Mechanistic overview of immune checkpoints to support the rational design of their combinations in cancer immunotherapy

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Checkpoint receptor blockers, known to act by blocking the pathways that inhibit immune cell activation and stimulate immune responses against tumor cells, have been immensely successful in the treatment of cancer. Among several checkpoint receptors of immune cells, cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death protein-1 (PD-1), T-cell immunoglobulin and ITIM domain (TIGIT), T-cell immunoglobulin-3 (TIM-3) and lymphocyte activation gene 3 (LAG-3) are the most commonly targeted checkpoints for cancer immunotherapy. Six drugs including one CTLA-4 blocker (ipilimumab), two PD-1 blockers (nivolumab and pembrolizumab) and three PD-L1 blockers (atezolizumab, avelumab and durvalumab) are approved for the treatment of different types of cancers including both solid tumors such as melanoma, lung cancer, head and neck cancer, bladder cancer and Merkel cell cancer as well as hematological tumors such as classic Hodgkin’s lymphoma. The main problem with checkpoint blockers is that only a fraction of patients respond to the therapy. Insufficient immune activation is considered as one of the main reason for low response rates and combination of checkpoint blockers has been proposed to increase the response rates. The combination of checkpoint blockers was successful in melanoma but had significant adverse events. A combination that is selected based on the mechanistic differences between checkpoints and the differences in expression of checkpoints and their ligands in the tumor microenvironment could have a synergistic effect in a given cancer subtype and also have a manageable safety profile. This review aims to help in design of optimal checkpoint blocker combinations by discussing the mechanistic details and outlining the subtle differences between major checkpoints targeted for cancer immunotherapy.

Key words: T cells, activation, exhaustion, checkpoints, PD-1, cancer immunotherapy

Introduction

Monoclonal antibodies targeting T-cell inhibitory checkpoint receptors have been highly successful in inducing antitumor immune responses and have shown enormous potential in the treatment of cancer (Figure 1). To date six checkpoint receptor blockers (commonly known as checkpoint blockers) are approved for treatment of melanoma, lung cancer, head and neck cancer, bladder cancer, Merkel cell cancer as well as classic Hodgkin’s lymphoma and 17 more are in advanced stages of clinical testing (Table 1 and supplementary Table S1, available at Annals of Oncology online) [1–28]. The greatest advantages of checkpoint blockers have been impressive durable response rates and manageable safety profile [29–31]. However, the achievements of cancer immunotherapy are eclipsed by low response rates with only a fraction of patients benefitting from therapy. Although the use of combinational immunotherapy as in the combination of ipilimumab and nivolumab increased response rates in metastatic melanoma patients, a sizeable proportion of patients remained unresponsive [32, 33]. More importantly, the
Figure 1. Milestones in the clinical development of checkpoint blockers. Source: References [1, 3, 5, 7, 8, 10, 11, 21–25, 28, 32, 33, 100–102].

Table 1. List of FDA approved drugs that target T-cell checkpoints as of 20 September 2017

<table>
<thead>
<tr>
<th>Drug; Brand name; Marketed by</th>
<th>Approved indications</th>
<th>Recommended dose and route</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTLA-4 blockers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ipilimumab; Yervoy; Bristol-Myers Squibb</td>
<td>Metastatic melanoma, metastatic non-small-cell lung cancer (NSCLC), renal cell carcinoma (RCC), classical Hodgkin’s lymphoma, head and neck squamous cell carcinoma (HNSCC), metastatic urothelial carcinoma, hepatocellular carcinoma (HCC), colorectal cancer with MSI-H and MMR aberrations</td>
<td>Metastatic disease: not more than four doses of 3 mg/kg i.v. for every 3 weeks&lt;br&gt;Adjuvant setting: 4 doses of 10 mg/kg i.v. for every 3 weeks, followed by 10 mg/kg i.v. for every 12 weeks, for up to 3 years</td>
<td>[1–3]</td>
</tr>
<tr>
<td><strong>PD-1 blockers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nivolumab; Opdivo; Bristol-Myers Squibb</td>
<td>Metastatic melanoma, metastatic NSCLC, renal cell carcinoma (RCC), classical Hodgkin’s lymphoma, head and neck squamous cell carcinoma (HNSCC), metastatic urothelial carcinoma, hepatocellular carcinoma (HCC), colorectal cancer with MSI-H and MMR aberrations</td>
<td>For melanoma, NSCLC, RCC, metastatic urothelial carcinoma, HCC and colorectal cancer with MSI-H and MMR aberrations: 240 mg i.v. infusion for every 2 weeks until disease progression or toxicity&lt;br&gt;For classical Hodgkin’s lymphoma and HNSCC: 3 mg/kg i.v. infusion for every 2 weeks until disease progression or toxicity</td>
<td>[4, 5, 10, 11, 13–15, 17, 18, 102]</td>
</tr>
<tr>
<td>Pembrolizumab; Keytruda; Merck</td>
<td>Metastatic melanoma, metastatic NSCLC, renal cell carcinoma (RCC), classical Hodgkin’s lymphoma, HNSCC, gastric cancer, solid tumors with MSI-H and MMR aberrations, metastatic urothelial carcinoma</td>
<td>200 mg i.v. infusion for every 3 weeks until disease progression, toxicity or up to 24 months</td>
<td>[6–9, 12, 16, 19, 100, 101]</td>
</tr>
<tr>
<td><strong>PD-L1 blockers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atezolizumab; Tecentriq; Genentech/Roche</td>
<td>Metastatic urothelial carcinoma, metastatic NSCLC</td>
<td>1200 mg i.v. infusion for every 3 weeks until disease progression or toxicity</td>
<td>[19–23]</td>
</tr>
<tr>
<td>Avelumab; Bavencio; Pfizer</td>
<td>Merkel cell carcinoma, metastatic urothelial carcinoma</td>
<td>10 mg/kg i.v. infusion for every 2 weeks until disease progression or toxicity</td>
<td>[26]</td>
</tr>
<tr>
<td>Durvalumab; Imfinzi; Astrazeneca</td>
<td>Metastatic urothelial carcinoma</td>
<td>10 mg/kg i.v. infusion for every 2 weeks until disease progression or toxicity</td>
<td>[27, 28]</td>
</tr>
</tbody>
</table>
immune-related adverse events commonly associated with checkpoint blockers such as diarrhea, colitis, myocarditis and endocrine disorders were found to be more severe in patients treated with the combination and the activity of the combination also may not extrapolate to lung cancer patients based on the available data [32–41]. As listed in Table 2, checkpoints inhibit immune responses through unique mechanisms; checkpoint receptors as well as their ligands are differently expressed on immune cells and the downstream signaling that follows the receptor activation is also varied. While some checkpoints inhibit immune cell activation in lymph nodes and peripheral tissues, others regulate activation only at peripheral tissues (Table 3). Similarly, some checkpoints induce tolerogenic phenotype in antigen presenting cells (APCs) and/or drive the priming of regulatory T cells (TRegs), whereas others are involved only in induction of T-cell dysfunction. The differences in checkpoint-mediated regulation could be the reason for varied response rates and adverse effects of checkpoint blocker combinations. A review discussing how the different checkpoints converge in inhibition of antitumor immune response could provide a platform for the development of effective checkpoint combinations. To our knowledge there have been very few attempts to compare different checkpoints and did not include all the major checkpoint targets in the discussion [42–46]. The present review therefore aims to provide a foundation for rational design of checkpoint combinations by discussing checkpoints that are currently evaluated in clinical trials such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death protein-1 (PD-1), T-cell immunoglobulin and ITIM domain (TIGIT), T-cell immunoglobulin-3 (TIM-3) and lymphocyte activation gene 3 (LAG-3). The basic concepts of T-cell activation in the lymph node, regulation of T-cell activity in peripheral tissues and the exhaustive/dysfunctional phenotype of T cells are discussed in the following sections to aid in the better understanding of checkpoint mechanisms.

Table 2. Checkpoints of T-cell activation

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CTLA-4</th>
<th>PD-1</th>
<th>TIGIT</th>
<th>TIM-3</th>
<th>LAG-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonyms</td>
<td>CD152</td>
<td>PDCD1 and CD279</td>
<td>WUCAM</td>
<td>HAVCR2</td>
<td>CD223</td>
</tr>
<tr>
<td>Gene location</td>
<td>Chromosome 2q33</td>
<td>Chromosome 2q37.3</td>
<td>Chromosome 3q13.31</td>
<td>Chromosome 5q33.3</td>
<td>Chromosome 12p13.32</td>
</tr>
<tr>
<td>Number of amino acids</td>
<td>223 amino acids</td>
<td>288 amino acids</td>
<td>344 amino acids</td>
<td>301 amino acids</td>
<td>498 amino acids</td>
</tr>
<tr>
<td>Signaling motif</td>
<td>Cytoplasmic tail</td>
<td>ITSM</td>
<td>ITT-ITIM</td>
<td>Tyrosine residues in the cytoplasmic tail</td>
<td>KEELE motif in the cytoplasmic tail</td>
</tr>
<tr>
<td>Ligands</td>
<td>CD80 and CD86</td>
<td>PD-L1 and PD-L2</td>
<td>PVR/CD155 and CD112</td>
<td>Galectin-9, Ceacam-1, HMG8L1 and phosphatidyserine</td>
<td>Galectin-3</td>
</tr>
<tr>
<td>Cells expressing receptor</td>
<td>Activated T cells, TRegs, exhausted effector T cells</td>
<td>Activated T cells, memory T cells, TH1 cells, TRegs, Tr1 cells, NK cells, NKT cells and exhausted effector T cells</td>
<td>Activated T cells, TH17 cells, TReg, DCS, NK cells, monocytes, and exhausted effector T cells</td>
<td>Activated CD4+ T cells, TRegs, Tr1 cells, activated CD8+ T cells, NK cells, DCS, B cells, and exhausted effector T cell</td>
<td>Activated CD4+ T cells, TRegs, Tr1 cells, activated CD8+ T cells, NK cells, DCS, B cells, and exhausted effector T cell</td>
</tr>
<tr>
<td>Cells expressing ligand</td>
<td>APCs, hematopoietic &amp; nonhematopoietic cells and tumor cells</td>
<td>APCs, fibroblasts, endothelial cells and tumor cells</td>
<td>APCs, tumor cells</td>
<td>APCs</td>
<td>Liver cells and tumor cells</td>
</tr>
<tr>
<td>Reference</td>
<td>[72–80]</td>
<td>[70, 88, 92–98]</td>
<td>[103–112]</td>
<td>[117–126]</td>
<td>[129–134, 143, 144]</td>
</tr>
</tbody>
</table>

T-cell activation

Activation of T cells involves presentation of antigens on MHC I/II molecules to T-cell receptor (TCR) on T cells by APCs such as dendritic cells (DCs), which results in phosphorylation of CD3ζ subunits and activation of zeta-chain associated protein kinase 70 (ZAP-70) that phosphorylates linker for activation of T cells, a scaffold protein for other signaling molecules, and activates PI3K-Akt-MTORC1, MAPK and NFKB pathways [47–56]. However, antigen recognition alone is not sufficient to activate T cells and requires a second activating signal (also known as costimulation), which most commonly originates from the interaction between CD28 receptors on T cells and B7-ligands (CD80/ CD86) on APCs; presentation of antigens to T cells in the absence of costimulation causes T-cell anergy and apoptosis [57]. In addition to CD28, several other receptors also function as costimulatory receptors for T-cell activation; the receptors and their respective ligands are listed in supplementary Table S2, available at Annals of Oncology online. Uncontrolled T-cell activation is also prevented by specialized receptors on T cells called ‘checkpoint receptors’, which act by binding to their cognate ligands on APCs (supplementary Table S2, available at Annals of Oncology online). Thus the fate of the T cell in lymph nodes depends on the degree of APC-antigen-mediated TCR activation, number of costimulatory receptors, presence of costimulatory ligands on APCs as well as on the number of checkpoint receptors (Figure 2). Interestingly, T-cell activity can also be regulated (activation or inhibition) outside the lymph node and T cells can interact again with a new APC or other immune cells before reaching the target site. Although the second interaction is not a compulsory event, colocalization of immune cells in tissue spaces introduces the possibility of interaction between T cell : APC, T cell : T cell, T cell : NK cell etc. The second interaction in the tissues could
decide whether the activated T-cell should proceed with effector functions or should be shut down and prevented from causing damage to the tissues (Figure 2). To explain the significance of second interaction, some immunologists proposed a two-step activation theory of T cells: the first step of activation takes place in the lymph nodes and the second step of activation takes place in the peripheral tissues. According to this model, T cells are primed and activated in the first stage but are not fully committed to the effector phenotype; complete differentiation and commitment to effector functions takes place only after the second interaction, also referred to as ‘second touch’ [58]. Regardless of whether there is enough experimental evidence to support the two-step activation model, the possibility of interactions between immune cells and the outcome cannot be ignored. Costimulatory receptors on T cells further activate the T cells and amplify the immune response whereas checkpoint receptors inhibit the T cells and dampen the response. Tumor microenvironment (TME) is enriched with mediators of immunosuppression, which induce the expression of checkpoint receptors on the surface of T cells and promote a dysfunctional/exhausted phenotype in T cells, which is briefly described in the following section [59–65].

### Table 3. Differences in effects of checkpoint blockade

<table>
<thead>
<tr>
<th>Check-points</th>
<th>Autoimmune phenotype of knock-out mice</th>
<th>Phases of immune response effected by checkpoint blockade</th>
<th>Cells effected by checkpoint blockade</th>
<th>Expected stimulation of immune response with checkpoint blockade</th>
<th>Expected safety of checkpoint blockade</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>Spontaneous and lethal; mice die within 3–4 weeks of age</td>
<td>Mainly early stage activation in lymph nodes; also affects exhaustion and activation in tissues</td>
<td>Activated T cells in lymph nodes and periphery, DCs and exhausted effector T cells</td>
<td>+++</td>
<td>+</td>
<td>[72–74, 81]</td>
</tr>
<tr>
<td>PD-1</td>
<td>Spontaneous but less severe</td>
<td>Mainly activation in tissues, exhaustion and also activation in lymph nodes</td>
<td>Activated T cells in lymph nodes and periphery, Tregs, macrophages, exhausted effector T cells, and NK cells</td>
<td>++</td>
<td>++</td>
<td>[89–91, 96, 97]</td>
</tr>
<tr>
<td>TIGIT</td>
<td>Mild; not spontaneous</td>
<td>Exhaustion and activation in tissues</td>
<td>Activated T cells in lymph nodes and periphery, DC phenotype, Tregs, activated T cells in periphery, exhausted effector T cells and NK cells</td>
<td>+</td>
<td>+++</td>
<td>[103–108, 113]</td>
</tr>
<tr>
<td>TIM-3</td>
<td>Mild; not spontaneous</td>
<td>Exhaustion and activation in tissues</td>
<td>DC phenotype, MDSCs, Tregs, activated T cells in periphery, exhausted effector T cells and NK cells</td>
<td>+</td>
<td>+++</td>
<td>[118, 120, 121–123]</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Mild; not spontaneous</td>
<td>Exhaustion and activation in tissues</td>
<td>Tregs, activated T cells in periphery, exhausted effector T cells and NK cells</td>
<td>+</td>
<td>+++</td>
<td>[135–140]</td>
</tr>
</tbody>
</table>

+, low; ++, moderate; ++++, high.

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**T-cell exhaustion**

Long-term exposure to antigens in the presence of inflammatory cytokines induces a distinct phenotype in T cells, characterized by loss of effector functions, sustained expression of inhibitory receptors, poor proliferative capacity and decreased cytotoxic functions [66–68]. Progressive loss of T-cell effector functions, termed ‘T-cell exhaustion’ is commonly seen during chronic viral infections and in cancer. The significance of T-cell exhaustion in cancer has rapidly gained importance and several studies have identified the presence of ‘exhausted’ T cells in TME [59–65]. Negative regulatory signals arising due to the activation of immunosuppressive checkpoints are thought to be the main mechanism for effector T-cell dysfunction in TME. Interestingly, exhausted T cells were found to have residual effector functions and the blockade of checkpoints of T-cell activity has been shown to rescue and reverse the phenotype in at least a subset of the cells [68–71]. Indeed, monoclonal antibodies targeting CTLA-4 and PD-1 checkpoint pathways have shown tremendous potential in the treatment of some of the cancers types with very poor prognosis (Table 1). The mechanisms of checkpoint blockers are...
debated as to whether their effects are through promotion of increased T-cell activation by APCs or through reversal of the phenotype of a subset of exhausted T cells or both. Strikingly, PD-1 receptor expression is considered as an important marker for degree of T-cell exhaustion and the cells with high T-bet (T-box transcription factor) and low-to-intermediate PD-1 expression are considered as reversible [67, 68]. The fact that PD-1 : PD-L1 pathway targeting antibodies have produced durable responses in some patients provides credence to the argument that reversal of T-cell exhaustion is one important mechanism by which antitumor immune response can be stimulated. However, although the response rates to PD-1 : PD-L1 targeting antibodies are encouraging, a significant fraction of patients still do not respond to therapy. The focus of ongoing research is to effectively treat these nonresponding patients. Increasing evidence indicates that exhausted T cells in the TME express multiple checkpoints and that blockade of a single checkpoint may not be sufficient to stimulate an immune response [59, 60]. Using immune profiling of peripheral blood from melanoma patients, Wherry and associates demonstrated that CD8 T cells expressing multiple checkpoints were the most responsive to anti-PD-1 therapy [69]. Deeper understanding of the regulation of T-cell activity by checkpoints with emphasis on similarities and differences between the mechanisms could therefore help in the development of a checkpoint blocker combination with higher response rates. Checkpoints commonly associated with T-cell exhaustion are

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**Figure 2.** Regulation of T-cell activation. The outcome of T cell priming in lymph node depends on the degree of TCR activation and on the levels of costimulatory/checkpoint receptor expression on T cells. Optimal TCR activation results in minimal CTLA-4 and PD-1 expression (checkpoints) and maximal CD28 expression (costimulatory receptor), driving the T cell toward an effector phenotype. Hyperactivation of TCR during priming induces expression of CTLA-4 and/or PD-1 and drives the T cells into anergy, dysfunction or differentiation into TRegs depending on the checkpoint receptor and the naive T-cell sub-type. Similarly, peripheral interaction between effector T cells and other immune cells such as DCs, macrophages, B cells and Th1 cells can also result in amplification or dampening of the effector responses depending on the TCR-antigen interaction and the subsequent costimulatory or checkpoint receptor activation.
discussed in the next section followed by factors relevant to checkpoint blocker combinations.

**Checkpoints of T-cell activation**

**CTLA-4**

CTLA-4, was discovered in 1987 by Brunet et al., through screening of mouse cytolytic T-cell derived cDNA libraries. The protein was found to be expressed mainly in activated lymphocytes and was co-induced with T-cell-mediated cytotoxicity [72]. Loss of *Ctla-4* in mice was reported to be associated with development of rapidly progressive and fatal disease characterized by massive lymphoproliferation, multiorgan tissue destruction and death by 3–4 weeks of age [73, 74]. Studies on human CD28/CTLA-4: B7-1 crystal structure revealed that during T-cell activation, CTLA-4 binds to B7 ligands on APCs with higher affinity and at a much lower surface density compared with costimulatory receptor CD28 and suppresses the activation by competing with CD28 for binding with B7 ligands [75]. CTLA-4 expression was shown to be minimal in resting murine T cells and induced at both mRNA and protein level in response to TCR activation [76, 77]. Unlike other effector cells, murine TRegs were shown to constitutively express CTLA-4 owing to their high expression of forkhead transcription factor FoxP3, a known regulator of CTLA-4 expression [42, 78–80]. CTLA-4 : B7-ligand interaction in mice was shown to cause sequestration of B7-ligands, transendocytosis and degradation of endocytosed ligands resulting in tolerogenic APCs due to significant depletion of the B7 ligands from the surface [81]. Furthermore, studies in murine T cells that followed intracellular events after CTLA-4 engagement also illustrated activation of cell-intrinsic signaling cascades and cross-talk with pathways such as MAPK, PI3K and NFkB that regulate cell survival and proliferation [82–87]. Anti-CTLA-4 antibodies were the first to show promising results in cancer treatment and three antibodies, ipilimumab, tremelimumab and MK1308 progressed into clinical trials. Tremelimumab and MK1308 are currently under development (supplementary Table S1, available at *Annals of Oncology* online), whereas ipilimumab is approved for treatment of unresectable stage III/IV metastatic melanoma and as adjuvant therapy for surgically treated ‘high-risk’ melanoma patients [1–3].

**PD-1**

PD-1 was first described as a cell surface receptor expressed commonly on T cells by Honjo et al. from studies on pathways of programmed cell death [88]. Unlike CTLA-4, the phenotype of PD-1 deficient mice is relatively mild, has delayed onset and is dependent on the genetic background of the mouse [89–91]. In humans, PD-1 is expressed on activated T cells and B cells, TRegs, natural killer T (NKT) cells, natural killer (NK) cells, activated macrocytes and on myeloid DCs. PD-L1 is the widely expressed ligand for PD-1, found on T cells, B cells, macrophages, DCs as well as nonhematopoietic cell types such as endothelial cells, fibroblastic reticular cells, epithelia, pancreatic islet cells, astrocytes, neurons and also on cells at sites of immune privilege including trophoblasts in the placenta and retinal pigment epithelial cells. PD-L2 has a comparatively narrow expression profile and is seen mainly on activated macrophages and DCs [92–95]. The interaction between PD-1 and its ligands PD-L1/ PD-L2 impairs T-cell survival and blocks the characteristic features of T-cell response such as cell proliferation, cytokine secretion and cytotoxic ability [96, 97]. In addition, PD-1 signaling was also shown to enhance FoxP3 expression in murine models and to regulate the differentiation of induced TReg (iTReg) cells [97]. Recently, PD-1 was shown to be expressed on murine and human tumor-associated macrophages and inhibit their phagocytic potency against tumor cells [98]. The PD-1 : PD-L1 pathway is a tissue protective pathway; tumor cells utilize this normal physiological mechanism by expressing PD-L1 and thereby develop resistance to antitumor immune responses [42]. Additionally, chronic exposure to increased levels of inflammatory cytokines and antigens can also result in increased expression of PD-1 and PD-L1, a characteristic feature of T-cell exhaustion and dysfunction [68]. PD-1-mediated signaling involves recruitment of phosphatases SHP1 and SHP2, dephosphorylation of effector kinases leading to inhibition of glucose consumption, cytokine production, and the proliferation and survival of T cells [99]. In contrast to CTLA-4, PD-1 blocks the proximal activation of PI3K/Akt; the extent of its T-cell inhibition thus depends on TCR signal strength and was shown to be abrogated in tumor-bearing mice, in presence of T-cell costimulation [70]. While PD-1 : PD-L1/PD-L2 pathway could be inhibited by blocking PD-1 or PD-L1 or PD-L2, PD-1 blockers are expected to have greater activation of immune response as they inhibit both PD-1 : PD-L1 and PD-1 : PD-L2 axis. PD-L1 blockade would only inhibit PD-1 : PD-L1 axis, but is expected to activate the immune response significantly because PD-L1 is broadly expressed, whereas PD-L2 blockade can only inhibit PD-1 : PD-L2 axis and due to the low expression of PD-L2 ligands, PD-L2 blockers are not expected to significantly increase the immune response. PD-1 : PD-L1 pathway has been the most promising target for cancer immunotherapy and two anti-PD-1 antibodies (pembrolizumab and nivolumab), and three anti-PD-L1 antibodies (atezolizumab, avelumab and durvalumab) are approved for the treatment of various types of cancer [4–28]. Further, eight monoclonal antibodies against PD-1 and five against PD-L1 are under clinical development (supplementary Table S1, available at *Annals of Oncology* online). Strikingly, anti-PD-1 therapy is the first cancer treatment to receive approval for patients with solid tumors that are positive for microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) abnormalities [100–102].

**TIGIT**

TIGIT is an inhibitory receptor recently discovered by scientists from Genentech and independently by other researchers through genomic search for T-cell-specific genes with protein domain structures representative of potential inhibitory receptors [103–105]. In humans, TIGIT expression is mainly seen on resting TReg cells, Tr1 cells, memory T cells, activated T cells, exhausted CD8+ T cells, TFH cells, NKT cells and NK cells, but it is not detected on naïve CD4+ T cells [103–108]. TIGIT has greater affinity towards poliovirus receptor (PVR; also known as CD155 and nectin-like protein 5) ligands and like CTLA-4 it acts mainly by competing with the costimulatory receptors CD226 and CD96.
expressed on T cells for PVR and PVRL2 ligands (also known as CD112 and nectin 2) [109–112]. Studies in mice showed that TIGIT–PVR interaction inhibits the activation, proliferation and differentiation of T cells and at the same time activates the survival pathways thereby ensuring long-term survival of inhibited T cells. The interaction of PVR on murine DCs with TIGIT was shown to induce a tolerogenic phenotype in DCs by skewing the cytokine secretion profile of DCs towards decreased IL-12 production and increased IL-10 production [103]. Activation of TIGIT on human NK cells reportedly resulted in decreased IFN-γ production, cytotoxic granule polarization and NK-cell cytotoxicity [105]. Finally, experiments in mice revealed shift in cytokine balance, promotion of Th2 phenotype and suppression of Th1- or Th17-phenotype upon TIGIT engagement on Tregs [113]. Intracellular TIGIT signaling has been mostly demonstrated in NK cells where its intracellular domain was shown to associate with β-arrestin 2, recruit SHIP1 (SH2-containing inositol phosphatase 1) and regulate PI3K, MAPK and NF-κB signaling cascades [114–116]. Genentech is leading the development of anti-TIGIT antibodies for the treatment of cancer and its molecule MTIG7192A/RG6058 is currently in clinical trials (supplementary Table S1, available at Annals of Oncology online).

TIM-3

TIM-3, was identified by Kuchroo and associates in 2002, as a cell surface receptor selectively expressed on differentiated CD4+ Th1 cells [117]. Apart from Th1 cells, TIM-3 is also expressed on activated CD8+ T cells, Th17 cells, TRegs and other innate immune cells such as DCs, NK cells and monocytes [118]. The ligands for TIM-3 include galectin-9, a C-type lectin, carinoembryonic antigen-related cell adhesion molecule 1 (Ceacam-1), high mobility group box1 protein (HMGB-1) and phosphatidylserine [119]. Unlike the ligands for other checkpoints such as TIGIT, the ligands for TIM-3 have a broad expression profile and are expressed on a variety of cell types including cancer cells [118]. TIM-3 has been shown to regulate the proliferation and cytokine release of Th1 cells and Tim-3−/− mice were found to be refractory to induction of antigen-specific tolerance [120, 121]. Studies in mice also implicated TIM-3 in expansion of TRegs and myeloid-derived suppressor cells (MDSCs) and inhibition of the phagocytic activity and maintenance of peripheral tolerance by DCs [122, 123]. Additionally, TIM-3 was also found to be involved in T-cell exhaustion; high TIM-3 expression has been observed on CD8+ T cells from tumor-bearing mice as well as from cancer patients [124–126]. Human TIM-3 does not have a conventional signaling motif in its cytoplasmic tail and depends on the conserved tyrosine residues in the cytoplasmic region for initiation of signaling cascade. BAT-3 (HLA-B–associated transcript 3) plays an important role in the control of TIM-3 signaling; under basal conditions when TIM-3 is not activated by its ligand, BAT3 is bound to TIM-3 and promotes the TCR signaling by blocking SH2 domain interaction with inhibitory Src kinases. TIM-3 activation leads to phosphorylation of tyrosine residues mediated release of BAT-3 from TIM-3 tail and inhibits TCR signaling [44, 127, 128]. The clinical development of anti-TIM-3 antibodies is currently being pursued by Novartis, Tesaro and by Roche (supplementary Table S1, available at Annals of Oncology online).

LAG-3

LAG-3 (also known as CD223) was discovered more than 25 years ago as a CD4-related molecule found on activated T cells as well as NK cells [129]. In addition, LAG-3 expression is also seen on both natural and induced TRegs, T17 cells, exhausted T cells, B cells and DCs. The ligands for LAG-3 receptor include MHC class II molecules expressed on APCs, Galectin-3 and liver sinusoidal endothelial cell lectin (LSECtin), a member of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) family of molecules, expressed in liver as well as several tumor subtypes [130–134]. Unlike other negative regulators of immune response, the effects of LAG-3-mediated suppression are comparatively mild, as the LAG-3-deficient mice develop autoimmune disorders only when tested under permissive genetic background [135–138]. LAG-3 is thought to function in coordination with other checkpoints such as PD-1 to promote tolerance, T-cell dysfunction and exhaustion [44, 68, 139]. Studies in human peripheral blood mononuclear cells showed that the negative effects of LAG-3 on effector T-cell functions were due to its association with CD3 and the subsequent LAG-3 : CD3 ligation-mediated inhibition of T-cell proliferation, cytokine production and calcium flux [140]. Interestingly, LAG-3 has differential effects on T-cell subtypes; while it inhibits the activity of effector T cells, LAG-3 promotes the suppressive activity and the IL-10 secretion capacity of TRegs as seen by studies in mouse as well as human cells [141, 142]. The mechanisms involved in these varying effects and the signaling events that occur after LAG-3 activation are not completely understood. While the detailed mechanisms are yet to be elucidated, the KIEELE motif in the cytoplasmic region was shown to be critical for the inhibition of CD4+ effector T cells [44, 143, 144]. Anti-LAG3 antibodies are under clinical development and recently, anti-LAG-3 (BMS986016) plus anti-PD-1 (nivolumab) combination was reported to show encouraging efficacy in melanoma patients who progressed after anti-PD-1 therapy (supplementary Table S3, available at Annals of Oncology online) [145, 146]. Apart from BMS, anti-LAG-3 antibodies developed by Roche, Novartis and Tesaro are also in clinical trials for treatment of cancer (supplementary Table S1, available at Annals of Oncology online).

Perspective

Immunotherapy has transformed cancer treatment in the past decade with dramatic increase in durable response rates. While other strategies like cancer vaccines, indoleamine oxidase inhibitors and chimeric antigen receptor engineered T cells (CAR-T cells) are also promising, the review only focused on discussing checkpoint blockers because they are the most successful class of immunotherapeutics and are approved for multiple tumor-types both as monotherapy and in combination [94, 147]. By discussing the mechanistic differences between checkpoints, the review aims to support the development of future combinations of checkpoint blockers. As pointed out in the previous sections, checkpoint receptors play a significant role in the initial T-cell activation in lymph nodes, second activation in tissues and also in T-cell exhaustion. A key point to be noted here is that though there is some overlap in inhibitory functions, each checkpoint receptor also has distinct functions (Figure 3). For example,
CTLA-4 is primarily involved in regulation of T-cell activation in lymph nodes/tissues and in TReg-mediated suppression of DC activity. CTLA-4 is not expressed on NK cells and therefore does not regulate NK-cell functions. The fact that CTLA-4 knockout mouse has a lethal autoimmune phenotype indicates its predominant role in priming and tolerance to self-antigens [73, 74].

PD-1 receptors on the other hand are expressed on activated T cells as well as NK cells, and therefore regulate T-cell activation in lymph nodes/tissues, differentiation into TRegs as well as NK-cell activity. PD-1 knockout mouse also develops autoimmune phenotype but the onset is delayed and is less severe compared with phenotype of CItla-4 /− mouse, suggesting that PD-1 has basal role in induction of tolerance to self-antigens and priming, and that it mainly regulates the immune responses in peripheral tissues [89–91]. TIGIT receptors are expressed on activated T cells and NK cells; they shift the cytokine profile of DCs from Th1-promoting towards TReg promoting and also regulate T- and NK-cell effector functions. Milder phenotype of Tigit /− mouse suggests less critical role of TIGIT in the induction of tolerance to self-antigens and points to its importance in peripheral sites.
Inhibitor of effect T cells and NK cells in nonlymphoid/peripheral tissues due to cell–cell interactions as well as exhaustion of effect T cells due to continuous antigen release under immuno-suppressive conditions appear to be important mechanisms operating in the TME. Blockade of PD-1: PD-L1 pathway has been the most successful cancer immunotherapy strategy to date possibly because it abrogates both peripheral inhibition and T-cell exhaustion and also affects the priming of Tregs. CTLA-4 pathway blockade acts mainly by increasing the T-cell activation in lymph nodes, which is possibly responsible for severe immune-related side-effects but not sufficient for stimulating antitumor immune responses in most patients. A combination of CTLA-4 and PD-1: PD-L1 pathway blockers was expected to increase immune cell activation in lymph nodes as well as peripheral tissues and also reverse T-cell exhaustion. The combination did show remarkable success in melanoma patients but was associated with severe immune-related adverse events and also was unable to achieve similar response rates in lung cancer patients [32–36]. The reasons for this varied success of the combination could be due to differences in the pathways that lead to inhibition of effector cells in TME. The degree of T-cell exhaustion as well as the characteristic checkpoints in melanoma patients may differ from lung cancer patients. Tumor mutation load, neoantigen generation, rate of antigen release and immunogenicity has been reported to vary between cancer subtypes and between cancer patients; these differences could lead to disparities in priming of naïve T cells, activation of immune response and in degree of T-cell exhaustion [148–154]. Analysis of tumor samples from non-small-cell lung cancer patients showed that the PD-1 expressing CD8+ T cells had lowest percentages of other inhibitory receptors such as CTLA-4, whereas analysis of immune cells from melanoma patients showed that PD-1+ CD8+ T cells also expressed other checkpoints such as CTLA-4 and were in fact the most responsive to anti-PD-1 therapy, pointing toward the significance of differences in checkpoint expression and the mechanisms of T-cell exhaustion operating in the TME [59, 69]. TIGIT expression was reported to correlate significantly with PD-1 expression in tumor samples from lung cancer patients and the combination of anti-TIGIT and anti-PD-1 antibodies showed synergistic effects in mouse tumor models suggesting that targeting TIGIT and PD-1 could be advantageous in lung cancer [106]. Along those lines, TIM-3 expression was shown to be altered in CD8+ T cells following PD-1 therapy indicating the benefits of combining anti-PD-1 and anti-TIM-3 antibodies [69]. Degree of effector T-cell infiltration into tumor sites is another factor that influences the response to immunotherapy and the combination that can increase tumor infiltration of T cells could have synergistic benefits. For example, in a relatively small group of metastatic renal cell carcinoma patients, a combination of anti-vascular endothelial growth factor (anti-VEGF) antibody, known to increase vascular permeability and anti-PD-L1 antibody was shown to significantly increase CD8+ T cells in tumor tissues and induce tumor regression [155, 156]. An effective combination could thus be cancer-specific and dependent on multiple factors such as status of immune response, mechanisms operating in the TME and the levels of checkpoint receptor co-expression. Assays such as whole exome sequencing, immunohistochemical analyses and peripheral blood immunophenotyping could be employed to analyze biomarkers that help in understanding patient-specific mechanisms of immuno-suppression such as neoantigen expression, presence of antigen-specific T cells, relative expression of checkpoint receptors on effect T cells, neutrophil-to-lymphocyte ratio and serum lactate dehydrogenase, and the information could be used in rational selection of checkpoint combinations (supplementary Table S4, available at Annals of Oncology online) [100–102, 153, 154, 157–168]. In cancer types where lack of sufficient priming of naïve T cells and activation of immune responses are expected, a combination of CTLA-4 plus PD-1: PD-L1 pathway blockers could be useful, whereas when the lack of immune response is predominantly due to checkpoints such as TIGIT, TIM-3 and LAG-3, then combining anti-PD-1 with anti-TIGIT, anti-TIM-3 or anti-LAG-3 could be helpful. Similarly, when the immune-related adverse events cannot be tolerated, combining anti-PD-1 therapy with targets that can have relatively milder phenotypes such as TIGIT, TIM-3 or LAG-3 would be favorable. In summary, a combination that considers the subtle differences in checkpoint expression and control as well as the information on subtype-specific immunosuppressive pathways in TME may help address the issue of improving response rates to cancer immunotherapy.

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