The PD1:PD-L1/2 Pathway from Discovery to Clinical Implementation

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The immune system maintains a critically organized network to defend against foreign particles, while evading self-reactivity simultaneously. T lymphocytes function as effectors and play an important regulatory role to orchestrate the immune signals. Although central tolerance mechanism results in the removal of the most of the autoreactive T cells during thymic selection, a fraction of self-reactive lymphocytes escapes to the periphery and pose a threat to cause autoimmunity. The immune system evolved various mechanisms to constrain such autoreactive T cells and maintain peripheral tolerance, including T cell anergy, deletion, and suppression by regulatory T cells (TRegs). These effects are regulated by a complex network of stimulatory and inhibitory receptors expressed on T cells and their ligands, which deliver cell-to-cell signals that dictate the outcome of T cell encountering with cognate antigens. Among the inhibitory immune mediators, the pathway consisting of the progranule cell death 1 (PD-1) receptor (CD279) and its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) plays an important role in the induction and maintenance of peripheral tolerance and for the maintenance of the stability and the integrity of T cells. However, the PD-1:PD-L1/L2 pathway also mediates potent inhibitory signals to hinder the proliferation and function of T effector cells and have inimical effects on antiviral and antitumor immunity. Therapeutic targeting of this pathway has resulted in successful enhancement of T cell immunity against viral pathogens and tumors. Here, we will provide a brief overview on the properties of the components of the PD-1 pathway, the signaling events regulated by PD-1 engagement, and their consequences on the function of T effector cells.

Keywords: PD-1, PD-L1, T cell responses, T cell tolerance, T cell exhaustion, cancer immunology, cancer immunotherapy

INTRODUCTION

The field of T-cell costimulation started with the “two-signal” theory of lymphocyte activation that was originally proffered to distinguish self from non-self. This model explains the process of activation or anergy when a naïve T cell confronts an antigen (1, 2). As per this model, two signals from antigen-presenting cells (APCs) are required for effective activation of a naïve T cell. The first signal confers specificity to the immune response and involves antigen recognition, provided by the interaction of antigenic peptide-major histocompatibility complex (MHC) with the T cell receptor (TCR). The second antigen-independent signal is the “costimulatory signal,” delivered by costimulatory molecules expressed on APCs to receptors expressed on T cells. If a T cell
discovery only antigen-specific TCR stimulation in the absence of costimulation, it will become unresponsive (anergic) to subsequent antigenic challenge (3, 4). Later, negative costimulatory (i.e., coinhibitory) signals were also found to exist. Receptors delivering coinhibitory signals function as immune checkpoints and play a decisive role in maintaining peripheral tolerance and impeding autoimmunity (5–8).

The best-studied pathway for T cell costimulation includes B7-1/B7-2–CD28/CTLA-4 superfamily, which is essential for T cell activation and tolerance (9–13). While both the receptors and ligands of this superfamily are structurally type I transmembrane protein with a single IgV extracellular domain that predominantly mediates the receptor–ligand interaction, the ligands also contain an IgC domain on their outer surface. The immune system functions by maintaining an intricate balance between CD28/costimulation-mediated T cell activation and CTLA-4/immune checkpoint-mediated inhibition. Identification of the programmed cell death 1 (PD-1) as another inhibitory receptor and inclusion of its ligands as additional members of the B7-1/B7-2–CD28/CTLA-4 family (14, 15) re-established the importance of immune checkpoints to safeguard the maintenance of T cell tolerance. Since the beginning of its discovery, costimulation had been of therapeutic interest because it was thought to provide a way to promote T cell activation to enhance antitumor responses. But with the discovery of CTLA-4 as a potent inhibitory immune checkpoint, the notion about cancer immunotherapy was modified and the preferred approach was understood as not to activate the immune system to attack cancer but to remove the coinhibitory signals that block antitumor T cell responses. Based on the same concept, the PD-1/PD-L1 coinhibitory pathway was exploited therapeutically resulting in remarkable outcomes with 20–90% response rates in multiple clinical trials and various types of cancer (16–19).

**DISCOVERY OF THE PD-1:PD-L1 PATHWAY**

While studies have shown that PD-1–PD-L interaction is important to maintain a balance between peripheral tolerance and autoimmunity, it also impairs viral and tumor immunity, promoting chronic infection and tumor progression. PD-1 is a 288 amino acid protein mostly expressed on the surface of activated T cells (20–23). In 1992, PD-1 was identified as an apoptosis-associated molecule (24). In an attempt to identify gene(s) important for apoptosis, Tasuku Honjo and colleagues at Kyoto University performed subtractive-hybridization assay and PD-1 cDNA was found to be encoded by all of the isolated clones. However, its overexpression had no effect on apoptosis in the studied cell lines (23). In 1999, the same group demonstrated PD-1 to be a negative regulator of immune responses by studying PD-1-deficient mice, which developed an autoimmune phenotype with delayed onset, organ-specific effects and incomplete penetrance. While CTLA-4 deficiency caused the rapid-onset of systemic autoimmunity, PD-1 deficiency resulted in spontaneous development of lupus-like arthritis, splenomegaly, glomerulonephritis, increased number of B-lymphocytes and myeloid cells, and increased serum IgA, IgG2b and IgG3 in C57BL/6 mice. Interestingly, PD-1 deletion in Balb/c background resulted in a distinct autoimmune phenotype as early as of 5 weeks of age, with dilated cardiomyopathy, gastritis, and high circulating level of troponin reactive IgG1. PD-1 deficiency induced subacute type I diabetes in non-obese diabetic (NOD) mice whereas lethal myocarditis was observed in mice with MRL background (8, 25–27). Introduction of the lpr mutation, which causes absence of Fas-mediated apoptosis pathway (B6-lpr/lpr-PD-1−/−), expedited the commencement and severity of the disease. However, no disease was developed in Balb/c-PD-1−/− RAG−/− mice, showing the importance of lymphocytes for disease development. To study the response to autoantigens, PD-1-deficient 2C TCR transgenic mice were bred to the autoreactive background (H−2k) and the offspring displayed splenomegaly, growth retardation, and lethal graft versus host disease (GVHD) (7). The group collaborated with Genetics Institute at Cambridge, MA, USA, in an attempt to identify the ligand of PD-1.

Almost in parallel, Liping Chen's group, then at Mayo Clinic, identified PD-L1, which was named B7-H1, as a molecule with homology to B7-1 and B7-2 (28). The group did not discover that B7-H1 is a ligand for PD-1 but reported that B7-H1 costimulates T cells via a receptor different from CD28, CTLA4, or ICOS and delivers an activation signal to T cells, which leads to IL-10 production, but not to detectable levels of IL-2. A third, independent research group led by Gordon Freeman at Dana–Farber Cancer Institute identified by database search a B7-like molecule that did not interact with CD28, CTLA4 or ICOS. The group collaborated with Genetics Institute at Cambridge, MA, USA, in order to identify its receptor. Through these interactions with the two independent groups, the researchers at Genetics Institute found that this B7-1 like molecule was a ligand for PD-1, and was then named PD-L1 (Pdcd1lg1, CD274) for PD-1 ligand 1 (14). The collaboration further identified the second PD-1 ligand, named PD-L2 (Pdcd1lg2, CD273) (15).

**MOLECULAR STRUCTURE**

Programed cell death 1 is composed of a single N-terminal IgV-like domain sharing 21–33% sequence identity with CTLA-4, CD28, and ICOS, about 20-amino acid stalk separating the IgV domain from the plasma membrane, a transmembrane domain, and a cytoplasmic tail containing two tyrosine-based signaling motifs. Since PD-1 lacks the membrane proximal cysteine residue, which is essential for homodimerization, it is believed to exist as monomer on the cell surface (29). Unlike CD28 and CTLA-4, PD-1 tail does not contain any SH2- or SH3-binding motifs. Instead, it contains an N-terminal sequence VDYGEL, forming an immunoreceptor tyrosine-based inhibitory motif (ITIM), which is required for recruiting SH2 domain-containing phosphatases (30) and a C-terminal sequence TEYATI, forming an immunoreceptor tyrosine-based switch motif (ITSM), essential for the inhibitory function of PD-1 (31, 32). The ligands of PD-1 (PD-L1 and PD-L2) are type I transmembrane glycoproteins, containing IgC and IgV domains. The amino acid identity between PD-L1 and PD-L2 is about 40%, while PD-Ls and B7s have about 20% similarity. Human and murine orthologs of PD-Ls display about 70% identity. The crystal structure analysis shows that PD-1 utilizes its front β-face
stimulate PD-L1 expression on B (CD19+/monocyte (55, 56). PD-L1 expression on human T cells are induced by com-
while PD-L2 expression is confined in medullary stromal cells (54). In the thymus, PD-L1 is expressed mostly in the cortex, marrow derived mast cells, and more than 50% of peritoneal B1 expression is restricted to activated DCs, macrophages, bone
ects the eye from activated T cells (50–53). In contrast, PD-L2 pigmented epithelium (RPE) and PD-1–PD-L1 interaction pro-
functions in the placenta to induce fetal–maternal tolerance (48, shown to be expressed on placental syncytiotrophoblasts and islets, astrocytes, neurons, and keratinocytes (36). It has also been expressed on T and B cells, DCs, macrophages, mesenchymal stem cells, bone marrow-derived mast cells (35). In addition, PD-L1 is expressed on a wide variety of non-hematopoietic cells including lung, vascular endothelium, fibroblastic reticular cells, liver non-parenchymal cells, mesenchymal stem cells, pancreatic islets, astrocytes, neurons, and keratinocytes (36). It has also been shown to be expressed on placentalar syncytiotrophoblasts and functions in the placenta to induce fetal–maternal tolerance (48, 49). PD-L1 is expressed constitutively in the cornea and retinal pigmented epithelium (RPE) and PD-1–PD-L1 interaction pro-
tects the eye from activated T cells (50–53). In contrast, PD-L2 expression is restricted to activated DCs, macrophages, bone marrow derived mast cells, and more than 50% of peritoneal B1 cells (54). In the thymus, PD-L1 is expressed mostly in the cortex, while PD-L2 expression is confined in medullary stromal cells (55, 56). PD-L1 expression on human T cells are induced by common γ chain cytokines IL-2, IL-7, and IL-15, whereas IL-21 can stimulate PD-L1 expression on B (CD19+) cells from peripheral blood mononuclear cells (PBMCs). LPS or BCR activation also result in induction of PD-L1 and PD-L2 in human B cells (14, 15, 28). IFN-γ, but not tumor necrosis factor (TNF)-α, treatment results in the expression of both ligands in human monocytes. IL-10 can also induce the expression of PD-L1 on monocytes, while IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) stimulate PD-L2 expression on DCs (57). IFN-γ can also regulate PD-L1 expression in non-lymphoid cells. Endothelial cells constitutively express PD-L1 on their surface and in vitro treatment with IFN-γ causes its rapid upregulation (58). In addition, MyD88, TRAF6, MEK, and JAK2 are also known to play important role in signaling pathways involved in PD-L1 expression (59–61). PD-Ls are also expressed on various tumor cells. PD-Ls mediate potent inhibitory signals after ligation with PD-1, causing a detrimental effect on antitumor immunity by allowing the tumor cells to escape immunosurveillance (62–64).

EFFECTS OF PD-1 ON SIGNALING PATHWAYS

Identification of PD-Ls and confirmation of their interaction with PD-1 established PD-1 as a negative regulator of immune responses (14, 15). Unlike other members of CD28 family, PD-1 transduces signal only when cross-linked together with B- or T-cell antigen receptor. PD-1-mediated signaling inhibits T lymphocyte glucose consumption, cytokine production, prolif-
eration, and survival. CD28 costimulation (14) or IL-2 (65) can override PD-1-mediated inhibition. PD-1 engagement prevents the expression of transcription factors associated with effector cell function, including GATA-3, T-bet, and Eomes (66). Upon TCR stimulation, the tyrosine residues in the ITIM and ITSM motifs on the cytoplasmic tail of PD-1 become phosphorylated, recruiting SHP-1 and SHP-2, which in turn, dephosphorylate proximal signaling molecules downstream of the TCR and CD28. Positional matemutasgenesis studies have shown that the ITSM motif is critical for the inhibitory function of PD-1 (22, 67). Specifically the ITSM tyrosine (Y248) of PD-1 associates with SHP-2 and is mandatory for PD-1-mediated inhibition of PI3K/Akt activation (22, 68). PD-1 ligation causes diminished phosphorylation of CD3, ZAP70, and protein kinase Cβ (69). It can also inhibit Erk activation, which can be overcome through IL-2, IL-7, and IL-15 signaling (70). In B cells, PD-1 engagement inhibited B cell receptor-mediated Ca2+ mobilization and phosphorylation of Igβ, Syk, PLC-γ2, and Erk1/2, and these effects were dependent on SHP-2 recruitment to the ITSM tyrosine of PD-1 (67).

A puzzling question has been surfaced about the role of SHP-2 versus SHP-1 in the inhibitory function of PD-1. Recruitment of SHP-2 to the cytoplasmic tail of PD-1 has been well documented in B cell line (67), Jurkat T cells (15), and primary human T cells (22, 71). SHP-1 may also be a potential candidate for interaction with PD-1 cytoplasmic tail, as found by yeast two-hybrid screening. SHP-1 functions as a negative regulator of cell activation and its expression is largely confined to hematopoietic cells (72). SHP-1-deficient mice display prolonged phosphorylation of the TCR/CD3 complex and increased activation of Lck, Fyn, and other proximal TCR signaling components (73–75). In contrast, the role of SHP-2 in T cells is different. SHP-2 is omnipresent and SHP-2 deficiency results in embryonic lethality in mice. SHP-2 mostly has been depicted as a positive regulator of cell activation and appears to be necessary for optimal induction of MAPK/Erk pathway (76). SHP-2 can recruit insulin receptor substrate to insulin receptor (77) and Grb2 to both platelet-derived growth factor receptor (78) and erythropoietin receptor (79, 80). A live cell imaging study determined that SHP-2, but not SHP-1, is the
phosphatase that interacts with PD-1 upon TCR-mediated activation in live cells (81). This work showed that PD-1 is translocated to dynamic TCR microclusters and accumulates at the signaling central supramolecular activation cluster (c-SMAC). SHP-2 is shortly recruited to PD-1 there after in the microclusters and associates with ITSM of PD-1 (81). Using site-directed mutagenesis and stable expression of mutagenized PD-1 constructs in Jurkat T cells, it was determined that although only mutation of PD-1 Y248 abrogated interaction with SHP-2, both Y248 and Y223 are actively involved in the inhibitory effects of PD-1 on IL-2 production (82).

Effects of PD-1 on TCR Signaling and Functional Outcomes

Programed cell death 1 ligation attenuates TCR-mediated signaling at a proximal level and impairs the activity of two signaling cascades, the PI3K/Akt and the Ras/MEK/Erk pathway (68, 83), which are co-required to initiate T cell activation (Figure 1). One of the many mechanisms via which PD-1 inhibits activation of the PI3K/Akt pathway includes PTEN phosphorylation and phosphatase activity, which is regulated by CK2 (84). CK2 mediates phosphorylation of PTEN C-terminus serine/threonine cluster S380/T382/T383, which, aids PTEN protein stability, while reducing PTEN lipid phosphatase activity against the substrate PIP3 (85, 86). During TCR/CD3- and CD28-mediated stimulation, PTEN is phosphorylated by CK2 (84), which stabilizes PTEN and suppresses its phosphatase activity. In contrast, PD-1 inhibits the stabilizing phosphorylation of the Ser/Thr cluster within the C-terminus domain of PTEN, resulting in increased PTEN phosphatase activity. The other major signaling pathway targeted by PD-1 is the Ras/MEK/Erk pathway (69, 83). The activation of Ras and its downstream MEK/Erk MAP kinase pathway in T cells comprises of the Ca²⁺ and DAG-mediated activation of RasGRP1 (87–89) downstream of PLC-γ1 (90), which is inhibited by PD-1 (83). Other signaling events initiated by TCR ligation are also attenuated by PD-1 ligation including activation of ZAP70 and PKCθ (69).

PD-1 Targets the Cell Cycle

A major downstream target of the synergistic effect of PI3K/Akt and Ras/MEK/Erk activation in T cells is the cell cycle machinery. Primary T lymphocytes naturally reside in the G0 phase and lack expression of cyclins, which are required to interact with cyclin-dependent kinases (Cdks) to form cyclin–Cdk holoenzyme complexes that drive cell cycle progression (91–93). p27kip1, a
member of the Kip/Cip family of Cdk inhibitors, interacts with Cdk2 and is abundantly present in T cells. Ubiquitin-dependent degradation of p27Kip1 is required to initiate cell cycle progression and entry to S phase by allowing activation of Cdk2. This event is predominantly mediated by Skp1-Cullin-F-box (SCF) family ubiquitin ligase, SCF^Rap2 (94). TCR/CD3 and CD28 costimulation regulates the transcriptional induction of Skp2, a substrate of SCF^Rap2 ubiquitin ligase, and this process requires simultaneous activation of PI3K/Akt and Ras/MEK/Erk pathways (95). Ligation of PD-1 during the T cell stimulation causes abrogated expression of Skp2, resulting in increased p27Kip1 level and Cdk2 inhibition (83). The impaired Cdk2 activity inhibits Rb phosphorylation, impacting its interaction with chromatin remodeling proteins. Inhibited Cdk2 also fails to phosphorylate the checkpoint inhibitor Smad3, upregulating its transcriptional activity (96) and resulting in the increased abundance of the G1 phase Cdk inhibitor, p15^INK4B, and the loss of the Cdk-activating phosphatase Cdc25A (83, 97).

PD-1 Reduces the Threshold of TGF-β-Mediated Signaling

One major consequence of PD-1-mediated Cdk2 inhibition and subsequent reprogramming of Smad3 transcriptional events is the conversion of naïve T cells into induced T regulatory cells. Regulatory T cell populations are critical for the maintenance of peripheral tolerance, are potent inhibitors of immune responses and play an important role in the prevention of graft rejection (98, 99). Foxp3^+ Tregs can be divided into two subsets: natural Tregs (nTregs) and induced Tregs (iTregs). CD4^+Foxp3^+ nTregs arise as committed regulatory cells from thymus (100), while iTregs (or adaptive Tregs) develop in the periphery from CD4^+Foxp3^− naïve T cells in a TGF-β- and IL-2-dependent fashion (101–107). PD-1 regulates the function of Smad3 and synergizes with TGF-β-mediated signals (83). This synergizing effect on naïve T cells promotes the differentiation of Tregs cells (108), thereby suppressing generation and function of T effector cells (Teff) via a cell extrinsic mechanism. In addition, generation, function, and fate of Tregs cells requires the αLβ2 (LFA-1) integrin, whose activity is dependent on small GTPase Rap1 (109–112). Importantly, PD-1 does not inhibit Rap1 activation (83), indicating that PD-1 also supports the pathways required for Tregs to perform their immunosuppressive functions. Experiments with PD-L1-deficient APCs resulted in minimal conversion of naïve CD4^+ T cells to iTregs. PD-L1-Ig has also been shown to increase Foxp3 expression and suppressive function of established iTregs through attenuation of Akt-mTOR signaling and concomitant upregulation of PTEN signaling events that are known to drive generation of iTregs (108, 113–115).

PD-1 Alters the Metabolic Program of Activated T Cells

Upon activation, signals from the CD28 costimulatory pathway and the γ-chain signaling cytokines promote naïve T cells to switch their metabolism from oxidative phosphorylation to glycolysis, which is required to support their growth, proliferation, and effector functions (116–119). Divergence in the metabolic reprogramming is critical for imprinting distinct T cell fates. Namely, preferential switching to glycolysis accompanies effector T cell differentiation (120) and switching to fatty acid oxidation (FAO) causes the conversion of T effector to T memory cells (121). Furthermore, imposing FAO by pharmacologic means boosts the generation of Treg cells (122). Studies investigating metabolism profile of T cells receiving PD-1 signals have shown that PD-1 ligation disengaged them from glycolysis, glutaminolysis, or metabolism of branched chain amino acids, but induced increased rate of FAO (123). While PD-1 ligation inhibited the expression of receptors and enzymes involved in glycolysis and glutaminolysis, it increased the expression of carnitine palmitoyl transferase (CPT1A), the rate-limiting enzyme of FAO. T cell activation causes an increase in extracellular acidification rate (ECAR), an indicator of glycolysis, and in oxygen consumption rate (OCR), an indicator of oxidative phosphorylation. PD-1 engagement results in lower ECAR and OCR, but higher OCR/ECAR ratio compared with T cells stimulated without PD-1 ligation (Figure 1). By altering the metabolic programs of T cells, PD-1 ligation seems to generate a more oxidative environment (123, 124).

CLINICAL IMPLICATIONS OF PD-1

Role of PD-1 in Chronic Viral Infection: T Cell Exhaustion

Programed cell death 1 has unique regulatory roles in the control of virus-specific immune responses, and these regulatory functions have been studied extensively during chronic viral infections. The CD8^+ effector T cells behave differently in acute and chronic viral infections. During acute infection, naïve antigen-specific CD8^+ T cells get activated, proliferate, and differentiate into effector CD8^+ T cells and efficiently clear the virus. Most of these virus-specific effector CD8^+ T cells then become apoptotic and a very small number (5–10%) of long-lived memory cell population arises, which is protective against secondary infection (125). However, during chronic viral infection, sustained antigenic stimulation engenders the loss of effector T cells and their failure to develop into memory CD8^+ T cells (Figure 2A). Under these conditions, T cells become unresponsive to viral antigens and persist in a non-functional, exhausted state (TEX), in which they are unable to clear virus effectively (126).

During the development of exhaustion, loss of effector functions happens in a hierarchical manner: IL-2 production, high proliferative capacity and in vitro cytolytic activity are lost first, followed by impairment in the production of TNF-α, IFN-γ, and degranulation (127, 128). Although incapable of functioning as effector or memory, TEX cells are not functionally dormant. Instead, they commit to the containment of chronic infections, because depleting CD8^+ T cells including TEX during simian immunodeficiency virus (SIV) infection results in rapid increase in viral titers and progression to AIDS (129, 130), suggesting an important role for the residual function of SIV-specific TEX in maintaining a host–pathogen equilibrium and contributing to the containment of the chronic infection. TEX cells often retain the capacity to produce low levels of IFN-γ and/or beta chemokines...
and express high levels of granzyme B. In addition, one subset of T^E_{EX} retains some residual cytotoxicity (127, 131, 132). High granzyme B expression is an interesting feature of T^E_{EX}, given that the ex vivo killing capacity of these cells is impaired compared with T^E_{EFF} (131, 132). Thus, while T^E_{EX} cells exhibit impaired effector functions, some residual functionality remains, and this may be important in a host–pathogen equilibrium. In addition to PD-1, T^E_{EX} cells expressed higher levels of other inhibitory receptors (e.g., Tim-3, Lag-3, and CD160) as well (131). However, blockade of PD-1 is sufficient to induce reinvigoration of a significant fraction of this cell population, which selectively expresses T-bet^{Hi}, Eomes^{Hi}, PD-1^{int} and has the ability to proliferate after PD-1 blockade. In contrast, T^E_{EX} cells exhibiting Eomes^{Hi}, PD-1^{Hi} are unable to respond after PD-1 blockade (132). Similar subsets of T^E_{EX} defined by reciprocal patterns of T-bet, Eomes, and/or PD-1 expression have been found in human patients with HCV and HIV infection (133, 134). In these patient populations, PD-1 blockade resulted in augmentation of pathogen-specific T cells and decrease of viral load (46, 47).

**Role of PD-1 in Transplantation and Autoimmunity**

PD-L1 is expressed on a wide variety of non-hematopoietic cells and plays a key role for the maintenance of self-tolerance (Figure 2B). PD-1 and PD-L1 levels increase after heart allotransplantation and their levels correlate with the likelihood of rejection, while the use of a PD-L1-Ig fusion protein decreased rejection (135). This finding was of great interest as PD-L1 is expressed in endothelial cells, which are located between the graft cells and the immune cells and suggests a potential target to decrease the rates...
of graft rejection. Similarly, GvHD occurring after bone marrow transplantation (BMT) has been associated with expression of PD-1 in the infiltrating cells. However, deficiency of the PD-1 pathway has also been related to higher mortality resulting from GvHD (136, 137).

Programed cell death 1 appears to be of major clinical relevance in autoimmune diseases, such as diabetes mellitus type I (DM I) and systemic lupus erythematosus (SLE). PD-L1 is expressed in pancreatic beta islet cells and limits the activation and harmful cytotoxic function of self-reactive T-cells against islet cells, thereby protecting from autoimmune damage. Treatment of non-obese mice with PD-1- and PD-L1-blocking antibodies caused faster development of DM I, while treatment with PD-L2 blocking antibody had no effect (138, 139). In SLE, data that associates polymorphisms of the PD-1 gene with susceptibility to the disease in humans (140) are in line with evidence that mice deficient in PD-1 develop manifestations that resemble SLE, including glomerulonephritis and arthritis (7). Involvement of the PD-1 pathway in other autoimmune diseases, namely multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease, is also suggested by studies in animals and attributed to either absence or non-functionality of regulatory T-cells (115).

Role of PD-1 in Antitumor Immunity

The expression of PD-L1 and PD-L2 on APC after exposure to IFN-γ and the expression of PD-L1 in cancer cell lines (141) led to the hypothesis that blockade of the PD-1/PD-L1 inhibitory pathway might induce antitumor immunity. The hypothesis that engagement of PD-1/PD-L1 pathway might dampen immune responses for tumors was confirmed by the observation that overexpression of PD-L1 on a mouse mastocytoma cell line inhibits CD8+ T cell cytolytic activity through PD-1 ligation, which intensifies tumor growth and invasiveness (142). Studies in various types of human cancers have confirmed that tumors exploit PD-1-mediated immune suppression to escape immune surveillance. A wide variety of solid tumors, including urothelial, ovarian, breast, cervical, colon, pancreatic, gastric, melanoma, glioblastoma, non-small cell lung cancer (NSCLC), and hematologic malignancies have been found to express PD-L1 and to a lesser extent PD-L2, which correlate with adverse prognosis (143–149). Importantly, the presence of PD-L1 within the tumor microenvironment (TME) also correlates with a better clinical response to PD-1/PD-L1 checkpoint blockade therapy (17, 18, 150). In addition to cancer cells, PD-L1 and PD-L2 are also expressed in other cellular components of the TME including macrophages (mostly M2), myeloid DCs, myeloid suppressor cells (MDSC), stromal fibroblasts, and endothelial cells (Figure 2C). Similarly to cancer cell-specific expression, PD-L1 expression on tumor-infiltrating immune cells correlates with clinical responses to PD-1/PD-L1 blockade therapy. Conversely, lack of PD-L1 upregulation in tumor cells or tumor-infiltrating immune cells correlates with lack of therapeutic response and disease progression (151).

PD-L1 expression on cancer cells can be mediated by cell intrinsic mechanisms activated by oncogenic mutations (152). PD-L1 expression on cancer cells and tumor-infiltrating immune cells can also be induced by local inflammation, i.e., type I/II IFN-gamma released by activated T cells, a condition termed “adaptive immune resistance” (Figure 2C) (153). It should be noted that reported studies use a different cutoff of PD-L1 expression level to define positivity and variable approaches regarding evaluation of PD-L1 expression only on cancer cells or also on tumor-infiltrating immune cells (17, 18, 150). Use of different antibodies for histopathological assessment of PD-L1 expression may also lead to variable conclusions. Regardless of these confounding factors, there is an unequivocal conclusion that the degree of PD-L1 expression in the TME positively correlates with clinical response.

When the PD-1/PD-L1 pathway is active in the TME, it promotes survival of cancer cells via antiapoptotic signals mediated via PD-L1 (141, 154) and inhibits the activation of signaling pathways, which are critical for survival, expansion, and differentiation of T cells that recognize tumor antigens. The imbalanced activation of signaling events in T cells results in tumor tolerance by inhibiting T effector and memory cell generation and promoting the differentiation of TEx and TReg cells (Figure 3, left side). Importantly, high expression level of PD-L1 has been detected on tumor-infiltrating T cells, compared with T cells in normal tissues and peripheral blood from the same patients and healthy donors, and correlate with an exhausted phenotype and an impaired effector function (155). Blocking the PD-1/PD-L1 pathway by anti-PD-1 or anti-PD-L1 antibodies suppresses cancer cell survival, reverses the effects of PD-1 on T cell signaling, and promotes the generation of T effector and memory cells while preventing the differentiation of TEx and TReg cells. Together, these cell signaling and functional programs enhance antitumor T cell responses, leading to tumor regression and rejection (Figure 3, right side). It remains to be deciphered whether the therapeutic outcome of PD-1 blockade is different between patients with oncogenic PD-L1 expression versus immunogenic PD-L1 expression, in which PD-L1 is expressed on cancer cells and immune cells, respectively.

TAKING THE BENCHWORK TO CLINIC

Targeted therapy against PD-1/PD-L1 has shown significant clinical activity in a variety of cancers including solid tumors and hematologic malignancies such as melanoma, renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma, gastric cancer, hepatocellular carcinoma, ovarian cancer, cervical cancer, uterine cancer, breast cancer, colorectal cancer, prostate cancer, bladder cancer, Merkel cell carcinoma, Hodgkin’s lymphoma (HL), diffuse large B cell lymphoma, and follicular lymphoma (16–19, 145, 150, 156–174).

PD-1 Blockade in Melanoma

The use of antibodies that block immune checkpoints in the treatment of solid tumors was officially established in the armory of anticancer therapies in 2010 when ipilimumab, a CTLA-4 inhibitor, showed to improve survival in metastatic melanoma and led to the FDA approval of ipilimumab for the treatment of melanoma (175). Based on the fact that – similar to CTLA-4 – PD-1 is a coinhibitory receptor, antibodies have been developed
with the goal to inhibit the PD-1:PD-L1 pathway (Table 1). These antibodies have generated remarkable responses in a wide spectrum of cancers (Table 2) and have shown better clinical benefit and better toxicity profile than CTLA4-blocking antibodies (158, 170).

Nivolumab, an IgG4 PD-1 antibody, binds to PD-1 with high affinity and specificity and was the first PD-1 blocking agent to demonstrate clinical activity in several different types of cancers, including melanoma, RCC, and NSCLC in a phase I/II trial completed in 2012 (18). In a phase Ib dose escalation study, 32% of the patients with advanced melanoma developed durable remission, which correlated with expression of PD-L1 in the tumor cells defined as positive at a minimum level of 5% (18, 168). Subsequently, in a phase III study, which compared nivolumab with dacarbazine in patients with melanoma without B-Raf mutation, nivolumab was associated with a survival benefit (73 versus 42%) and higher objective response rate (40 versus 14%). The response rate of patients with PD-L1-positive tumors (defined as PD-L1-positive tumor cells >5%) was also better than in patients with PD-L1-negative/intermediate tumors (53 versus 33%) (150). In the second phase III trial, nivolumab was compared with chemotherapy in patients with advanced melanoma non-responsive to ipilimumab (or ipilimumab and BRAF inhibitor in BRAF-mutant tumors). A response rate of 32% was noted in the nivolumab treatment group versus 11% in the chemotherapy treatment group. This study also found PD-L1 as a response-predictive biomarker with 44% response rate in PD-L1 positive versus 20% in the PD-L1 negative tumors (171). Based on these outcomes, FDA approved nivolumab on December 22, 2014 for the treatment
of patients with melanoma, whose disease state has progressed after prior treatment.

Pembrolizumab is a very high affinity humanized IgG4 antibody directed against human PD-1. Randomized trials have been performed in both ipilimumab naïve (NCT01866319) and previously treated patients (NCT01704287). Promising results in preliminary studies (165, 166) led to a phase II dose escalation trial comparing two dose levels of pembrolizumab to chemotherapy in ipilimumab-refractory melanoma patients (176). This study showed clear benefit for both groups that received pembrolizumab with 6-month progression-free survival (PFS) of 34 and 38% compared to the PFS of 16% of the chemotherapy group. A subsequent phase III trial compared the treatment outcome of two different administration schedules of pembrolizumab to ipilimumab in patients with advanced melanoma and provided evidence of improved survival rate in both pembrolizumab treatment groups compared to the ipilimumab group (74 and 68 versus 58%) (158). On September 4, 2014 pembrolizumab was approved by FDA for the treatment of advanced melanoma in patients previously treated with ipilimumab or a BRAF inhibitor in BRAF V600 mutation positive patients. To date, both nivolumab and pembrolizumab have also been approved by FDA and used for treatment in NSCLC, head and neck cancer, RCC, and Hodgkin lymphoma.

**PD-1 Blockade in NSCLC**

The success of PD-1 blocking antibodies in NSCLC has made headlines since checkpoint blockade was thought to be responsive solely in immunogenic tumors like melanoma and RCC. A phase I dose-evaluating study of nivolumab has shown responses in patients with squamous and non-squamous histology, with or without EGFR or KRAS mutations, with or without tumor PD-L1 expression, and across different dose levels (18). On the basis of these preliminary results, two randomized phase III studies were undertaken. One compared nivolumab to docetaxel in advanced squamous NSCLC and found an improved median overall survival (OS) (9.2 versus 6.0 months) (172). In this study, tumor expression of PD-L1 determined at three different expression cutoff levels (1, 5, and 10%) had neither prognostic nor predictive of treatment benefit. The second study followed the same design and studied responses of patients with non-squamous NSCLC. In this patient group, nivolumab also resulted in improved median OS benefit compared to docetaxel (12.2 versus 9.4 months) while the OS at 1 year was 51% in the nivolumab group versus 39% in the docetaxel group (177). Correlations between therapeutic benefit and PD-L1 expression on tumor cells at the same cutoff levels (1, 5, and 10%) was also studied. In contrast to the observations in patients with advanced squamous NSCLC (172), patients with PD-L1-positive non-squamous NSCLC tumors had therapeutic benefit over those with PD-L1-negative tumors and this was observed across all three PD-L1 expression levels. Nivolumab was approved by FDA in March, 2015 for treatment of squamous NSCLC, and eventually for all patients with advanced NSCLC progressing after platinum-based chemotherapy on October 9, 2015. Almost in parallel, FDA also approved Pembrolizumab on October 2, 2015 for PD-L1-positive NSCLC, based on a large clinical trial, which assessed efficacy and safety of pembrolizumab in patients with advanced NSCLC (17). As in previous studies, therapeutic benefit was correlated with tumor PD-L1 positivity, which in this study was defined at the >50% cutoff. Importantly, this study also provided evidence that a striking survival benefit was observed in patients who received pembrolizumab without prior treatment with chemotherapy.

**PD1:PD-L1 Blockade in Other Cancers**

More than 100 trials are currently investigating the use of PD-1 blockade agents as monotherapy or in combination with chemotherapeutic agents, targeted therapies, or alternate immunotherapy modalities for multiple tumor types (http://clinicaltrials.gov).

For RCC, immunotherapy has always been considered as a primary therapeutic strategy because of its immunogenic nature. The rationale for treatment with PD-1:PD-L1 blockade in RCC was further supported by the excessive PD-L1 expression in inflamed and cancerous kidney tissues (178). A randomized phase II trial comparing different doses of nivolumab in advanced RCC patients has shown a long-lasting objective response in about 22% of the patients (179). Currently, a combinational treatment regimen of nivolumab with either sunitinib or pazopanib is being developed, which has shown better efficacy, but higher toxicity (180). The efficacy of pembrolizumab is also currently being evaluated in a phase I/II trial in treatment naïve metastatic RCC patients in combination with pazopanib or axitinib.

One of the most impressive responses has been observed in HL, in which PD-1 blockade with nivolumab resulted in response rate of 87% (19, 181, 182). This outcome is based on the molecular upregulation of the PD-1:PD-L1 pathway through amplification of 9p24.1, which increases the gene dosage of PD-L1 and PD-L2 together with Jak2 in nodular sclerosing HL (183).
TABLE 2 | Examples of clinical trials with antibodies blocking the PD-1-PD-L1 pathway.

<table>
<thead>
<tr>
<th>Cancer types</th>
<th>Blocking agents</th>
<th>Clinical response rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Melanoma</strong></td>
<td></td>
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<tr>
<td></td>
<td>Nivolumab</td>
<td>12.8% in treatment-refractory metastatic melanoma, 28% in advanced melanoma, 40% in</td>
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<td></td>
<td></td>
<td>melanoma treated in combination with ipilimumab, 20% in nivolumab followed by</td>
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<td></td>
<td></td>
<td>ipilimumab, 40% in previously untreated melanoma without BRAF mutation, 57.6% (nivolu-</td>
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<td></td>
<td></td>
<td>mumab plus ipilimumab) versus 19% (ipilimumab) versus 43.7% (nivolumab) in untreated</td>
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<td></td>
<td></td>
<td>stage III or IV melanoma</td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>38% in comparison to chemotherapy (14%), 26% in ipilimumab-refractory advanced</td>
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<tr>
<td></td>
<td></td>
<td>melanoma</td>
</tr>
<tr>
<td></td>
<td>Atezolizumab</td>
<td>21% objective response rate</td>
</tr>
<tr>
<td></td>
<td>MDX-1105</td>
<td>17.3% objective response rate</td>
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<tr>
<td><strong>NSCLC</strong></td>
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<tr>
<td></td>
<td>Nivolumab</td>
<td>12.8% in treatment-refractory metastatic NSCLC, 18% in advanced NSCLC, 14.5% in</td>
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<tr>
<td></td>
<td></td>
<td>refractory NSCLC, 17% in previously treated NSCLC, 20% in advanced squamous cell</td>
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<td></td>
<td></td>
<td>NSCLC, higher overall survival (12.2 months) versus docetaxel treatment (6 months)</td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>63 versus 0% in stage IV NSCLC patients with high and low non-synonymous mutation</td>
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<td></td>
<td></td>
<td>burden, 19.4% in advanced NSCLC of unselected population, 45.2% objective response</td>
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<td></td>
<td></td>
<td>rate in PD-L1+ population</td>
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<td></td>
<td>Durvalumab</td>
<td>14% objective response rate in unselected population and 23% in PD-L1+ population</td>
</tr>
<tr>
<td></td>
<td>Atezolizumab</td>
<td>15% objective response rate in unselected population and 38% in PD-L1+ population</td>
</tr>
<tr>
<td></td>
<td>MDX-1105</td>
<td>10.2% in NSCLC</td>
</tr>
<tr>
<td><strong>Renal cell cancer</strong></td>
<td>Nivolumab</td>
<td>Higher overall survival (25 months) and better objective response rate (25%) in</td>
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<tr>
<td></td>
<td></td>
<td>comparison to everolimus treatment (19.6 months and 5% ORR)</td>
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<td></td>
<td>Atezolizumab</td>
<td>21% overall response rate</td>
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<tr>
<td></td>
<td>MDX-1105</td>
<td>11.7% response rate</td>
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<tr>
<td><strong>Breast cancer</strong></td>
<td>Atezolizumab</td>
<td>19% objective response rate</td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>18.5% response rate</td>
</tr>
<tr>
<td><strong>Small cell lung cancer</strong></td>
<td>Nivolumab</td>
<td>18% objective response rate in monotherapy and 17% objective response rate in</td>
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<tr>
<td></td>
<td></td>
<td>combination</td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>35% response rate</td>
</tr>
<tr>
<td></td>
<td>Atezolizumab</td>
<td>21% objective response rate</td>
</tr>
<tr>
<td><strong>Head and neck</strong></td>
<td>Durvalumab</td>
<td>12% objective response rate</td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>24.8% objective response rate observed in both HPV+ and HPV− patients</td>
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<tr>
<td></td>
<td>Atezolizumab</td>
<td>19% objective response rate</td>
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<tr>
<td><strong>Hepatocellular carcinoma</strong></td>
<td>Nivolumab</td>
<td>19% objective response rate</td>
</tr>
<tr>
<td><strong>Gastric cancer</strong></td>
<td>Nivolumab</td>
<td>31% response rate</td>
</tr>
<tr>
<td></td>
<td>Atezolizumab</td>
<td>21% overall response rate</td>
</tr>
<tr>
<td><strong>Ovarian cancer</strong></td>
<td>Nivolumab</td>
<td>15% response rate, responses lasted up to 17 months</td>
</tr>
<tr>
<td></td>
<td>Avelumab</td>
<td>14.7% objective response rate</td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>11.5% response rate</td>
</tr>
<tr>
<td></td>
<td>Atezolizumab</td>
<td>21% overall response rate</td>
</tr>
<tr>
<td></td>
<td>MDX-1105</td>
<td>5.9% response rate</td>
</tr>
<tr>
<td><strong>Bladder cancer</strong></td>
<td>Atezolizumab</td>
<td>26% objective response rate in unselected population and 43% in PD-L1+ population</td>
</tr>
<tr>
<td></td>
<td>Pembrolizumab</td>
<td>25% objective response rate in unselected population and 38% in PD-L1+ population</td>
</tr>
<tr>
<td><strong>Mismatch repair-deficient carcinoma (colorectal and other)</strong></td>
<td>Pembrolizumab</td>
<td>40% objective response rate in repair-deficient CRC, 0% in repair-sufficient CRC, 71% in mismatch repair-deficient non-colorectal carcinomas</td>
</tr>
<tr>
<td><strong>Merkel cell carcinoma</strong></td>
<td>Pembrolizumab</td>
<td>71% objective response rate</td>
</tr>
<tr>
<td><strong>Hodgkin’s lymphoma</strong></td>
<td>Nivolumab</td>
<td>87% objective response in relapsed or refractory Hodgkin’s lymphoma</td>
</tr>
<tr>
<td></td>
<td>Pembrolizumab</td>
<td>66% overall response rate</td>
</tr>
</tbody>
</table>

Currently, ongoing clinical trials are investigating PD-L1 blocking antibodies. Such antibodies, specifically MPDL3280A (Atezolizumab) and MEDI4736 (Durvalumab) are being evaluated in metastatic melanoma. Interestingly, Atezolizumab was associated with good responses and less pulmonary toxicity compared to PD-1 antibodies (184). In a randomized phase II trial, Atezolizumab is being compared with platinum-based chemotherapy and docetaxel after platinum failure in NSCLC. Durvalumab in combination with an EGFR inhibitor is being compared to chemoradiation in stage III NSCLC, where an objective response rate of 14% has been noticed across all histologies (185, 186). Atezolizumab is also currently being investigated as monotherapy or in combination with bevacizumab in comparison to a control group of sunitinib in treatment-naive locally advanced or metastatic RCC. In a recent study, one complete and two partial responses were observed in patients with recurrent or metastatic triple negative breast cancer who are PD-L1 positive. PD-L1 blockade therapy also appears to be effective in bladder
cancer. In a phase I study of atezolizumab in advanced bladder cancer, an objective response rate of 43% has been observed in tumors expressing high levels of PD-L1 (160).

**Combination Approaches**

The combination of checkpoint blockade was first tested in advanced melanoma patients treated with nivolumab and ipilimumab and the resulting clinical activity was phenomenal (187). In a phase II study, the objective response rate for nivolumab plus ipilimumab was 59% in comparison to 11% with ipilimumab alone (188). Most recently, a phase III study of nivolumab plus ipilimumab versus nivolumab versus ipilimumab was performed in treatment naïve advanced melanoma patients (170). Again, the response rate was 57.6% for the combination therapy in comparison to 43.7% for nivolumab and 19% for ipilimumab monotherapies. The improved outcomes of the combination therapy over ipilimumab alone appear to be sustained within the 2-year follow-up of patients with combination therapy (189). The combination approach was also tested in patients with metastatic RCC. In this patient group, ipilimumab plus nivolumab in two different dose levels gave a response rate of 43 and 48%, respectively (190). Comparison between the combination checkpoint immunotherapy and sunitinib in advanced RCC is under investigation.

In NSCLC, a phase III study of nivolumab plus ipilimumab versus nivolumab monotherapy versus chemotherapy is currently undergoing (NCT02477826). Promising results were also reported from a phase I study of combination of Durvalumab and tremelimumab in NSCLC. Also, another phase III study in untreated, advanced NSCLC has recently begun with durvalumab plus tremelimumab versus durvalumab versus chemotherapy (NCT02453282). The combinatorial studies examining efficacy and safety of these drugs are also been undertaken in several other malignancies, including SCLC (191), gastric, and bladder cancer (NCT01928394). Because of different cellular expression/localization of PD-1 and PD-L1 in normal tissues, the tolerability and efficacy in combination of PD-1 plus PD-L1 is also being investigated (NCT02118337).

**Determinants of Response**

In order to understand how PD-1 blockade imparts tumor rejection, it is critical to identify the cell population(s) that are targeted and altered during antibody treatment (192). The presence of PD-L1 within TME in more than 1% of tumor cells has been shown to correlate with a better clinical response to PD-1/PD-L1 checkpoint blockade therapy. In contrast, lack of PD-L1 upregulation in tumor cells or lack of tumor-infiltrating immune cells has been observed in most progressing patients (18, 151, 193). However, studies in RCC have determined that detectable tumor expression of PD-L1 can be documented only in a small fraction of patients (20–30%), yet, a higher number of patients with PD-L1 negative RCC responded to PD-1 blockade (194).

As mentioned above, in addition to tumors, PD-L1 expression on tumor-infiltrating immune cells, mainly myeloid APC (macrophage and myeloid DCs) correlates with clinical responses to PD-1/PD-L1 blockade therapy (151). Based on these findings, it is possible that therapeutic PD-1 blockade might work more effectively if the tumors have already been identified by the host immune system and PD-L1 expression in cancer and innate immune cells is the consequence of local IFN-γ production by tumor-activated T cells (151, 193). Thus, one key approach to understand which cell types are important for tumor rejection is to determine location, density, and phenotype of the immune cells inside the TME and their spatiotemporal expression of PD-1 and PD-L1. Techniques to achieve this goal include, but not limited to, slide-based quantitative immunohistochemistry (IHC) and quantitative multiplexed IHC in situ gene expression assay (193, 195–197). The use of different anti PD-1 and anti PD-L1 antibodies, the different cutoff points to measure expression, the different cell types in which expression is being evaluated and the different scoring systems used by various pathology laboratories has caused difficulty in harmonizing the IHC readouts. The Cancer Immunotherapy Trials Network has started to review the immunodynamic effects of checkpoint inhibitors with the goal to identify and define immune assessment modalities and sites, both systemic and intratumoral, which are critical to the therapeutic success (198). Refining immune endpoints will provide the tools for the design of improved clinical trials, for selection of appropriate candidate patients for PD-1-based immunotherapy, and for assessment of induction and maintenance of therapeutic response.

**CONCLUSION AND FUTURE DIRECTIONS**

Programed cell death 1 is involved in the induction and maintenance of peripheral tolerance and plays a crucial role in the regulation of autoimmunity, transplantation immunity, infectious immunity, and tumor immunity. Currently, in parallel with the development of new discoveries about the molecular mechanisms of PD-1 function, clinical trials of combinatorial approaches are emerging. Such studies aim to maximize therapeutic antitumor benefit by blocking PD-1 together with other checkpoint inhibitors – such as CTLA-4, LAG3, TIM3, or by blocking PD-1 while engaging activating receptors of the TNF superfamily with agonist antibodies. Furthermore, PD-1 blockade together with chemoradiotherapy is anticipated to extend the therapeutic benefits of PD-1 checkpoint inhibition to a higher number of patients.

**AUTHOR CONTRIBUTIONS**

KB prepared the main body of the manuscript and figures. TA participated in the preparation of the clinical section of the manuscript. VB supervised the work and participated in the preparation of the manuscript and figures.

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PD-1 Pathway and Its Clinical Applications

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The PD-1 Pathway and Its Clinical Applications


**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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