furthermore, the presence of MDSCs in TME is also documented to induce primary resistance against anti-PD-1 therapy in non-small cell lung carcinoma. Thus, MDSCs in TME and anti-PD-1 has an inverse correlation in immunotherapy.[82] Similarly, the presence of other immunosuppressive factors such as cytokines,[83] tumor associated macrophages (TAMs)[84] and indoleamine 2,3-dioxygenase (IDO)[85] are also responsible for resistance to anti-PD-1 immunotherapy.

Oncogenic mutations — In general, the various oncogenic signaling pathways has well-known contribution in TME. Mutations in these signaling pathways can cause irregularities in the expression pattern of immunosuppressive cells in TME that may produce resistance in immunotherapy.[86] EGFR pathway is a well-known pathway in tumor biology, mutations in EGFR were studied to suppress the expression of PD-L1 that also contribute resistance.[87] However, the loss of PTEN is also linked with the resistance, due to the secretion of VEGF or by the production of immunosuppressive cytokines.[70] Thus, the oncogenic mutations may also be responsible for the primary resistance in anti-PD-1/PD-L1 immunotherapy.

Acquired resistance

Acquired or adaptive resistance is through immuno-editing, depletion of memory T cells and activation of inhibitory signaling. In few reported case studies, initially the host cell responds to immunotherapy but its long term use develop resistance. This type of resistance mechanism is quiet similar to the primary resistance that developed by immuno-editing[55] (Fig. 4).

Immunoediting — The immunity raised in anti-PD-1/PD-L1 immunotherapy prevents the tumor progression and ultimately results in tumor sub-clones that can prevents the anti-tumor immunity and develop acquired resistance.[88] However, the B2M mutation in MHC class I receptor has been noticed in pre-immunotherapy samples that was responsible for primary resistance in anti-PD-1/PD-L1 immunotherapy. But, in some cancers (viz., colorectal, melanoma and lung cancers) the B2M mutations are acquired during the course of the immunotherapy.[74,89,90] In the patients with acquired resistance, tumors were no longer responded to the IFN-γ. After long term use of this immunotherapy, JAK1/2 homozygous mutations were detected in melanoma samples.[89]

Depletion of memory T cells — After removal of antigen, cytotoxic T cells were transformed into memory cells that can be reactivated upon re-occurrence of antigen. Memory T cells were stored in an inactive form, on reoccurrence of antigen they become active and play important roles in immune response. On the other hand, the long term use of anti-PD-1/PD-L1 immunotherapy develop resistance through depletion of memory T cells.[91]

Activation of inhibitory signaling — Anti-PD-1/PD-L1 immunotherapy blocks multiple immune checkpoints, but still the tumor cells were escaped from their encounter through the activation of several inhibitory signaling pathways. During the course of this immunotherapy, the compensatory inhibitory signaling checkpoints (viz., TIM3, CD73, PD-L1, etc.) are activated in TME making the task difficult by PD-1/PD-L1 blockage. This type of resistance is generally acquired through the occurrence of metabolic alterations due to the long term use of anti-PD-1/PD-L1 immunotherapy.[92—94] Thus, both primary and secondary mechanisms depend on multiple factors within the TME and effectively reduces the efficacy of anti-PD-1/PD-L1 immunotherapy against multiple cancers. Scientists and clinicians has to be come forward and think more on this serious issue. Combination therapy may overcome this to enhance the effectiveness of anti-PD-1/PD-L1 immunotherapy.[30]

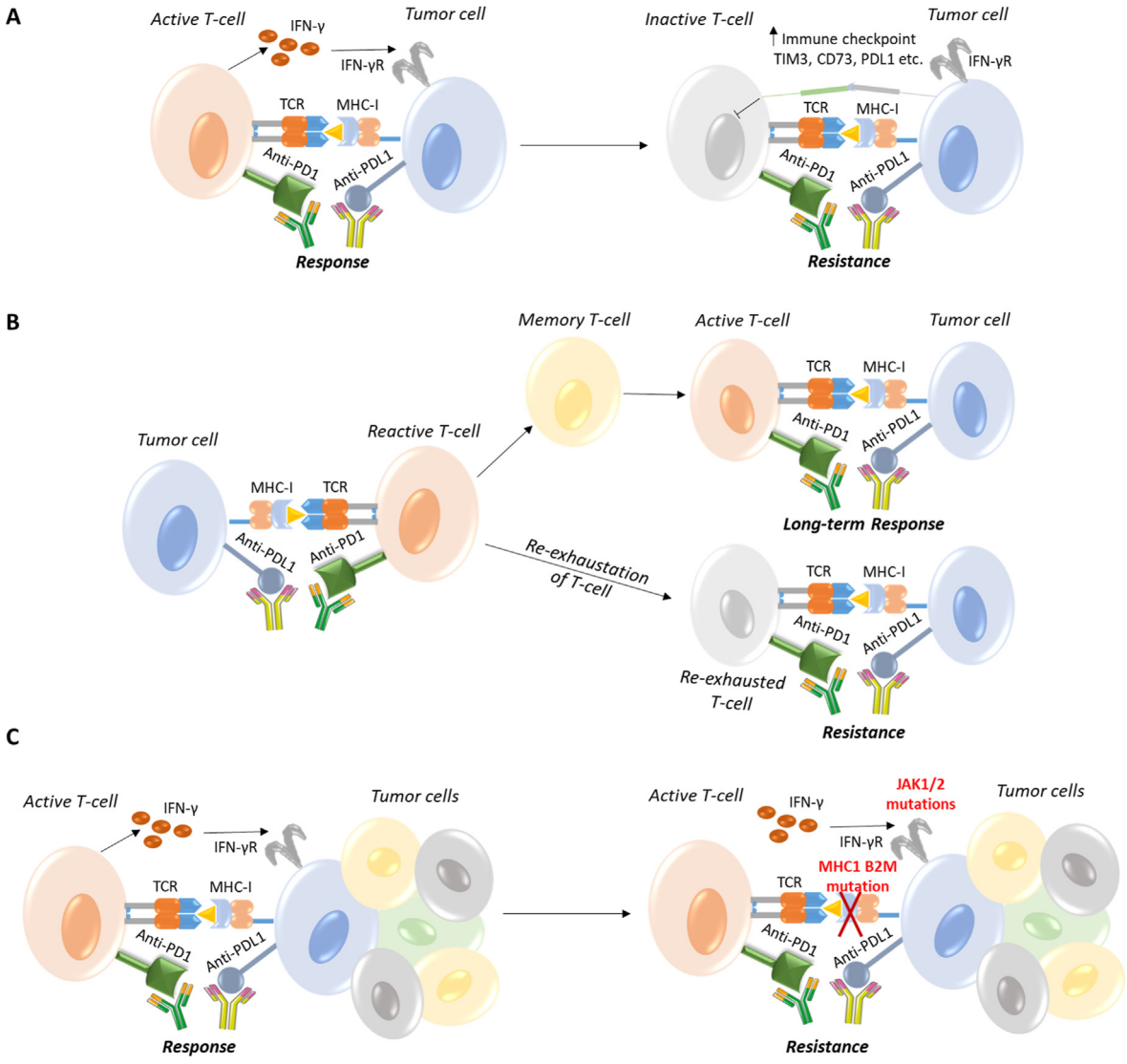

Figure 4 Acquired resistance mechanisms. The figure depicts acquired resistance mechanisms of anti-PD-1/PD-L1 immunotherapy occurs due to **(A)** immunoediting, **(B)** depletion of memory T cells, and **(C)** activation of inhibitory signaling.

How to overcome resistance against anti-PD-1/PD-L1 immunotherapy?

Induction of T cell infiltration, T cell priming, enhancement of immunosuppressive TME and preventing the depletion of memory T cells can be employed to overcome the resistance against anti-PD-1/PD-L1 immunotherapy.[95] Currently, various possible strategies are pursuing for clinical development and approaches to overcome resistance (Table 2).

Priming, infiltration and exhaustion of T cell — The cytotoxic chemotherapeutic agents promote the release of antigen from tumor cells. These agents can induce the damage through apoptosis, and the conversion of dendritic cells to antigen presenting cells for T cell priming.[96] Decitabine helps in increasing the antigen recognition by enhancing the expression of MAGE-A3 in esophageal malignancy.[97] Few cytotoxic chemotherapeutic agents (viz., cyclophosphamide and gemcitabine) deplete the MDSCs, Tregs and destroy the tumor cells.[98,99] The synergistic effect is observed in patients who have received chemotherapy in combination with anti-PD-1/PD-L1 immunotherapy.[96,100]

The multi-peptide vaccine in combination with anti-PD-1/PD-L1 immunotherapy is studied to improve the overall survival of melanoma patients, by enhancing T cell priming.[101] Similarly, oncolytic virus also promote the release of tumor associated antigens and simultaneously increases the T-cell priming to prevent development of resistance against anti-PD-1/PD-L1 immunotherapy.[102–104] Radiation therapy in combination with this immunotherapy is also one more option to combat resistance and enhance efficacy. This combination therapy promotes destruction of tumor cells by enhancing the release of IFN-γ and T cell priming.[105,106] Radiotherapy followed by use of pembrolizumab treatment also enhances the overall survival of non-small cell lung cancer patients.[107]

Furthermore, TLRs (viz., TLR3 and TLR9) targeting drugs prevent the development of resistance in anti-PD-1/PD-L1

Table 2 Combinatorial therapeutic strategies to overcome the resistance against anti-PD-1/PD-L1 based immunotherapy.

Therapeutic strategy used	Therapeutic combination	Clinical/Pre-clinical Tumor (cell) type	Outcome	Clinical Phase (NCT no.)	Ref.
Promotion of T-cell priming					
Chemotherapy	Pembrolizumab + Carboplatin + Paclitaxel	NSCLC	Improved survival	III (NCT02775435)	272
	Pemetrexed + platinum- drugs + Pembrolizumab	NSCLC	Improved survival	III (NCT02578680)	100
Oncolytic virus vaccine	T-VEC + Pembrolizumab	Melanoma	Study in progress	III (NCT02263508)	102,103
	MPV + Nivolumab	MART-1 (+) ve tumor	Reduced toxicity	NA	104
	Neoantigen vaccine + Pembrolizumab	Melanoma	Reduced growth	I (NCT01970358)	104
Radiotherapy + Chemotherapy	Radiotherapy + Pembrolizumab	NSCLC	Regression	I (NCT01295827)	109
TLR agonist	ARNAX + PD-L1 Ab	Multiple cancers (EG7)	Regression	—	108
	Lefitolimod + PD-1/PD-L1 Ab	CRC (CT26), BALB/c, B cell lymphoma, A20	Regression	—	109
Interferon-α	IFN-α anti-PD-L1 fusion protein	Multiple cancers (A20, MC38, B16F10 & L929)	Regression	—	111
Back-pedal of T-cell exhaustion					
Immune checkpoint blockade	Anti-TIM3 Ab + Nivolumab	NSCLC	Reduced resistance	NA (NCT02281214)	92—94
	Anti-TIGIT + PD-1 Ab	GBM	Improved survival	—	115
Co-stimulatory agonist	Anti-CD40 + PD-1 Ab	RCC	Regression (Reduced PD-1)	—	116
Promotion of T-cell infiltration					
Co-stimulatory agonist	Ab-guided LIGHT fusion protein + PD-L1 Ab	Adenocarcinoma	Regression (T cell infiltration)	—	112
Improving immune-suppressive TME					
TGF-β blockers	TGF-β blockers + PD-L1 Ab	Multiple cancers (EMT6 & MC38)	Regression (T cell infiltration)	-	120
	SRK-181-mIgG1 + PD-1 Ab	Multiple cancers (EMT6, S91 & MBT2)	Regression (T cell infiltration)	—	119
Cytokine receptor blockers	CSF1R blocker + PD-1 Ab	Adenocarcinoma	Regression	—	118
	Anti-CCR4 mAb	Melanoma	Regression (Tregs depletion)	—	121
	Anti-CXCR2 mAb + PD-1 Ab	Rhabdo-myosarcoma	Regression	—	122
PI3K inhibitor	PI3K inhibitor + PD-1 Ab	Myeloid carcinoma (B16F10)	Regression	—	123
Epigenetic modulators	DZNep + 5-AZA-dC + PD-1 blocker	Ovarian cancer	Regression	—	124
IDO inhibitor	INCB23843 + PD-L1 Ab + CTLA4 Ab	Myeloid carcinoma B16F10	Regression	—	125
Adenosinergic pathway inhibitor	Ciforadenant + PD-L1 Ab	Multiple cancers (MC38)	Regression	-	126,127
	Ciforadenant + Atezolizumab	RCC	Regression	I (NCT02655822)	128
	MEDI9447 + PD-1 Ab	Breast cancer (4T1 and MDA-MB-231)	Regression	I (NCT02503774)	129
Other combinatorial approaches					
Oncogenic pathway inhibitor	Vemurafenib + PD-1/PD-L1 Ab	BRAF mutated	Regression	—	130
	Dabrafenib + Trametinib + Pembrolizumab	Metastatic melanoma	Durable response	I (NCT02130466)	131

Microbiota	Bifidobacterium + PD-L1 Ab	Melanoma	Regression	–	[132]
	Fecal transplant + PD-L1 Ab	Melanoma	Regression	–	[133]

Abbreviations: CTLA4: Cytotoxic T lymphocyte associated antigen 4; GBM: Glioblastoma multiforme; IDO: Indoleamine 2,3-dioxygenase; MPV: Multi-peptide vaccine; Ab: Antibody; mAb: Monoclonal antibody; NSCLC: Non-small cell lung cancer; PD1: Programmed cell death 1; PDL1: Programmed cell death ligand 1; PI3K: phosphatidylinositol-3-kinase; RCC: Renal cell carcinoma; TGF- β: Transforming growth factor beta; TIM3: T-cell immunoglobulin and mucin-domain containing 3; TLR: Toll like receptor; TME: Tumor microenvironment; Treg: Regulatory T cell.

immunotherapy.[108,109] High expression of CTLA4 is considered as a negative regulator of T cell priming. Anti-CTLA4 antibodies in association with anti-PD-1/PD-L1 antibodies improves the patients overall survival.[110] However, IFN-γ is critical for T-cell priming. To enhance the T cell priming, researchers have developed a fusion protein (anti-PD-L1-IFN-α) that directly delivers the IFN-α to the tumor cells. Its intravenous administration enhances tumor regression in mice model.[111] Taken together, all these studies conclude that enhancement of T cell priming can reduce the resistance of anti-PD-1/PD-L1 immunotherapy. Promoting T-cells infiltration in combination with PD-1/PD-L1 blockade helps to build up sufficient amount of anti-tumor immunity. Anti-EGFR guided LIGHT fusion protein was tested in pre-clinical studies, which shows sufficient amount of increment in T-cell infiltration.[112] Additionally, the adoptive cell transfer in combination with anti-PD-1/PD-L1 immunotherapy promotes T-cell infiltration and cytotoxicity.[113]

Reversal of T cell exhaustion can be achieved by targeting immune checkpoints or expanding the co-stimulatory signals that may to lower the resistance of PD-1/PD-L1 blockade.[114] The PD-1/PD-L1 blockade in combination with other ICBs (viz., TIM3 and TIGIT blockade) is already proven to improve the overall survival rate in multiple studies.[93,115] Additionally, the CD40 antagonistic antibody is tested to reverse the T cell exhaustion and provide cytotoxicity to the tumor cells.[116]

Improving immunosuppressive TME — TME consists of multiple immunosuppressive cells where tumor associated macrophages (TAMs) is one of them, which can be targeted to improve the efficacy of anti-PD-1/PD-L1 immunotherapy.[117] Furthermore, in pre-clinical studies the anti-CSFIR medications in combination with this immunotherapy were also evaluated for tumor regression.[118] However, the cytokines present in TME helps not only to suppress anti-tumor immunity but also prevent the cytotoxic effects of $CD8^+$ T cells.[119] Targeting cytokines (TGF-β-1) in combination with PD-1/PD-L1 blockade results in the increment of T-cell infiltration, anti-tumor immunity and reduction in the resistance to immunotherapy.[120] Furthermore, the inhibition of chemokine receptors also help to strengthen the efficacy of PD-1/PD-L1 blockade.[121,122] Various other combinatorial strategies (viz., inhibition of PI3K-γ,[123] CXCL9 and CXCL10) in combination with PD-1/PD-L1 blockade can improve the efficacy and decrease the resistance against this immunotherapy.[124]

Metabolic components also play crucial roles in the immunosuppressive TME. Metabolite IDO has potential to impact TME by allowing the conversion of tryptophan to kynurenine. IDO inhibitors in combination with anti-PD-L1 and anti-CTLA4 antibodies is documented to improve T-cell infiltration and production of interleukin-2. This strategy may also work to prevent the resistance and improve the efficacy of anti-PD-1/PD-L1 immunotherapy.[125] However, the adenosine is a metabolite of adenosinergic pathway which has important role in TME, but its accumulation reduces the anti-tumor immunity. Its production can be targeted by inhibiting CD73 [126,127]. Recently, a clinical study of A2AR antagonist in combination with PD-1/PD-L1 blockade is performed in renal cell carcinoma (RCC) patients, which results in significant reduction of their resistance.[128,129]

Other combinatorial approaches — Targeting oncogenic signaling (MAPK) can mediate the production of immunosuppressive cytokines and abrogates the resistance to PD-1/PD-L1 blockade. Targeting BRAF mutation improves T cell infiltration and interferon-γ production in animal models.[130] Recently, a triple combination therapy (anti-BRAF, anti-ERK and anti-PD-1) was clinically studied to improve the anti-tumor immunity.[131] Other strategies to overcome the resistance issue includes probiotic administration, dietary modifications and fecal microbial transplantation are in the progressive stage of pre-clinical studies. Still much more clinical studies are needed to be performed in near future.[132,133]

Breast cancer: classification and regulation of PD1/PD-L1

Transformation of cells in the epithelium lining of breast lobules, ducts or glandular tissue leads to the onset of BCa. It accounts for major cancer related deaths in women worldwide and is considered to be the most prevalent form of cancer according to statistics that stated 7.8 million women diagnosed with breast cancer in 2015. Women disability-adjusted life years (DALYs) due to BCa was found to be maximum in comparison to other types of cancers. According to WHO in 2020, globally ~2.3 million diagnosed cases and ~6.8 L deaths in women were witnessed.[5]

Classification

BCa includes a wide range of phenotypically different diseases. To optimize relevant prognosis and therapy, it is crucial to differentiate between the diverse subtypes. It can be differentiated according to the presence/absence of intrinsic genes and on the basis of different clinical stages of the disease.[134]

Molecular basis

Gene expression profile has identified different intrinsic genes that distinguish BCa on the basis of expression of estrogen receptors (ER; the luminal cluster), HER2 (human epidermal growth factor 2) and triple negative/basal cluster. BCa usually divided into four types viz., Luminal BCa, HER2-enriched BCa, triple negative BCa and Normal-like BCa.[134]

Luminal BCa — encompasses two subtypes: Luminal A and Luminal B. Both of these phenotypes are progesterone receptor (PR) and/or estrogen receptor (ER) positive, however, type A is always HER2 negative but type B could be either HER2 negative or positive. Moreover, in Luminal A, Ki-67 expression is low whereas in Luminal B it is high. About 40% of all BCa Luminal A and Luminal B is less than 20%. Owing to the slow growing rate and low grade of Luminal A, hormonal therapy is the most common prescribed treatment, whereas Luminal B has fast growing rate which makes prognosis difficult.[134]

HER2-enriched BCa — accounts 10%—15% of all breast cancers and is ER and PR negative, has high levels of HER2 with enhanced proliferation gene clusters and decreased basal/luminal clusters. It has limited prognosis due to its high growing rate. Therapeutic strategies targeting HER2 protein (trastuzumab, pertuzumab and iapatinib) has shown successful results.[135]

Triple-negative or basal-like BCa — makes up to 20% of all breast cancers and is called triple negative because all three chief hormones signaling, ER, PR and HER2 are absent in this phenotype. It is mostly observed in African-American women, younger population (aged <40 years) and in women harboring mutation in BRCA1 gene. It is highly aggressive and has been denoted as a high grade cancer. Some of the associated histology includes medullary and ductal carcinoma. The absence of targeting proteins has narrowed the scope of non-invasive treatment in TNBC limiting it to chemotherapy. Like Normal, breast cancer is characteristically similar to luminal A subtype and expresses low levels of proliferation gene cluster[134] (Fig. 5).

Basis of different clinical stages

Post-diagnosis, the BCa treatment modality is highly dependent on specific disease stages. On the basis of anatomical phase, the International Union for Cancer Control (UICC) and American Joint Committee on Cancer (AJCC) has used the TNM (Tumor, Node, and Metastasis) classification system to segregate BCa into five stages (0—IV) depending on the size of primary tumor (T), condition of the regional lymph nodes (N), and presence of any related metastasis (M).[134]

Stage 0 — is associated with Ductal Carcinoma *in situ* (DCIS) where cancer cells starts developing inside the breast duct.[134]

Stage I — is further divided in two stages: IA and IB, among which stage IA has no nodal participation and T less than 20 mm, whereas IB has 0.2—2 mm nodal micrometastasis.[134]

Stage II — is also known as the invasive stage where T ranges between 20 and 50 mm. The early stage (IIA) involves metastasis in level I and II of the ipsilateral axillary lymph node with less than 20 mm T or no nodal participation with 50 mm T. IIB involves spread of tumor to the level I and II of the ipsilateral axillary lymph node with 20—50 mm T or no nodal involvement with greater than 50 mm T[134]

Stage III or locally advanced BCa — comprises of tumor with diameter greater than 2 inches with prominent metastasis in underarm or other lymph nodes and other neighboring tissues. Stage IIIA involves spread in ipsilateral Level I and II lymph node with fixed metastasis, in IIIB the primary tumor invasion in chest wall and skin is observed and stage IIIC consist of tumor of any size in ipsilateral level III or supraclavicular lymph node or internal nodes.[136]

Stage IV — includes all other distant tumors formed due to the invasion of BCa cells in other organs.[134,136]

PD-L1 expression

Density and composition of TILs show significant variation between and within the tumors due to unknown reasons.[137] Presence of increased TILs at tumor site are associated with greater efficacy to immune checkpoint blockade.[53] Breast tumor microenvironment are highly heterogeneous and along with cancer cells it consists of various immune cells such as $CD8^+$ T cells, $CD4^+$ T-helper cells, T-regs, gamma-delta T cells, macrophages, mast cells, NK cells and B

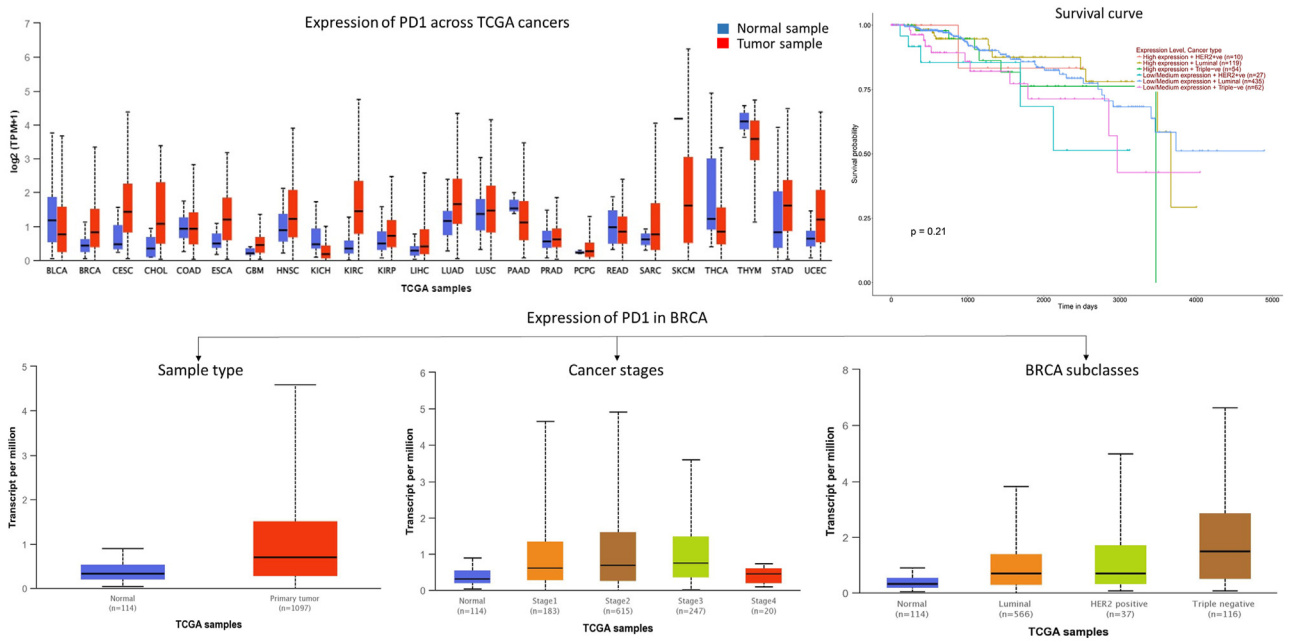

Figure 5 Expression of PD-1 in different TCGA cancer tissues, its expression and survival curve in BCa. Left upper panel depicts bioinformatics based Pan-Cancer analysis of PD-1 expression in different TCGA cancer tissues. Lower panel specifically shows its expression according to sample type, stages and subclasses of BRCA. And, upper right panel shows the survival curve of PD-1 expression *vs.* BRCA subclasses in TCGA cancer tissues. TCGA dataset were analyzed by using UALCAN (http://ualcan.path.uab.edu/analysis.html) platform. Abbreviations: BLCA, bladder urothelial carcinoma; BRCA, breast carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; THCA, thyroid carcinoma; THYM, thymoma; STAD, stomach adenocarcinoma; and UCEC, uterine corpus endometrial carcinoma.

cells.[137] Notably, both tumor cells and immune cells along with stromal cells can express PD-L1 [138]. Multiple studies have confirmed that, TNBC and HER2 positive BCa are frequently enriched by higher TILs and PD-L1 upregulation.[139] Frequency of PD-L1 is higher in inflammatory BCa. Basal like BCa is associated with higher PD-L1 expression than luminal subtype.[140] Also, axillary lymph nodes which are most common metastatic sites in BCa, express higher level of PD-L1 compared with primary BCa.[138] In case of male BCa, lower expression of PD-1 was observed than female BCa and associated with a higher tumor grade. However, expression of PD-L1 in these two groups is comparable. Additionally, fewer TILs are found in male BCa which indicate male BCa patients may show different response rate to checkpoint inhibitors than female patients.[141] Expression of higher level of PD-L1 is associated with negative prognostic features such as ductal types, high grade, large size of tumors, ER^-, PR^-, $HER2^+$, high proliferation index as well as aggressive subtypes.[142] Higher expression of PD-1 and PD-L1 mRNAs are associated with favorable prognosis.[143] Higher response rate to anti PD-1/PD-L1 therapy is associated with PD-L1 positivity and varies greatly among patients. A study showed that, in advance TNBC, the response rate to anti PD-1/PD-L1 therapy is 5%—23%. Expression of PD-L1 in BCa subtypes varies with molecular heterogeneity, use of different Ab clones.[144]

PD-1/PD-L1 regulation

Adipocytes which are abundant in breast tissue, play a major role towards the development and progression of BCa by secreting various tumor promoting soluble factors such as cytokines, hormones, lipid metabolites, ROS and ECM components. Pre-adipocytes are the important source of local estrogens and may induce the risk of obesity associated BCa in post-menopausal women. Whereas mature adipocytes promote tumor invasiveness by releasing VEGF-A and IL-1β. Recent studies showed that these adipocytes express PD-L1 and directly interact with T cell to prevent its function. A study also finds a sub-population of endogenous PD-L1 are internally located in adipocytes, indicating operation of a different signaling pathway to influence anti-tumor response.[145]

Gain of copy number at chromosome 9P24.1 is observed in 4%—25% of TNBC tumors. This region encodes JAK2, PD-L1 and PD-L2. Amplification of this region is associated with elevated PD-L1 expression in TNBC. Also, amplification of JAK2 leads to induced expression of PD-L1 via JAK2/STAT pathway which is common in TNBC.[146] PTEN/PI3K pathway plays a major role in BCa. Loss of PTEN can induce PD-L1 expression through activation of PI3K signaling. PI3K mutation has been identified upon 30%—40% ER/PR negative cases in BCa. Also, constitutive expression of STAT1 and STAT3 are necessary for maintaining the expression of PD-

L1. STAT1 and STAT3 translocate from cytosol to nucleus where heterodimer of pSTAT1 and pSTAT3 binds to the promoter of PD-L1 and maintains its steady state expression in BCa. Combined inhibition of IL-6/JAK/STAT pathway along with PD-L1 may increase the efficacy of checkpoint inhibitors.[147,148]

Epigenetic modification of DNA is important for early development of BCa where aberrant DNA methylation and repression of histone modification are the major clinical and histo-pathological features of BCa. A recent study found that CpG motifs of PD-1 promoter region in BCa are hyper-methylated. Repression of histone methylations including H3K9me3 and H3K27me3 were also reduced in PD-1 promoter region. Anti-PD-1 therapy can be used to reverse this methylation status of PD-1 in BCa.[149] TET2 protein also inhibits transcription of PD-L1 gene by recruiting HDAC I/II to the promoter region of PD-L1 gene in BCa.[150] Inhibition of HDAC also increases the expression of PD-L1 in TNBC and may be a novel therapeutic approach to treat TNBC.[151]

N-glycosylation of PD-L1 is essential for its activation and interaction with PD-1, which makes it ideal for therapeutic target. GSK3β prevents the glycosylation of PD-L1 by inducing phosphorylation dependent proteasomal degradation of PD-L1. However, EGF/EGFR signaling stabilized the expression of glycosylated PD-L1 via upregulation of B3GNT3 glycosyl transferase and inactivate GSK3β.[152] 2-Deoxy Glucose (2-DG) reduces the expression of PD-1 on cell surface and induces PD-L1 trapping inside the ER. 2-DG can also de-glycosylated PD-L1 which relieves immunosuppression and reverses the PARP induced PD-L1 upregulation in TNBC.[153] During post-transcriptional modification E2 stabilizes PD-L1 mRNA and increases its protein expression by PI3K/AKT pathway in ER-α positive BCa.[154] PD-L1 expression is regulated by epigenetically including post-translational histone modification in Breast Cancer Stem Cells (BCa-SC). Lower distribution of the repressive histone in PD-L1 promoter and over expression of histone acetylation enzymes upregulate PD-L1 expression in BCa-SC.[155] Crk is an important regulator of cell proliferation, migration, and invasiveness. It was reported that Crk depleted tumors also have decrease expression of PD-L1 in breast *de novo* carcinoma.[156] Furthermore, inhibition of palmitoylation of PD-L1 via inhibition of palmitoyl transferase ZDHHC9 or by site specific mutation sensitized BCa to kill T cells. Additionally, lipid metabolism can influence immune escape of tumor cells through PD-L1 palmitoylation. Thus, it may be a good approach to improve therapeutic efficacy.[157] Amplification of MUC1 gene and activation of MUC1-c transmembrane subunit activates oncogenic signaling pathways such as PI3K/AKT and MEK/ERK in BCa. MUC1 activates PD-L1 transcription through MYC and NF-κB in basal TNBC cells and induces EMT transcription factor ZEB-1 which suppresses the expression of miR-200, a negative regulator of PD-L1.[158] PD-L1 expression is also upregulated by progranulin or PGRN in TAM via JAK/STAT3 pathway and leads to their polarization towards M2 macrophage phenotype for suppression of T cell proliferation and function in BCa.[159]

Overexpression of endoplasmic reticulum oxido-reductase (ERO1α) increases the expression of PD-L1 in TNBC via two ways. First, during oxidative protein folding and second, by enhancing ROS level which induce HIF1α in response to hypoxic tumor microenvironment and enhance both mRNA and protein level of PD-L1.[160] ERα negatively regulate PD-L1 gene transcription where ERα positive BCa patients showed lower expression of PD-L1 and ERα negative BCa led to immune evasion by expressing higher level of PD-L1.[161] MDR1 gene encodes P-glycoprotein (P-gp) which acts as an ATP efflux pump and presents in normal tissues to protect them from toxic compounds. P-gp is also overexpressed in cancer cells and makes them resistant against drugs. Notably, a study found that interaction of PD-1 with PD-L1 enhances MDR1/P-gp in BCa.[162]

PERK-P-eIF2α could downregulates PD-L1 expression and CXCL5 in TNBC.[163] Syntenin 1 plays an important role in migration and invasion in BCa. It enhances apoptosis of $CD8^+$ T cells through PD-L1 by inducing Tyr^{705} STAT3 phosphorylation.[164] Tumor derived IL-18 also enhances the expression of PD-1 on immunosuppressive NK cells in TNBC.[165] In TNBC cells, PD-L1 transcription is regulated by lipid kinase PIPK1-γ. IFN-γ and PMA activate NF-κB that induces PIPK1-γ expression also in turn enhances the PD-L1 level in TNBC.[166] Tumor derived MDSC also activates PI3K, NF-κB and AKT signaling pathways in PD-1 (−)/PD-L1 (+) Treg cells that in turn shows strong immunosuppressive effect by inhibiting IFN-γ secretion and T cell proliferation in BCa.[167]

Depletion of Alix, which negatively regulates EGFR, significantly increases the IFN-γ induced PD-L1 expression in BCa.[168] It was also identified that, NPM1 activates and upregulates PD-L1 expression in TNBC. Additionally, PARPi downregulates PD-L1 expression by interacting with NPM1 which suggests PD-L1 blockade and PARPi combination therapy may have better effects.[169] Recent studies demonstrated that BET proteins are critical regulator of PD-1/PD-L1 axis. BRD2, BRD3 and BRD4 are important for the expression of PD-L1 and highly enrich at the promoter site of CD274 in TNBC. Additionally, expressions of both BRD2 and BRD4 correlate with the expression of PD-1 mRNA in activated T cells and may be a good target to overcome T cell exhaustion.[170]

In Claudin-low BCa high EMT score observed, it was found that in this subset of BCa, EMT upregulates PD-L1 expression by PI3k/AKT and ERK/MEK dependent pathway and helps immune evasion.[171] In breast CSC, Notch3 signaling is crucial for the expression of PD-L1 and maintains the stemness of breast CSC partially through the Notch/mTOR pathway.[172] PD-L1 also maintains the expression of OCT4 and Nanog through PI3K/AKT pathway and stemness factor BMI1.[173] In TNBC-CSC, WNT signaling upregulates the expression of glycosylated PD-L1 expression which in turn participates in EMT process and stem like TNBC phenotype.[174] Targeting both CMTM6 and CMTM7 in SNAIL dependent EMT can also decrease surface expression of PD-L1 in TNBC[175] (Fig. 6).

Involvement of exosomes

In B cells, ecm-myCAF promotes the expression of PD-1 in Tregs which in turn converts ecm-myCAF to TGFβ-myCAF that ultimately leads to resistance in immunotherapy.[176] Another study indicates that there may be a crosstalk between PD-L1 pathway and HER2 which is responsible to develop drug resistance against anti-HER2 drug.[177] In the cancer genome atlas (TCGA), FAK shows positive

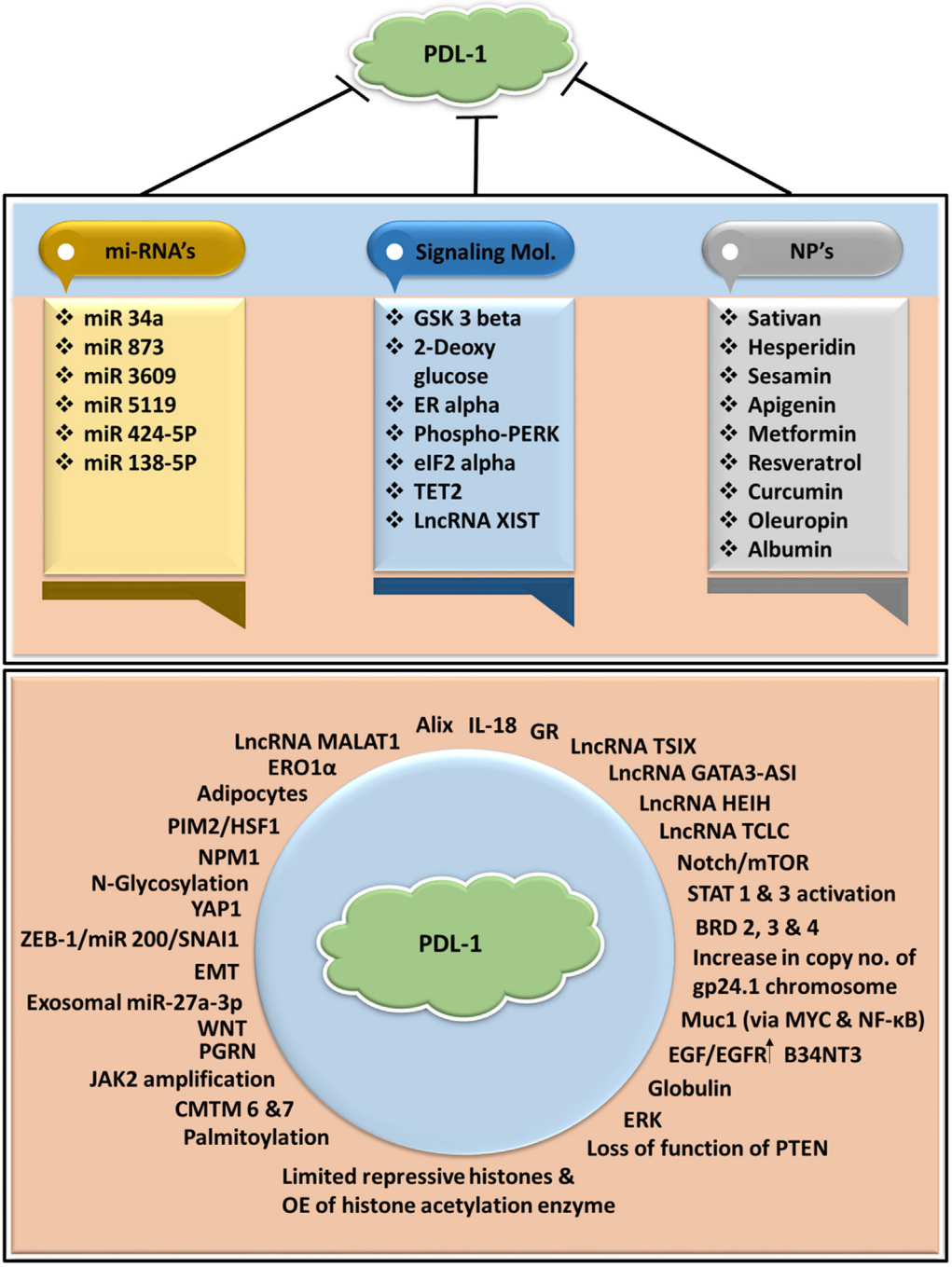

Figure 6 Therapeutic intervention of PD-L1 in BCa. The mi-RNA, signaling molecules and natural products (upper panel) responsible for downregulation while the molecules depicted in the lower panel upregulate the expression of PD-L1 in breast cancer.

correlation with PD-L1. A report stated that anti-PD-L1 atezolizumab inhibits FAK induced motility and PD-L1 expression in TNBC.[178] PD-L1 can also be expressed on the surface of exosomes and play an important role in attenuating anti-tumor response. Blockade of PD-L1 secreting exosomes may improve the efficacy of anti-PD-L1 therapy.[179] In addition, another study found that exosomal PD-1 secreted from activated T cell can attenuate PD-L1 on other exosomes or surface of tumor cells and induce PD-L1 internalization. Exosomal PD-1 inhibits PD-1/PD-L1 signaling via clathrin mediated endocytosis of PD-L1 and protects T cells from immune dysfunction in TNBC.[180] Furthermore, TGF-β promotes tumor derived exosomal PD-L1 to inhibit $CD8^+$ T cell killing activity.[181] CAFs derived exosomes can also induce high level of PD-L1 expression in BCa cells. To find the underlying mechanism a study observed that, breast cancer cell lines treated with CAF derived exosomes significantly increased miR-92 which then downregulated its target gene LATS2 and subsequently enhanced YAP1 nuclear translocation. YAP1 then promoted

PD-L1 transcription by binding to the PD-L1 enhancer region and ultimately caused immune suppression in BCa.[182]

Micro RNA (miRNA) and lncRNA play crucial role

miRNAs act as post-transcriptional modulators by binding to the 3′UTR region of target genes. PD-L1 also has been identified as the target of several miRNAs. In TNBC, PD-L1 and glycolytic enzyme LDHA both are upregulated. Recently, it was revealed that 3′UTR region of both these molecules have binding sites for miR-34a, so they both act as ceRNA and regulate the expression and function of each other. Targeting both of them may be useful to treat TNBC.[183] Multiple evidence suggests that EMT process is associated with PD-L1 expression. miR-873 acts as tumor suppressor in many cancers and regulates stemness of BCa and drug resistance by regulating PD-L1/PD-1 signaling.[184] It was also reported that various EMT related TFs such as ZEB-1/miR-200 or SNAI1 upregulate the expression of PD-L1 in BCa and make them resistant to CTLs mediated lysis.[185] A study identifies ADAM10 and ADAM 17 (metalo-proteases) regulate PD-1/PD-L1 pathway by proteolytic cleavage of PD-L1.[186] Lower expression of miR-3609 expression is associated with BCa and miR3609 targets PD-L1 in BCa.[187] MiR-195/miR-497 also regulates CD274 expression by binding to the 3′UTR in TNBC.[188]

In TNBC patients, resistance of PD-1 blockade may be due to upregulation of LINKA which degrades intrinsic tumor suppressor Rb, p53 and PLC via K48-linked poly-ubiquitination.[189,190] Lnc RNA TCL6 was also associated with PD-1 and PD-L1 expressions in BCa.[191] Two lnc RNAs XIST and TSIX have important role in X chromosome inactivation. Recent studies found double dose of the X chromosome genes favor cancers. In BCa presence of multiple X chromosomes are reported. According to XIST/TSIX/PD-L1 model hypothesis, high PD-L1 level in cancer cells maintains increased expressions of both OCT4 and NANOG to sustain cancer stemness and tumor renewal through PI3K/AKT/mTOR pathway. Also, high levels of OCT4 and NANOG result in downregulation of XIST and upregulation of TSIX which suggests that XIST acts as a tumor suppressor in BCa.[192] PD-L1 expression is also regulated by miRNA-lncRNA network "miR-182-5P/XIST/MALAT1/PD-L1" in BCa. Bioinformatics based studies predicted strong binding affinity between PD-L1 and two lncRNAs, XIST and MALAT1. However, when PD-L1 was transfected with si-XIST or si-MALAT1 it was observed that, knockdown of MALAT1 downregulates PD-L1 expression, whereas knockdown of XIST upregulates PD-L1 in BCa. miR-182-5P upregulates lncRNA MALAT1 and PD-L1 and downregulates XIST in the same breast cancer cell. Silencing of miR-182-5P, MALAT1 and TSIX also down-regulate the PD-L1 expression in MDA-MB-231 cells.[193] Also silencing of HEIH lncRNA can suppress the expression of PD-L1 in TNBC.[194] Another study showed that lncRNA GATA3-ASI positively regulates CSN5 which in turn deubiquitinylates and stabilizes PD-L1 protein. Also GATA3-ASI ubiquitinylates tumor suppressor GATA3 protein that degrades PD-L1 in TNBC and helps in tumor progression.[195]

In comparison to all the different subtypes of breast cancer, the lack of hormonal receptors has made TNBC prognosis extremely difficult and leads to short survival time of the patients. The mortality rate of TNBC patients post five years of diagnosis was found to be 40% and about 46% of the patients develop remote metastasis. Post-metastasis the median survival duration is around 13.3 months with 25% post-surgery recurrence. Moreover, within the first three months of tumor recurrence, mortality rate increases to 75%.[196,197] In this context, endocrine therapy is not possible hence much comprehensive approach is needed. Cancer immunotherapy is one of the strategies which can hypothetically lead to the suppression of all type of cancers irrespective of their phenotypical features. Therefore, in the next section of this review we are interested to discuss about the cancer immunotherapy achieved via inhibition of PD-1/PD-L1 signaling pathway for the treatment of TNBC.

Targeting PD-1/PD-L1 in TNBC — the clinical perspectives

This immunotherapy is also used for the treatment of other cancers and recently it is under clinical development in different stages against multiple cancers. To date, various anti PD-1/PD-L1 antibodies have been approved by FDA for the treatment of cancer. In this study authors also found that PD-L1 expression is positively correlated with the formation of multicellular aggregates.[198] In this section, we are highly interested to focus our discussion on anti-PD-1/PD-L1 immunotherapy for TNBC.

PD-1/PD-L1 inhibitors developed for BCa monotherapy (viz., pembrolizumab, atezolizumab, avelumab) are also used in combination with other combination therapies are under clinical trials.[199] Combination therapies are only hope and checkmate for TNBC. Treatment with atezolizumab induces the transition of PD-L1 from a random coil and α helical structure to β sheet conformation.[200] Combination of atezolizumab with nab-paclitaxel shows good efficacy with PD-L1 positive tumor in TNBC. Recently this combination therapy got FDA approval for the treatment of advanced or metastatic TNBC.[201] Various studies were reported about several immune related adverse events against immune checkpoint blockers although majorly cause mild and reversible effects. Clinicians should be conscious to the toxicity profile of checkpoint inhibitors for better treatment efficacy.[202] One important point to take into consideration is that blocking of PD-L1 leads to increase rate of fetal resorption and miscarriage. So, during pregnancy, if PD-L1/PD-1 inhibitors are used in cancer immunotherapy then proper reproductive safety must be taken.[203]

A study reported that atezolizumab not only block PD-L1/PD-1 interaction but also PD-L1/CD80 where free CD80 expression downregulated by CTLA-4. These studies proposed that with the increase of PD-1 on T cells, PD-L1 may be switched from CD80 binding positive regulator to PD-1 binding negative regulator where CD80 acts as a rheostat for PD-1/PD-L1 interaction. It may be possible that CD80 overexpressing patient would be less responsive to PD-1 or PD-L1 inhibitors. So selective blocker of PD-1/PD-L1 pathway may be more effective.[13] Another inhibitory receptor LAG3 (lymphocyte activation gene 3) on activated T cells can also found to be upregulated in some cancer.

Interestingly, it was observed that approximately 50% ER (−), PD-L1 (+) cases, LAG3 was co-expressed with PD-L1. So, combination of both anti-LAG3 and anti PD-L1 therapy may be a helpful strategy to treat breast cancer.[204]

In a BCa model, T-PNU is capable to overcome the resistance by inducing a strong anti-tumor potency. Combination with anti-PD-1 may be a promising strategy for the treatment of BCa.[205] Tumorigenicity is completely abrogated when adjuvants with immune stimulatory molecules (B7-1) and a cell surface anchored GM-CSF. Use of the combination therapy for PD-L1 blockade and cellular vaccination expressing glycolipid protein as another potential treatment strategy for BCa.[206] A study showed that in lung metastasis of BCa, anti-tumor response of T cell can be induced by tumor cell released CD3-HAC in the aid of MSC.[207] A study showed that sympathetic denervation can downregulate expressions of immune checkpoint molecules including PD-L1 and PD-1 and suppressed the proliferation and growth of breast cancer cells. In addition, induced parasympathetic stimulation can reduce PD-L1 and PD-1 expression.[208]

It was reported that traditional Chinese medicine Sativan can inhibit MAPK/PI3K/AKT pathway and showed inhibitory effect on EMT and PD-L1 expression. Interestingly, miR-200c can inhibit both PD-L1 expression and EMT and sativan can upregulate the expression of miR-200c in TNBC.[209] Metformin can also be suggested for the activation of CTLs by blocking PD-1/PD-L1 signaling in BCa.[210] Resveratrol may be able to disrupt N-glycan branching, preventing dimerization of PD-L1 and hamper the correct localization of PD-L1 to the plasma membrane which ultimately prevent the interaction between PD-1 and PD-L1.[211]

The nutri-epigenetic role of oleuropin was found in olea europaea where they showed that oleuropin can regulate miR-194, PD-L1 and lncRNA XIST triad. miR-194 increases PD-L1 expression and downregulates XIST. Oleuropin on the other hand can decrease the expression of both miR-194 and PD-L1 and enhance XIST level in TNBC.[212] Another study demonstrated the correlation between nutritional status and immune checkpoint molecules in BCa patients. Previous studies showed that Globulin (GLB) promotes the expression of PD-1 but suppressed NK cells function to induce cell mediated cytotoxic effect. However, Albumin (ALB) stimulates the expression of MHC-II and activates T cells. Based on this, they reported increased levels of ALB and Albumin/Globulin ratio (AGR) were related with better outcomes in various cancers including BCa. Although high GLB levels positively correlated with increased PD-1 mRNA expression and poor survival of BCa patients.[213] Naturally occurring or genetically modified oncolytic viruses have shown promising results by stimulating anti-tumor immunity. Maraba virus can induce PD-L1 expression in TNBC. CF33-hNIS-delta F 14.5 can also increase the level of PD-L1 on BCa. These can be used in combination with anti PD-L1 mAb in BCa.[214] Resistance against anti-PD-1/PD-L1 immunotherapy is a critical issue in TNBC treatment. Monotherapy is preferred in early stages of TNBC treatment although a few cases of primary resistance, but later they develop acquired resistance. It is clinically less effective against TNBC. To tackle this issue, number of combinatorial strategies were developed and are under clinical trials for the treatment of TNBC (Table 3).[215]

Mono-therapy

Applications of anti-PD-L1 antibodies are under extensive clinical investigation for the treatment of BCa. Pembrolizumab and avelumab antibodies are studied extensively. Pembrolizumab is registered under four different clinical trials (viz., NCT01848834, NCT02693535, NCT02447003 and NCT02555657). In the phase I of NCT01848834, pembrolizumab recipients were PD-L1 positive and expressed baseline level of CA125 with 22% overall response rate (ORR) with 2.9 months of progression-free survival (PFS) and 11.3 months of overall survival (OS).[216,217] Among the phase II registered trials, NCT02693535 reported 37% disease control (DC) and 21% objective response with median PFS and OS of 10.6 and 30.6 weeks respectively,[218,219] whereas in NCT02447003, 61.8% recipients were PD-L1 positive, and among the total and PD-L1 (+) population, the DCR was 7.6% and 9.5%, and ORR was 5.3% and 5.7%, respectively. In addition, the median PFS and OS were 2 and 9 months with a 6 months rate of 14.9% and 69.1%, respectively.[220,221] NCT02555657 (622 participants) registered in phase III trial concluded that no significant changes in OS upon treatment with pembrolizumab (12.7 months) in comparison to chemotherapy (11.6 months).[222,223] Avelumab trial (NCT01772004) reported that ORR was 3.0% in BCa and 5.2% in the TNBC subgroup, tumor shrinkage was observed in 27.9% of BCa and 45.7% TNBC subgroup and DCR was 28.0% in BC and 31.0% TNBC subgroup.[224,225] Combination therapy is being effectively explored to enhance the efficacy of anti-PD-1/PD-L1 antibodies. Some of the combination includes synergistic use of anti-PD-1/PD-L1 antibodies with signaling inhibitors/monoclonal antibodies/agonists, vaccines, natural killer (NK) cells and other chemotherapeutics. Significant number of studies are currently enrolled in different phases of clinical trials.

Combination therapy

Anti-PD-1 antibody plus signaling inhibitors/mAbs/agonists—Nivolumab, an anti-PD-1 antibody is widely used in combination with different inhibitors. Combination of monoclonal antibodies (mAbs) and agonists are currently enrolled in seven clinical trials for the treatment of breast cancer including TNBC. The phase I/II trial of nivolumab in combination with HDAC inhibitor (viz., romidepsin and cisplatin), actively recruiting participants for trial, preliminary result showed 44% ORR and 4.4 months median PFS with 10.3 months median OS.[226,227] In another complete trial this antibody was used along with VEGFR inhibitor (cabozantinib) in which adverse events were observed in 100% participants and clinical benefit rate was found to be 22.2% with a median PFS and OS of 4.4 months and 6.9 months, respectively.[228,229] Other trials include the use of nivolumab with CD122 agonist (NKTR-214),[230] Anti-CD38 mAb (daratumumab),[231] BTC targets EphA2 (BT5528),[232] PI3K inhibitor (IPI-549)[233] and PPARα antagonist (TPST-1120)[234] are still under clinical investigation, however the result of these studies has not been published yet.

Anti-PD-L1 antibody plus signaling inhibitors/mAbs—Pembrolizumab, atezolizumab, avelumab and

Table 3 Clinical trials of anti-PD-1/PD-L1 based monotherapy and combination strategies for TNBC treatment.

Strategy	PD-1/PD-L1 antibody used	Target (Inhibitor/ Antibody used)	Clinical status (Phases)	No. of enrollments	Status	Outcomes	NCT No.	Ref.
Monotherapy								
PD-L1 Ab	Pembrolizumab	—	Ib	297	C	Improved survival	NCT01848834	216,217
	Avelumab	—	Ib	1756	C	Improved response	NCT01772004	224,225
	Pembrolizumab	—	II	3581	R	—	NCT02693535	218,219
	Pembrolizumab	—	II	254	C	Improved response	NCT02447003	220,221
	Pembrolizumab	—	III	622	C	Improved survival	NCT02555657	222,223
Combination Therapy								
PD-1 Ab + Pathway inhibitor/mAb/ Agonists	Nivolumab	CD122 agonist (NKTR-214)	I/II	557	A	—	NCT02983045	230
		CD38 mAb (Daratumumab)	I/II	105	C	Adverse events	NCT03098550	231
		BTC (EphA2: BT5528)	I/II	116	R	—	NCT04180371	232
		HDAC (Romidepsin, Cisplatin)	I/II	51	A	—	NCT02393794	226,227
		PI3K (IPI-549)	I	219	A	—	NCT02637531	233
		PPARα antagonist (TPST-1120)	I	138	R	—	NCT03829436	234
		VEGFR (Cabozantinib)	II	18	C	Improved response	NCT03316586	228,229
PD-L1 Ab + Pathway inhibitor/Ab	Pembrolizumab	AXL (Bemcentinib)	II	29	T	Adverse events	NCT03184558	246
		CD73 Ab (CPI-006)	I	378	R	—	NCT03454451	247
		CDK (Dinaciclib)	I	32	A	—	NCT01676753	235
		IDO (INCB024360)	I/II	444	C	Improved duration and overall response	NCT02178722	236
		PARP (Olaparib)	II/III	1225	A	—	NCT04191135	248
		PARP (Niraparib)	I/II	122	C	Improved response, safety and tolerance	NCT02657889	237,238
		PARP (Talazoparib)	I	110	R	—	NCT04158336	249
		VEGFR (Lenvatinib)	II	590	A	—	NCT03797326	239,240
	Atezolizumab	AKT (Ipatasertib, Paclitaxel)	III	242	A	—	NCT04177108	250
		HDAC (Entinostat)	I/II	88	U	—	NCT02708680	241,242
		PARP (Rucaparib)	I	29	C	Dose dependent toxicity	NCT03101280	251
		PI3K (PI-549, Nab-Paclitaxel)	II	90	R	—	NCT03961698	252
		VEGFR (Cabozantinib)	I/II	1732	R	—	NCT03170960	253
	Avelumab	GITR mAb (TRX518) & Cyclophosphamide)	I/II	10	T	Adverse events	NCT03861403	254
		PTK7 Ab (PF06647020)	I	138	C	Dose dependent toxicity	NCT02222922	243
	Durvalumab	PARP (Olaparib)	I	3	C	Improved response	NCT03544125	244,245
			I/II	54	A	—	NCT03594396	245
			II	28	R	—	NCT03801369	255
PD-L1 Ab +	Pembrolizumab	PVX-410	I	20	A	—	NCT03362060	258

vaccines		P53 specific vaccine	I	19	A	–	NCT02432963	[256,257]
		WT1 specific vaccine	I/II	90	R	–	NCT03761914	[259]
	Durvalumab	PVX-410	I	22	A	–	NCT02826434	[260]
		Neo antigen DNA virus	I	13	A	–	NCT03199040	[261]
		Nab-Paclitaxel + Neo antigen vaccine	II	70	R	–	NCT03606967	[262]
Atezolizumab	Neo antigen vaccine	I	770	R	–	NCT03289962		[263]
PD-L1 Ab + NK cells	Avelumab	FT-516	I	12	A	–	NCT04551885	[264]
		HA-NK + IL15 + Vaccine + Chemo radiation	I/II	79	U	–	NCT03387085	[265]
PD-L1 Ab + Chemotherapy	Atezolizumab	Nab-Paclitaxel	I	240	C	Improved tolerance	NCT01633970	[266]
	Pembrolizumab	Abemaciclib	I	100	A	–	NCT02779751	[269]
	Pembrolizumab	Nab-Paclitaxel, Doxorubicin	I	60	C	Dose dependent toxicity	NCT02622074	[272]
	Pembrolizumab	Paclitaxel or Capecitabine	I/II	29	A	–	NCT02734290	[270]
	Pembrolizumab	Eribulin	I/II	258	C	Improved response and survival	NCT02513472	[273]
	Nivolumab	Cyclophosphamide, Cisplatin or Doxorubicin	II	84	A	–	NCT02499367	[274]
	Durvalumab	Nab-paclitaxel + Std. EC	II	174	C	Improved response	NCT02685059	[275]
	Atezolizumab	Nab-paclitaxel	III	902	C	Improved progression free survival	NCT02425891	[267]
	Pembrolizumab	Nab-paclitaxel + Carboplatin followed by EC	III	1174	A	–	NCT03036488	[271]
	Atezolizumab	Nab-Paclitaxel & carboplatin followed by DC/EC/FEC	III	278	A	–	NCT02620280	[268]

Abbreviations: A: Active; BTC: Bicycle Toxin Conjugate; C: Completed; DC: Doxorubicin + Cyclophosphamide; EC: Epirubicin + cyclophosphamide; FEC: Fluorouracil + Epirubicin + Cyclophosphamide; GITR: Glucocorticoids induced TNFR related protein; HA-NK: High affinity NK; HDAC: Histone deacetylase; LA: Locally advanced; NR: Not yet recruiting; PI3K: Phosphoinositide 3-kinase; PPARα: Peroxisome proliferator-activated receptor; VEGFR: Vascular endothelial growth factor; R: Recruiting; TNBC: Triple negative breast cancer; and ST: Solid tumors; U: Unknown.

durvalumab are anti-PD-L1 antibodies being clinically studied in association with inhibitory molecules and antibodies for the treatment of TNBC. Pembrolizumab in conjugation with different signaling inhibitors are registered in seven trials among which its conjugation with CDK inhibitor dinaciclib leads to adverse effect in all participants. This trial is active in phase I, but not presently recruiting.[235] A complete trial showed conjoint use of pembrolizumab and IDO inhibitor, INCB024360 resulted in 10% ORR and 36% DCR.[236] Another complete trial of pembrolizumab with PARP inhibitors, niraparib reported 21% ORR and 49% DCR.[237,238] Pembrolizumab with VEGFR inhibitor lenvatinib showed 29% ORR and 58% DCR with 1 CR and 8 partial responses (PRs).[239,240] Another anti-PD-L1 antibody, atezolizumab has five registered trials in which its cooperation with HDAC inhibitor extinostat showed that in participants with prior TNBC treatment no increase in median PFS was noted for entinostat + atezolizumab (1.68 months) administration in comparison to atezolizumab and placebo (1.51 months). Moreover, entinostat + atezolizumab lead to an increase in adverse effects (notable outcome of death).[241,242] Completed trial of avelumab combined with Anti-PTK7 Ab (PF06647020) reported 22% ORR.[243] Lastly, the use of durvalumab and PARP inhibitor (olaparib) showed that 2 participants received more than 6 months clinical benefit and one had more than 18 months prolonged CR.[244,245] The clinical results of other combinations of pembrolizumab with AXL inhibitor (bemcentinib),[246] anti-CD73 Ab (CPI-006)[247] and PARP inhibitors (olaparib[248] and talazoparib[249]); atezolizumab with AKT inhibitor (ipatasertib),[250] PARP inhibitor (rucaparib),[251] PI3K inhibitor (IPI-549 and nab-paclitaxel)[252] and VEGFR inhibitor (cabozantinib)[253]; avelumab with anti-GITR mAb/ago (TRX518, cyclophosphamide)[254]; and durvalumab with PARP inhibitor (olaparib)[245,255] are not yet published.

Anti-PD-L1 antibody and vaccines —Anti-PD-L1 antibody in cooperation with different vaccines is another approach of combination therapy and seven trials of three different anti-PD-L1 antibodies (pembrolizumab, durvalumab and atezolizumab) has been registered. In a preliminary result active trial of pembrolizumab in conjugation of p53 specific vaccine showed that clinical benefit was achieved by 2 participants as a result of consistent p53-oriented, T cell ($CD8^+$) mediated elevated immune response for more than 6 months. Regression of the tumor was also observed.[256,257] The clinical status of the combinations, pembrolizumab with PVX-410[258] and WT1 specific vaccine[259]; durvalumab with PVX-410,[260] neo antigen DNA virus,[261] nab-paclitaxel and neo antigen vaccine[262]; and atezolizumab with neo antigen vaccine[263] are currently in progress.

Anti-PD-L1 antibodies and NK cells —Synergistic therapy of avelumab and NK cells is registered in two trials, avelumab conjointly with FT-516[264]; HA-NK + IL15 + vaccine and chemo-radiation.[265] However, trials are still under clinical investigation and results are not published yet.

Anti-PD-L1 antibody plus chemotherapy —Conjoint application of anti-PD-L1 antibody and chemotherapy has ten registered clinical trials. Atezolizumab, pembrolizumab, nivolumab and durvalumab are the antibodies involved. Completed trial of atezolizumab along with nab-paclitaxel reported 39.4% ORR and 9.1 months median response with a 51.5% DCR. OS and PFS were 14.7 and 5.5 months, respectively.[266] Another completed study showed atezolizumab + nab-paclitaxel delayed the progression of metastatic TNBC without causing major adverse effects.[267] The initial data of an active trial, investigating the combined effect of atezolizumab, nab-paclitaxel and carboplatin followed by DC/EC/FEC, reported pathological complete response (PCR) for atezolizumab combined treatment was 86.9% whereas for only chemotherapy it was 72.0%.[268] Preliminary result of an active clinical trial of pembrolizumab with abemaciclib showed an increase in adverse effects.[269] Pembrolizumab-paclitaxel and capecitabine resulted in PRs in three and metaplastic pathology in two. Another subject showed stable disease and 48 weeks ongoing response[270]. Active trial of pembrolizumab with nab-paclitaxel + carboplatin followed by EC showed that PCR of pembrolizumab-chemotherapy group was 64.8% whereas in the placebo-chemotherapy group it was 51.2%.[271] Completed trial of pembrolizumab and nab-paclitaxel/doxorubicin reported 60% PCR and OR ranging from 80% to 100%.[272] In pembrolizumab and eribulin combination, ORR was found to be 25.8% for patients with no prior treatment and 21.8% for patients with prior treatment history.[273] Nivolumab and cyclophosphamide/cisplatin/doxorubicin are in active trials and preliminary data show an overall ORR of 20% with 11 PRs and 2 CRs. Moreover, two recipients had >24 weeks SD leading to 23% benefit rate. The ORR was 8% for nivolumab-irradiation/nivolumab-cyclophosphamide, 23% for nivolumab-cisplatin and 35% for nivolumab—doxorubicin combination treatment whereas nivolumab alone lead to 17% ORR.[274] Durvalumab in conjugation with nab-paclitaxel + Std. EC has a completed clinical trial status and showed that a decrease in more than 90% B cells and 50% natural killer cells and $CD4^+$ T lymphocytes upon administration of the second phase drug.[275] In this section, we have mostly discussed about the clinical development and status of anti-PD-1/PD-L1 immunotherapy based treatment of TNBC. Both monotherapy as well as combination therapies are preferred, out of them combination therapy is much preferred in view of above discussed resistance issue. The significant efficacy of different PD-1/PD-L1 inhibitors presents a future of non-invasive BCa therapy and has great potential in reducing patient morbidity. In comparison to targeted cancer therapies, utilizing the elevation of immunogenicity is much generalized and is hypothesized to work in every cancer type.[95] In near future, this wing is expected to contribute greatly in clinical cancer treatment.

Biomarkers for response to immunotherapy in TNBC

To reduce the toxicities and enhance the benefits of immunotherapy, the distinction between responders and non-responders is needed and immunological biomarkers are important. The well-known characteristic biomarkers in TNBC are high expression of PD-L1, tumor-infiltrating lymphocytes (TILs), huge mutational burden in tumor cells, mismatch repair (MMR) deficiency and microsatellite instability. These marks may act like possible determinants for the effectivity of immunotherapy. However, the

experimental reliability and benefits of these immunological biomarkers in TNBC are still not clear.[276–280] Here, in the following sections we are going to discuss about these biomarkers.

Tumor-infiltrating lymphocytes (TILs)

The broad heterogeneity of TNBC is known to emerge from TME. TILs composed of distinct level of monocytes and lymphocytes infiltration, are two major components of TME. At present, the multiple evidences show the existence of type, density and position of TILs in TNBC for the disease prognosis and progression.[281] Recently, the quantitative measurement of higher TILs level in 17 studies for their involvement in the prognosis of TNBC is studied.[282] However, another report indicates that the high level of TILs is inversely proportional to the risk of distant recurrence.[283] TILs are also known as a possible factor in chemotherapy. A strong relationship between the high expression of immunological biomarkers and high pathological complete response (pCR) or better outcome is observed in TNBC patients who is treated with neo-adjuvant chemotherapy. However, another report suggests a non-dependent relationship between number of intra-tumoral lymphocytes and pCR in both training as well as validation cohorts. It is observed that pCR rate in lymphocyte-predominant breast cancer (LPBC) patients is 42% and 40% in training and validation cohorts respectively. However, the pCR rate in infiltration lacking lymphocyte breast tumors is 3% and 7% for training and validation cohorts respectively. This study concludes that the pCR rate is higher in LPBC than the infiltration lacking breast cancer patients.[284] Furthermore, the two major clinical trials (ECOG 2197 and ECOG 1199) showed high TILs (sTILs is 80% and iTIL is 15%) in TNBC patients in combination with chemotherapy. The raised sTILs percentage is directly proportional to the disease prognosis, recurrence and death of patients. This study indicates that the high sTIL percentage acts as a major prognostic factor in TNBC.[285] The higher stromal TILs level is also observed in the KEYNOTE-086 (using pembrolizumab) clinical trial which shows better overall response rate in metastatic TNBC.[220] Thus, all these studies conclude that TILs is a potential immune related biomarker to predict the response of immunotherapy in TNBC.

PD-L1 expression

PD-L1 is known to bind with PD-1 and this interaction leads to negative regulation of T cell functions. According to TCGA, the expression of PD-L1 in TNBC is much higher than other breast cancer subtypes. A meta-analysis of 7877 cases taken from 6 different studies also finds the high expression of PD-L1 that leads to the prevention of host anti-tumor immune response and make the cancer more malignant and aggressive.[286,287]

Treatment of TNBC patients by targeting PD-1/PDL-1 axis is under multiple clinical trials where preclinical results were effective and promising against TNBC and substantially different in early *vs*. advanced TNBC subtypes. In the early TNBC patients with high PD-L1 expression were reported with high pCR and prolonged survival time. However, the better outcome with reduced survival time is reported in PD-L1 positive advanced TNBC patients than the PD-L1 negative patients. Recently, a phase III clinical trial (KEYNOTE-522) tested the use of pembrolizumab in combination with chemotherapy in 602 early TNBC patients. Higher pCR rate was observed in pembrolizumab + chemotherapy treated early TNBC patient than the control group. Significant change is observed in both PD-L1 positive as well as negative TNBC patients where the rate of pCR change is higher in PD-L1 positive compared to the negative patient groups.[271] A phase 1 solid tumor study (JAVELIN) in which avelumab is used in metastatic TNBC having higher PD-L1 expression results in the higher ORR. Another clinical trial (KEYNOTE-119) uses the pembrolizumab in TNBC patients which gives higher ORR and survival in PD-L1 positive patients.[224] Thus, all these studies also suggest that the expression of PD-L1 in TNBC patients is effective and key biomarker for immunotherapy.

Immune gene signatures

Multiple immunological cell types have different immune gene signatures along with their different functions in TNBC. The inverse relationship is found between immune gene expressions, somatic copy number alteration levels and clonal heterogeneity in 193 TNBC samples. Lymphocyte-deficient TNBC is associated with less mutational burden that shows better prognosis than lymphocyte-rich TNBC. This suggests that immune-rich TNBC is associated with lesser clonal heterogeneity which is further validated with TCGA and METABRIC data sets.[288]

Several genes (viz., CTLA-4, LAG3, PD-L1, IDO1, etc.) are also associated with efficacy of immunotherapy response in TNBC patients. Beside these factors, tumor mutational burden is also a predictive biomarker for the immunotherapy.[289] A recent report suggests that patients having high TMB in TNBC are good responders. Another clinical trial (KEYNOTE-119) has found improvement in ORR and OS in patients with TMB $\geq$10 mut/Mb than the patients with TMB $<$10 mut/Mb.[290] The DNA mis-match repair (MMR) was also studied to be associated with tumor progression in multiple cancers along with breast cancer and can acts as a predictive biomarker for immunotherapy.[291] Recently, few clinical trials (viz., KEYNOTE-016, 012, 164, 028 and 158) showed the durable response of pembrolizumab in breast, colon, endometrial, gastric cancers etc. Some new biomarkers are also reported but those are still under validation and confirmation stages (viz., proto-oncogene MYC, KRAS, etc.).[292,293] Thus, all these findings conclude that immune related genes are important biomarkers for TNBC immunotherapy.

CAR-T cell in TNBC immunotherapy

Numerous preclinical therapeutic studies and clinical testing make significant progress in the development of CAR-T-cell based immunotherapy for the treatment of TNBC patients. Largely overexpressed TNBC specific antigens such as RTK family proteins, viz., AXL, Epidermal growth factor receptor (EGFR), c-Met, Receptor-tyrosine-

kinase-like orphan receptor 1 (ROR1); cell surface proteins, Chondroitin sulfate proteoglycan 4 (CSPG4), Intercellular adhesion molecule-1 (ICAM-1), Mesothelin, MUC-1, Tumor endothelial marker 8 (TEM-8), Folate receptor α, stress ligands, Natural killer group 2 member D (NKG2D); Integrin avb3 and disialoganglioside targeted by engineered CAR-T-cell are discussed below .[294]

MUC-1 — CAR-T cell targeting aberrantly glycosylated tMUC1, cleaved derivative (MUC1*), and aberrant glycosylated form Tn-MUC1[295,296] have potent antitumor activity, inhibitory effect on tumor growth as well as significant cytokine and chemokine secreting ability *in vitro* and *in vivo*.[294,297] Recently, in a Phase 1 clinical study, CAR-T cells targeting MUC1* (NCT04020575) and Tn-MUC1 (NCT04025216) has been investigated and a Phase 1/2 study (NCT02587689) is proposed to determine the antitumor activity of MUC1-CAR-T cells in TNBC patients.[297]

c-Met — Cytolytic activity and reduced tumor growth by cMet-CAR-T cells have been reported that ensures its safety for TNBC treatment.[297] Encouragingly in a Phase 1 study (NCT01837602) of mRNA-cMet-CAR-T based intra-tumoral injection[298] and another Phase 1 clinical trial (NCT03060356) of mRNA-CAR-T based intravenous injection[299] showed tolerable effect and well elicited inflammatory immune response in TNBC patients.

Mesothelin — Engineered CAR-T cells targeting mesothelin showed significant cytotoxicity, tumor regression and cytokine production in TNBC model *in vitro*. Interestingly, in a recent study *PD-1* knockout MSLN-CAR-T cells showed encouraging result in TNBC tumors.[300] A Phase 1 clinical trial testing (NCT02792114) is underway.[297]

ROR 1 — Tumor recognition ability and antitumor function of ROR1 based CAR-T cell in an *in vitro* vascularized 3 dimensional TNBC model has been reported. An early clinical trial (NCT02706392) in TNBC patients has been investigated.[301,302]

AXL — Third generation AXL-CAR-T cells showed efficient antitumor function *in vitro* and tumor growth inhibition *in vivo*.[303] Furthermore, a recent study demonstrated co-stimulated expression of cytokine receptor C74IL7 with AXL based CAR-T cell have better efficacy in TNBC cells *in vitro*.[304]

EGFR — EGFR specific third generation CAR-T cell showed significant cytotoxic activity and tumor growth reduction in both cell line derived and patient derived TNBC models.[305]

TEM 8 — Although initial studies showed both safety and killing ability of TEM 8-CAR-T cell in TNBC tumors,[306] however, other studies reported its off-target toxic effect that needs thorough investigation before proceeding to clinical studies.[307]

SSEA 4 — Reduced tumor burden and co-targeting ability in TNBC as well as in hematopoietic multi-potent progenitor cells in lungs and bone marrow by stage-specific embryonic antigen-4 (SSEA-4)-CAR-T cells have been reported.[308]

FR α — Potent antitumor activity of FR α-CAR-T cell was reported in xenograft mouse model of TNBC[309]

NKG2D ligands — *In vitro* cytolytic activity and reduced tumor growth were found by first generation and second generation NKG2D-CAR-T cells in TNBC models.[310] A Phase 1 clinical trial testing (NCT04107142) begins to start.[297]

Integrin avb3 — Tumor growth reduction and pro-inflammatory cytokine secretion were reported by second generation avb3 targeting CAR-T-Cells in an *in vitro* TNBC model.[311]

CSPG4 — Potent antitumor activity, cytotoxicity[312] and CAFs targeting activity in TNBC tumor microenvironment[313] has been reported by second generation CSPG4-CAR-T cells.

ICAM-1 — Significant cytotoxic activity of ICAM 1-CAR-T cells has been found in TNBC.[314]

Disialoganglioside (GD2) — Third generation GD2-CAR-T cell showed cell lysis activity, its increased persistence, tumor growth reduction and prevention of lung metastasis in TNBC.[315]

Concluding remarks

In past few decades, breast cancer has become the most common type of cancer among women. Despite progress in basic and translational research, breast cancer has very limited treatment options with various side effects due to non-targeted therapies. To counter this problem from theoretical point of view here the rapid growth of research in past few years have been summarized. The mechanism of immunotherapy to simulate body's own immune system is utilized so that they can recognize and destroy cancer cells effectively with low toxicity. Although this is not an easy task as cancer cells are very much successful to mimic body's own developmental pathways for their rapid growth and proliferation as well as creating immune suppressive situations. In addition, if immune cells are able to identify them as foreign, cancer cells treated them such a way that they lost their antitumor killing ability. Expression of PD-L1 on their surface is one of such mechanism that tumor cells used to evade immune cells. In normal condition, PD-L1 is expressed on immune cells and bind with PD-1 on lymphocytes to regulate the duration and magnitude of their activity and protect body from immune cell mediated tissue damage. PD-L1 also plays a vital role in feto-maternal tolerance during pregnancy. Expression of PD-L1 on placenta saves fetus from destruction by maternal immune cells and identified it as a foreign object that signifies the importance of this inhibitory axis in the evolution of placental organism development. However, cancer cells imitating these developmental conditions and took the advantage of immune suppressive environment by over expressing PD-L1. Miserably present on tumor cells PD-L1 perform their part of job that it does in normal condition, i.e., attenuate immune cell's function, activating various signaling pathways to make the environment hypoxic, which in turn induce polarization of immune cells towards more immune repressive in nature. Cancer cells also use PD-L1 to maintain their stemness that is one of the main reasons for tumor to relapse.

Blocking of PD-1/PD-L1 opens a new door in the treatment of cancer. Although initially anti PD-L1 antibodies show some positive result but not all breast cancer patients get benefit from it and with time shows resistance to this therapy. The reason may be that besides PD-1, PD-L1 can also binds to CD80 in cis on APCs and can attenuate both CTLA4/CD80 and PD-1/PD-L1 coinhibitory pathway but did

not hamper the costimulatory CD28/CD80 axis. Inhibiting PD-L1 can also block its cis interaction with CD80 and as a side effect, CTLA4/CD80 inhibitory signaling is activated and costimulatory CD28/CD80 signaling is disrupted. In addition, co-expression of PD-1 and PD-L1 are also found that leads to PD-1/PD-L1 interaction in cis and restrict trans PD-1/PD-L1 inhibitory signaling. Blocking both CTLA4 and PD-L1 interaction and particularly trans PD-1/PD-L1 signaling can be more effective to restore immune cell's function. Although recent research found significantly better results in PD-L1 positive patients that make PD-L1 as a better choice as biomarker for breast cancer treatment. Adding more complexity to these interconnected networks expression of PD-L1 was also found on tumor derived exosomes and have an important role in therapy resistance. These exosomal PD-L1 can easily migrates for draining to lymph nodes and also in PD-L1 negative cancer cells and induce immune suppression both locally and systemically. Thus, blocking of both exosomal and cell surface PD-L1 can show better outcomes. In addition, exosomal PD-L1 are also found in the blood of various cancers and correlate with disease progression. These data also indicate that investigation of the presence of circulatory exosomal PD-L1 in the blood of breast cancer patients can be used as biomarker that can be readily available and easy for detection. Moreover, activated T cells can also release PD-1 that inhibit PD-1/PD-L1 and restore immune cell's function. Thus, there are multiple layers of crosstalk present that are not fully uncover yet. To make this checkpoint inhibitors more effective detail knowledge of PD-1, PD-L1 and their interactions with other cells or molecules and especially with other checkpoint molecules are very important. Clear understanding of their dynamic expressions, interactions in normal and pathological conditions as well as how they regulated at genetic, epigenetic, post-transcriptional and post-translational levels are crucial to design agents that can block this inhibitory pathway in breast cancer. At the same time, learning from the cancer cells how successfully they mimic developmental pathways and make immune suppressive situations for their own benefit, we shall also investigate the underlying mechanisms of how body cells regulate the restoration of immune active environment from immune suppressive conditions after finishing their duty in case of wound healing and pregnancy when they are back to normal condition. Identifying components and signaling pathways that are involved in this intricate regulation surely highlights some promising ways to develop common therapeutic avenues in different types of cancer.

Author contributions

All authors contributed equally, read and approved the final manuscript.

Conflict of interests

The authors declare that they have no competing interests.

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