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Tackling molecular targets beyond PD-1/PD-L1: Novel approaches to boost patients' response to cancer immunotherapy

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ABSTRACT

In the new era of immunotherapy, which has changed the clinical oncology practice guidelines, there is a pressing need for finding novel approaches to tune up the clinical outcomes of immunotherapy and extend its benefits to a wider cohort of cancer patients. Several non-classical molecular immune targets beyond PD-1/PD-L1 signaling were shown to be engaged as feedback resistance circuits to shut down the antitumor immune response mediated by the classical immune checkpoint inhibitors. Those include T-cell inducible co-stimulator (ICOS), CD40, CD47, V-domain Ig suppressor of T-cell activation (VISTA), cyclin-dependent kinase (CDK)12, enhancer of Zeste homolog 2 (EZH2), toll-like receptors (TLRs) and OX-40 (CD134). Herein we critically discussed the latest studies concerned with understanding the mechanisms involved in the negative clinical response to classical immunotherapies and strategies to optimize the efficacy of cancer immunotherapy through novel combinatorial approaches.

1. Introduction

Since the employment of cancer immunotherapy as an evolving approach in the management of hard-to-treat cancers, more efforts are needed to understand the reasons behind achieving an outstanding response in certain patients, and the lack of response in others. This major problem has shown an unprecedented clinical concern in several types of cancer. There are many proposed mechanisms for the innate or acquired resistance to immunotherapy such as the genetic alterations affecting neoantigen or cell signaling pathways leading to the disruption of cytotoxic T-cells function and so decrease the formation of T-cell memory and lower the response rates (Jenkins et al., 2018). The resistance, low response rates, and immune-related toxicities represent the major obstacle for the optimal utilization of this innovative approach for cancer therapy. Thus, finding novel strategies to boost the percentage of immunotherapy responders is currently a major challenge to save lives of more patients especially those with advanced cancers (Tolba and Omar, 2018).

Cancer immunotherapy relies on targeting the programmed death (PD)-1 receptor, PD-1 ligand (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) the classical immune checkpoints that are evaded by cancer cells to escape from the immune surveillance (Zou et al., 2016). In October 2018, James P. Allison and Tasuku Honjo were awarded the Nobel Prize in medicine for the discovery of the negative immune regulatory role of CTLA-4 and PD-1. Ipilimumab was the first agent ever to offer a survival advantage in metastatic melanoma patients via targeting CTLA-4 and so the activation of the adaptive immunity (Lipson and Drake, 2011). The design of the first FDA-approved PD-1inhibitor, pembrolizumab, was based on the preclinical evidence that cancer cells can evade the immune system via the activation of PD-1/PD-L1 signaling (Brahmer et al., 2012; Patnaik et al., 2015). Since then, many PD-1/PDL1 inhibitors were approved to treat serval

Abbreviations: PD-1, programmed death-1 receptor; PD-L1, programmed death-1 ligand; CTLA-4, cytotoxic T-lymphocyte associated protein-4; ICOS, T-cell inducible co-stimulator; VISTA, V-domain Ig suppressor of T-cell activation; CDK12, cyclin-dependent kinase 12; EZH2, enhancer of Zeste homolog 2; TLRs, toll-like receptors; TNF, tumor necrosis factor; GITR, glucocorticoid-induced TNF receptor; αCD40, agonistic CD40 mAb; MTD, maximal tolerated dose; NSCLC, non-small cell lung cancer; NHL, non-Hodgkin's lymphoma; SCLC, small cell lung cancer; TNBC, triple negative breast cancer; BCG, Bacillus Calmette-Guerin; TAA, tumor-associated antigen

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malignancies (Abdin et al., 2018; Seidel et al., 2018). While opening the avenue as a new hope for cancer patients, the response rates of the treatment using these agents are still low with a high incidence of treatment-related grade 3 and 4 adverse reactions (Callahan et al., 2018).

Many innovative non-classical targets have been introduced in immunotherapy to boost the patient response and minimize the resistance. Among these targets are T-cell inducible co-stimulator (ICOS) (Hutloff et al., 1999; Fu et al., 2011), CD40 (Eliopoulos and Young, 2004; Byrne and Vonderheide, 2016), CD47 (Brown and Frazier, 2001; Majeti et al., 2009; Edris et al., 2012; Weiskopf et al., 2016; Zhang et al., 2016a), V-domain Ig suppressor of T-cell activation (VISTA) (Wang et al., 2011; Gao et al., 2017), cyclin-dependent kinase (CDK)12 (Blazek et al., 2011; Cheng et al., 2012; Wu et al., 2018), enhancer of Zeste homolog 2 (EZH2) (DuPage et al., 2015; Yang et al., 2015; Goswami et al., 2018), toll-like receptors (TLRs) (Trinchieri and Sher, 2007; Hayashi et al., 2011) and OX-40 (CD134) (Redmond et al., 2009; Croft, 2010; Weinberg et al., 2011). The modulation of these targets directly or indirectly affects the tumor microenvironment via simultaneously manipulating different immune regulatory pathway. These targets have diverse mechanisms and various limitations.

Therefore, this work critically explored the latest novel targets beyond PD-1/PD-L1 to understand the mechanisms involved in the negative clinical response for cancer immunotherapy. We also highlighted the justification for tackling these novel non-classical immune targets in combinatorial approaches aiming at overcoming the resistance encountered with the classical immune checkpoint inhibitors.

2. Targeting ICOS/ICOSL pathway

T-cell inducible co-stimulator (ICOS) belongs to CD28/B7/CTLA-4 superfamily which has a crucial role in activating T-cells (Hutloff et al., 1999). ICOS expression is induced only upon the activation of T-cells, while other co-stimulatory proteins such as CD28 is constitutively present on the surface of T-cells (Hutloff et al., 1999). ICOS is involved in several aspects of T-cell responses such as the regulation of Th2-type cytokines production and the coordination between T- and B-cells (Dong et al., 2001; Sperling and Bluestone, 2001; Harada et al., 2003; Mak et al., 2003).

Several studies have been concerned with understanding the mechanisms involved in the positive clinical response to anti-CTLA-4 agents to find ways to widen the benefit of this therapy and boost the percentage of responders. Liakou et al., reported in a pre-surgical clinical trial that the treatment of localized bladder cancer patients with anti-CTLA-4 consistently increased the percentage of effector $CD4^+$ T-cells that are characterized by upregulated ICOS expression and enhanced IFN-ɣ production in response to tumor antigens (Liakou et al., 2008). The treatment also boosted the ratio of $CD4^+ICOS^{hi}$ (effector)/ $CD4^+FOXP3^+$ (regulatory) T-cells in peripheral blood as well as tumor tissues isolated from treated patients (Liakou et al., 2008). It is noteworthy that the enhanced percentage of $CD4^+ICOS^{hi}$ was also observed in both cancerous and noncancerous prostate tissues from patients with radical cystoprostatectomy (Chen et al., 2009). No immune-related adverse drug reactions were observed in the tested patients that have a high percentage of $CD4^+ICOS^{hi}$ in nonmalignant tissues. However, further studies are recommended to elaborate on whether such changes may contribute to the possible immune-related adverse reactions of anti-CTLA-4 therapy.

Retrospective evaluation of a small subset of metastatic melanoma patients receiving ipilimumab indicated a sustained increase in $ICOS^+$ T-cells over the period of 3- months treatment which correlated with enhanced survival rates (Carthon et al., 2010). Moreover, the increased percentage of $ICOS^+$ T-cells in peripheral blood from anti-CTLA-4-treated patients correlates to the increased percentage of the same cells in the tumor tissues (Carthon et al., 2010). This further support the importance of $ICOS^+$ T-cells in the antitumor immune responses to anti-CTLA-4 as well as its potential merit as a biomarker to monitor the response to this type of immunotherapy.

Fu et al., reported in a preclinical study that ICOS/ICOSL signaling is essential for the best therapeutic effect of CTLA-4 mAb therapy, where ICOS knockdown led to a defect in the antitumor activity (Fu et al., 2011). This finding highlights the importance of ICOS/ICOSL signals to be tackled in combinatorial approaches to boost anti–CTLA-4 therapy efficacy.

Clinical studies are in the process of testing agonistic anti-ICOS mAb therapies. The first in class ICOS agonistic mAb JTX-2011 is tested in phase 1/2 trial (ICONIC) alone or in conjunction with immune checkpoint inhibitor therapy in patients with advanced/refractory solid malignancies (NCT02904226). Preliminary data generated from this study supported that treatment with JTX-2011 either alone or as adjuvant to nivolumab is well tolerated and displayed promising antitumor effects in heavily pre-treated gastric cancer and triple negative breast cancer patients(Yap et al., 2018). GSK3359609 is another ICOS agonistic mAb that is currently under investigation alone or concurrently with pembrolizumab in phase 1 multicenter study (INDUCE-1) in patients with advanced solid tumors (NCT02723955). The preliminary data showed that it is well tolerated (Angevin et al., 2017).

3. CD40 as a therapeutic target

CD40 is a costimulatory protein that is considered a promising therapeutic target for cancer immunotherapy. It belongs to the tumor necrosis factor (TNF) receptor superfamily and it is present on the surface of antigen presenting cells (APCs) and its activation leads to enhanced immune response. CD40 is also expressed by some tumor cells and its activation results in apoptosis (Eliopoulos and Young, 2004). However, the ligand (CD40 L) is expressed on T-cells (Hassan et al., 2014). CD40/CD40 L interaction is important for T-cell priming. Moreover, the stimulation of CD40 leads to upregulated expression of the ligands of other activation receptors including CD134 (OX-40), glucocorticoid-induced TNF receptor (GITR), CD137 (Summers deLuca and Gommerman, 2012). It is noteworthy that CD40 stimulation can bypass the innate immune sensors TLRs, the inflammasome, Type I IFNs, and STING to generate effective priming of adaptive T-cells and to modulate the tumor microenvironment (Byrne and Vonderheide, 2016).

Clinical studies for evaluating the efficacy of agonistic CD40 mAb (αCD40) as monotherapy in melanoma patients indicated modest antitumor response rates without immune-related adverse events (Vonderheide et al., 2007; Bajor et al., 2014). Vonderheide et al., reported on the results of phase 1 trial for αCD40 (CP-870,893 Pfizer/ Roche) in patients with advanced solid malignancies (Vonderheide et al., 2007). The maximal tolerated dose (MTD) of the CD40 mAb was 0.2 mg/kg. A partial objective response was observed in 27% of melanoma patients enrolled in the study (Vonderheide et al., 2007). In 2014 Bajor et al., reported an exceptional response in a patient with metastatic melanoma who administered a single intravenous dose of CD40 mAb (MTD). This patient showed complete remission after CD40 antibody therapy and single metastasectomy and the response was durable up to 9 years after therapy initiation (Bajor et al., 2014).

In an attempt to enhance the response to αCD40, preclinical studies investigated the efficacy of αCD40 combinations in murine models of pancreatic ductal adenocarcinoma. These tumors are highly challenging because of their nature as cold tumors that are devoid of T-cell infiltration and have high resistance to chemotherapy (Sausen et al., 2015). The response rate to the standard chemotherapy combination gemcitabine/nab-paclitaxel is only 23% in patients with metastatic pancreatic ductal adenocarcinoma (Von Hoff et al., 2013).

The combined treatment with αCD40 and double chemotherapy of gemcitabine/nab-paclitaxel in a murine model of pancreatic ductal adenocarcinoma resulted in M1-shift in the myeloid compartment, and Th1-shift in the T-cells with a significant reduction in Treg

compartment within the tumor tissue (Byrne and Vonderheide, 2016). This led to T-cell dependent tumor regression with a significant increase in the percentage of responders with successful tumor regression by 59.7% which culminated in an increased overall survival by 17% (Byrne and Vonderheide, 2016). In another preclinical study, Winograd et al. indicated that the treatment with a combination of αCD40/gemcitabine/nab-paclitaxel potentiated the effect of immune checkpoint inhibitors (PD-1 or CTLA-4 mAbs) resulting in a prominent tumor regression with a 2-fold increased survival rate in the treated mice. The same combination developed a durable antitumor response protected the mice from the subsequent tumor re-challenge (Winograd et al., 2015).

The promising outcomes from the preclinical studies supported the initiation of clinical trials. SEA-CD40 is an agonistic CD40 mAb that is currently tested in a phase 1 trial for patients with advanced solid malignancies (NCT02376699). Interim results showed that SEA-CD40 has a favorable safety profile with promising anticancer activity in patients with advanced solid tumors that received heavy pretreatments (Grilley-Olson et al., 2018). In another clinical study, the efficacy of the triple therapy combination of αCD40/gemcitabine/nab-paclitaxel is being tested in subjects with resectable pancreatic ductal adenocarcinoma (NCT02588443). Moreover, a phase 1/2 clinical study is testing the tolerability and antitumor activity of the CD40 agonist mAb ABB927 alone or combined with ABBV-181 (PD-1 mAb) in patients with advanced stage solid malignancies (NCT02988960). The activating anti-CD40 antibody APX005 M is being investigated as monotherapy or combined with nivolumab (anti-PD1) in a phase 1/2 study in patients with NSCLC or metastatic melanoma (NCT03123783).

4. Targeting CD47

Phagocytic cells including macrophages and dendritic cells express signal regulatory protein alpha (SIRP-α) which interacts with CD47 that is expressed on cancer cells resulting in inhibition of phagocytosis (Brown and Frazier, 2001; Jaiswal et al., 2010). The expression of CD47 is upregulated in non-Hodgkin's lymphoma (NHL) cells and is linked to adverse molecular features and bad prognosis (Chao et al., 2010). Studies also showed an upregulated expression of CD47 on the surface of cancer cells in small cell lung cancer (SCLC), glioblastoma and leiomyosarcoma (Edris et al., 2012; Weiskopf et al., 2016; Zhang et al., 2016a). The interruption of SIRP-α/CD47 interaction via the therapeutic targeting of CD47 using specific anti-CD47 monoclonal antibodies leads to reactivation of tumor cells phagocytosis and reduced tumor burden (Chan et al., 2009; Majeti et al., 2009; Edris et al., 2012; Weiskopf et al., 2016; Zhang et al., 2016a).

The administration of anti-CD47 synergized with rituximab (anti-CD20) leading to a superior therapeutic effect with successful elimination of lymphoma in both localized and disseminated NHL models (Chao et al., 2010). The mechanism of synergy between the two antibodies included both Fc receptor (FcR)-dependent (rituximab) and FcR-independent (anti-CD47) stimulation of phagocytosis which is valid for other cancer types (Chao et al., 2010). The ability of anti-CD47 to synergize with rituximab underpins the possibility of its synergistic interaction with other FcR activating clinically approved antibodies such as trastuzumab, cetuximab, and alemtuzumab which are used for HER2+ breast cancer, colon cancer, and CLL respectively. Studies on the humanized anti-CD47 antibody Hu5F9-G4 in non-human primates (cynomolgus monkeys) showed its safety when administered intravenously at doses that achieve therapeutic serum levels (Liu et al., 2015). Toxicology data from studies on cynomolgus monkeys indicated the occurrence of transient anemia which was alleviated via the use of a low priming dose before the administration of higher maintenance doses (Liu et al., 2015). Clinical trials are now in process for Hu5F9-G4 in patients with advanced-stage solid malignancies or acute myelogenous leukemia, (NCT02216409). The preliminary data from phase 1 study indicated that Hu5F9-G4 is well tolerated at 3 mg/kg/week, with a 1 mg/kg priming dose without the occurrence of grade 3 anemia. The study is currently in the process of testing the safety of higher maintenance doses (NCT02216409).

5. VISTA (V-domain Ig suppressor of T-cell activation)

The V-domain Ig Suppressor of T-cell Activation (VISTA) is a novel negative immune checkpoint modulator that shares homology with PD-L1 and is highly abundant in the tumor microenvironment. VISTA expression is observed at high levels in the hematopoietic cell compartment especially the myeloid cells (Wang et al., 2011). It serves as a potent suppressor of T-cell activation and upregulates the expression of Foxp3 (Flies et al., 2011; Wang et al., 2011). Targeting VISTA can offer a new approach to boost the response rate to immunotherapy. Given its expression on tumor-infiltrating leukocytes rather than being expressed on certain subsets of tumor cells, VISTA comprises an attractive therapeutic target for a wide spectrum of solid tumors.

In an attempt to understand the potential resistance mechanisms of prostate cancer to immune checkpoint therapy, gene expression analysis studies were done on prostate cancer specimens from ipilimumab-treated patients in comparison to pre-surgical biopsy specimens. The data showed an upregulated expression of PD-1, PD-L1 and VISTA in the prostate tumor microenvironment after ipilimumab therapy (Gao et al., 2017). The expression of VISTA was boosted on CD4+, CD8 + T-cells and CD68+ macrophages. Similar results were observed in blood monocytes and tumor specimens from metastatic prostate cancer patients that received ipilimumab therapy, which further support the role of VISTA as a compensatory inhibitory immune pathway in both localized and metastatic prostate cancer (Gao et al., 2017). Furthermore, studies on specimens from therapy-naïve gastric tumors indicated increased expression of VISTA in the immune cells infiltrating the tumor microenvironment (Boger et al., 2017). VISTA upregulated expression in immune cells was more prominent in KRAS- and PIK3CA-mutant gastric cancers (Boger et al., 2017). These findings highlight VISTA as a potential therapeutic target for the management of gastric cancer.

CA-170 is an orally bioavailable dual inhibitor for PD-L1 and VISTA immune checkpoints. This small molecule inhibitor is free from off-target effects against CTLA-4, LAG-3, BTLA, or B7/CD28 pathways. Preclinical studies for CA-170 showed its potent antitumor activity in immune competent syngeneic murine tumor models that are insensitive to anti-PD-1 such as melanoma(B16/F1) and breast cancer (4T1). Toxicity studies performed in rodents and non-human primates indicated the safety of CA-170 when administered orally at doses up to 1000 mg/kg/day for 28 days (Adusumilli et al., 2017). Supported by the promising results from the preclinical investigations, a phase 1 clinical trial was initiated for testing the efficacy of CA-170 in human subjects with lymphoma or advanced solid tumors such as TNBC, melanoma, and NSCLC (NCT02812875) (Lee et al., 2017).

6. Cyclin-dependent kinase (CDK)12

Cyclin-dependent kinases (CDKs) are considered key regulators of several cellular functions. These kinases fall into two categories; the first one is cell cycle-associated CDKs which include CDK1, CDK2, CDK4, and CDK6 that regulate the progression over cell cycle phases. The second category encompasses transcription-associated CDKs that include CDK7- 9 and CDK11-13 which regulate gene transcription. The CDKs are considered attractive therapeutic targets for wide-range of tumors because they are often dysregulated in cancer cells (Asghar et al., 2015). CDK12 associates in a heterodimeric complex with its activating partner cyclin K to regulate various cellular processes (Blazek et al., 2011; Cheng et al., 2012). CDK12 directly regulates the transcription through the phosphorylation of serine residues of the heptapeptide repeats (YSPTSPS) located within the C-terminal domain of RNA polymerase II that is crucial for transcriptional elongation (Bartkowiak et al., 2010; Blazek et al., 2011; Cheng et al., 2012).

CDK12 was previously reported to be implicated in the control of genetic stability through its regulatory function for the DNA damage response genes such as ATR and BRCA1(Blazek et al., 2011; Juan et al., 2016).

A novel subtype of prostate cancer (PC) that is characterized by biallelic loss of CDK12 was identified in an integrative genomic analysis study conducted on 360 specimens of metastatic castration-resistant PC (Wu et al., 2018). Specimens from these CDK12 mutant cases displayed an increased genomic instability and neoantigen burden as a result of fusion-induced chimeric open reading frames with subsequently enhanced tumor T-cell infiltration and clonal expansion. A positive response to PD-1 blockade (decline in PSA level) was recorded in 2 out of 4 metastatic castration-resistant PC patients in the same study which represented an outstanding response. Based on these facts, CDK12 loss in patients with metastatic castration-resistant PC presents as a promising prognostic biomarker for the potential merit of immune checkpoint immunotherapy (Wu et al., 2018). Supported by these findings a phase 2 clinical study (IMPACT) was recently started to test the combination therapy of nivolumab and ipilimumab PD-1 and CTLA4 immune checkpoint therapy in patients with metastatic PC or metastatic non-prostatic tumors with CDK12 loss mutations (NCT03570619).

The study of Wu et al., (Wu et al., 2018) also set the stage for future studies to investigate a new combinatorial approach using CDK12 inhibitors as potential sensitizers to enhance the response of prostate tumors to immune checkpoint antibodies therapy. CDK12 inhibitors demonstrated their promising anticancer effects either as monotherapy or in combination with PARP1/2 inhibitors. Although dinaciclib was initially described as CDK2 and CDK9 inhibitor, it displayed an inhibitory effect to CDK12 at a comparable potency. Dinaciclib treatment mimicked CDK12 silencing through downregulation of hormone receptor genes and decreased phosphorylation of RNA polymerase II CTD Ser2 (Johnson et al., 2016). Combination therapy of dinaciclib and veliparib (PARP1/2 inhibitor) showed efficient tumor growth inhibition in both BRCA1 wild-type or mutated patient-derived xenografts of TNBC by reversing resistance to PARP1/2 inhibitors (Johnson et al., 2016). Another small molecule inhibitor under development is THZ1, an inhibitor of CDK7, that was shown to inhibit CDK12 but at higher IC_{50} (Kwiatkowski et al., 2014). This agent showed promising effects in preclinical models of TNBC, lung cancer and leukemia (Lin et al., 2012; Huang et al., 2014; Gregory et al., 2015). Based on THZ1, the more selective and potent CDK12/13 inhibitor TZH531 was developed (Zhang et al., 2016b). This new agent showed promising proliferation inhibitory effects in Jurkat T-cell leukemia cells (Zhang et al., 2016b).

7. Enhancer of Zeste homolog 2 (EZH2)

Chromatin remodeling is vital for creating cellular identities. The epigenetic regulator EZH2 is a chromatin-modulatory enzyme that is implicated in the conservation of Treg cell identity during cellular activation. DuPage et al., showed that EZH2 is upregulated upon CD28-mediated activation in Tregs. EZH2 is a key factor for optimum Treg cell function as it forms a complex with Foxp3 which supports Foxp3-mediated gene expression patterns after cellular activation (Arvey et al., 2014; DuPage et al., 2015; Yang et al., 2015). On the other hand, EZH2 prevents the differentiation of T-cells into effector T-cells (Kanno et al., 2012; Tumes et al., 2013; Yang et al., 2015; Kwon et al., 2017). While EZH2 is crucial for immune homeostasis by prevention of autoimmunity (Coit et al., 2016; Sarmento et al., 2017), it negatively affects the anti-tumor immune response.

Goswami et al., (Goswami et al., 2018) recently reported that either the genetic depletion or the pharmacologic inhibition of EZH2 resulted in a robust antitumor immune response through the modulation of Tregs phenotype and function leading to enhanced T-cell-mediated cytotoxicity. It is noteworthy that ipilimumab treatment upregulated EZH2 in peripheral CD4 + T-cells from metastatic melanoma (Goswami et al., 2018) and prostate cancer patients (Subudhi et al., 2016). Treatment with the pharmacological EZH2 inhibitor CPI-1205 significantly boosted the antitumor effect of CTLA-4 mAb and improved the survival rate in murine models of bladder cancer (MB49) or melanoma (B16-F10) (Goswami et al., 2018). The combined treatment enhanced the ratio of effector T-cells/Tregs by reducing the level of FoxP3+ Tregs and augmenting the level of both ICOS + CD4+T-cells as well as IFNγ+ CD8+T-cells. It is noteworthy that the combination of ipilimumab/CPI-1205 displayed a favorable safety profile in the treated animals (Goswami et al., 2018). The preclinical evidence for the safety and efficacy of this combination supported the initiation of phase1/2 clinical trial (ORIOn-E) for CPI-1205 plus ipilimumab in patients with advanced-stage solid malignancies (NCT03525795). Another clinical study (ProSTAR) was initiated to test a combination of CPI-1205 with either enzalutamide or abiraterone/prednisone in patients with metastatic castration-resistant prostate cancer (NCT03480646) (Taplin et al., 2018). It is noteworthy that CPI-1205 is also being tested in patients with B-Cell Lymphomas (NCT02395601).

8. Toll-like receptors (TLRs)

Activators of innate immunity including TLR agonists are under extensive investigations for the treatment of solid malignancies (van Duin et al., 2006; Wang et al., 2016; Leung et al., 2017). TLR7 and TLR9 are mainly expressed in immune cells such as macrophages, natural killer (NK) cells and dendritic cells (DC) rather than tumor cells (Trinchieri and Sher, 2007). Preclinical investigation of the TLR7 agonist, 1V270 reported its ability to significantly suppress tumor growth in B-16 melanoma-bearing mice upon local intratumoral injection (Hayashi et al., 2011). The CpG oligonucleotide SD-101 is a TLR9 agonist that induces high levels of IFN type I which subsequently enhances the maturation and expansion of dendritic cells and B cells (Crittenden et al., 2015; Ribas et al., 2016). Combinations of TLR agonists and PD-1 immune checkpoint inhibitors were investigated since each agent target a different component in the tumor microenvironment where PD-1 blockers target the adaptive immunity and TLR agonists target innate immunity. Wang et al. indicated that intratumoral SD-101 reverts the resistance to anti-PD-1 treatment in murine models of CT26 and MCA38 colon carcinoma as well as TSA mammary adenocarcinoma (Wang et al., 2016). Sato-Kaneko et al. reported that local co-treatment with either 1V270 or SD-101 together with PD-1 inhibitor significantly increased both local and abscopal antitumor effects in preclinical models of head and neck cancers(Sato-Kaneko et al., 2017). In the same study, treatment with the TLR7 agonist 1V270 augmented the ratio of M1/M2 tumor-associated macrophages and enhanced the tumor infiltration with activated IFNγ-producing CD8+T-cells in addition to a systemic increase in the clonality of CD8+T-cells (Sato-Kaneko et al., 2017). It is noteworthy that TLR7 or TLR9 expression by tumor cells was reported to augment tumor growth upon treatment with TLR7/9 activators(Min et al., 2012; Kauppila et al., 2015). However, mRNA expression analysis of 967 human cancer cell lines indicated that only a few (SKMEL31, KR97, OUMS23, HT1197) were positive for the expression of TLR7/9 and none of them was derived from head and neck tumors (Sato-Kaneko et al., 2017). These data propose a new combinatorial approach for the management of head and neck cancers. SYNERGY-001 is an ongoing phase 1b/2 clinical trial testing TLR9 agonist SD-101 combined with pembrolizumab in patients with metastatic melanoma or metastatic head and neck malignancies. The preliminary data generated from the study showed an acceptable safety profile with promising response rates in the PD-1 inhibitor naïve patients (NCT02521870) (Leung et al., 2017).

Imiquimod is a synthetic imidazoquinolone amine that acts as a TLR7 agonist. It was approved by the FDA in 2004 for cutaneous malignancies such as actinic keratosis and superficial basal cell carcinoma (Bubna, 2015). Promising results were reported in the clinical study of combined local treatment with Bacillus Calmette-Guerin (BCG) and

imiquimod in melanoma patients (Kidner et al., 2012). In this study, the patients received an intralesional injection of BCG followed by topical 5% imiquimod cream after the development of an inflammatory response to BCG. The combination was well tolerated with a complete tumor regression in 5 out of 9 patients. Three patients had a complete response after surgical removal of the lesion, while one patient only had a partial response. Upon long-term follow up (35 months), 7 patients did not experience a relapse of melanoma (Kidner et al., 2012). An early phase 1 clinical trial was initiated at the end of 2017 to study the combination of Imiquimod and the immune checkpoint inhibitor pembrolizumab in patients with advanced stage IIIB-IV melanoma (NCT03276832). Moreover, a phase 2 clinical trial is currently ongoing to test the combined treatment of imiquimod with cyclophosphamide and radiotherapy for breast cancer with cutaneous metastasis (NCT01421017).

Zymosan is a promising immunomodulatory glucan that was studied against melanoma preclinically(Taghavi et al., 2018). Intraperitoneal treatment with zymosan(10 μg) significantly reduced tumor growth by 50% compared to control in B16F10 melanoma-bearing C57BL/6 mice. Zymosan treatment upregulated the expression of TLR2 and TLR 4 in the peritoneal macrophages in addition to boosting the serum levels of TNF-α in the treated mice compared to control untreated melanoma-bearing mice. Moreover, low doses of zymosan (0.1–10 μg/ml) resulted in a dose-dependent increase in the proliferation of the splenocytes ex-vivo (Taghavi et al., 2018). Care must be taken in the extrapolation of these trials to the clinical setting since the use of high doses (500 mg/kg) of zymosan can induce multiple organ failure (Volman et al., 2005).

TLR3 is highly expressed on dendritic cells (Bachem et al., 2010; Jongbloed et al., 2010) where its activation prompts dendritic cells maturation and cross-priming of tumor-specific T-cells. ARNAX is a specific TLR3 agonist that induces tumor-specific cytotoxic T-cells in both lymphoid tissues and tumor tissues. ARNAX does not induce systemic cytokine or IFN production. Therefore, it is anticipated to be devoid of severe adverse reactions that were encountered with the previously clinically tested poly(I:C) polyriboinosinic-polyribocytidylic acid (Lampkin et al., 1985). Testing of ARNAX in a preclinical murine model of ovalbumin -overexpressing lymphoma indicated that a combination of ARNAX along with tumor-associated antigen (TAA) showed promising outcomes and successfully overcame the resistance to anti-PD-L1 therapy(Takeda et al., 2017).

9. OX-40 (CD134)

OX40 (CD134) is an attractive target for immune response modulation. It is a TNR receptor that is mainly expressed on activated CD4+ and CD8 + T-cells and conveys a potent costimulatory signal upon its activation (Weinberg et al., 2011). Preclinical studies indicated that treatment with agonistic anti-OX40 antibodies augments antitumor immune response in preclinical models of breast cancer, prostate cancer, and melanoma (Sugamura et al., 2004). Activation of OX40 leads to upregulated expression of cytokines and pro-survival molecules implicated in T-cell expansion and differentiation in addition to the generation of memory T-cells (Watts, 2005; Redmond et al., 2009; Croft, 2010). However, monotherapy with anti-OX40 displayed limited efficacy which prompted the necessity for testing anti-OX40 mAb in combination with other immunotherapies with the aim of achieving a better therapeutic response.

Co-treatment with anti-OX40/anti-CTLA-4 augmented the anti-tumor activity and enhanced the survival in murine models of prostate cancer (TRAMP-C1) or sarcoma (MCA-205) compared to monotherapy groups (Redmond et al., 2014). Such effect was attributed to augmenting the expansion and differentiation of effector CD4+ and CD8 + T-cells in addition to boosting the production of distinct Th1 cytokines (IFNγ, IL-2) and Th2 cytokines (IL-4, IL-5, IL-13) (Redmond et al., 2014). Triple therapy with intratumoral CpG vaccine together with anti-OX40/anti-CTLA-4 was shown to boost the antitumor immunity in a murine lymphoma model via increasing the percentage of IFNγ+ T-cells infiltrating the tumors (Houot and Levy, 2009). Combined treatment with OX40 agonistic mAb and subcutaneous vaccination of irradiated GM-CSF-expressing glioma tumor cells exhibited synergistic antitumor activity and significantly boosted the survival of GL261 glioma-bearing mice (Jahan et al., 2018). The underlying mechanisms for such effect included an enhanced activity of Th1 CD4 + T-cells while reducing Th2 fraction in the brain. The combination therapy also reduced the expression of PD-1, TIM3, and LAG-3 on the intracranial T-lymphocytes which led to a successful reversal of T-cell immune exhaustion (Jahan et al., 2018). The addition of indoximod to a combination of anti-OX40 mAb and vaccine resulted in significant tumor regression in 60% of the treated myeloma-bearing C57BL/6 mice with subsequent improvement in the survival in comparison to monotherapy or dual combinations (Berrong et al., 2018). Activation of OX40 leads to upregulation of IFNɣ within the tumor microenvironment and in the periphery (Sugamura et al., 2004) which in turn leads to the induction of indoleamine 2,3-dioxygenase-1 (IDO) activity which develops an immune suppressive milieu (Spranger et al., 2013). Therefore the inhibition of IDO activity by indoximod synergized anti-OX40 mAb therapy(Berrong et al., 2018).

Agonistic anti-OX40 therapy is being investigated on the clinical level with promising outcomes. Results from the phase 1 clinical trial of a mouse mAb (9B12) which activates human OX40 supported its tolerability and promising efficacy with the resolution of at least one metastatic lesion in 12 out of 30 patients with advanced stage cancers (NCT01644968) (Curti et al., 2013). MEDI6469 is another murine anti-OX40 mAb that is being tested alone and in combination with other immunotherapies in phase 1b/2 clinical trial (NCT02205333). In patients with advanced solid malignancies, MEDI6469 is tested as a co-treatment with anti-CTLA-4 tremelimumab or anti-PD-L1 MEDI4736. However, in patients with diffuse large B cell lymphoma, it is tested in combination with anti-CD20 rituximab (Powderly et al., 2015). MEDI6469 is also being tested clinically for metastatic colorectal carcinoma patients in conjunction with surgery or radiotherapy (NCT02559024). The therapeutic efficacy of MEDI6469 is being investigated in combination with stereotactic body radiation in patients with breast cancer metastasized to the lungs or liver that is progressive on regular systemic therapeutic protocols (NCT01862900). Triple therapy with anti-OX40, cyclophosphamide and radiotherapy is being tested in a phase 1b study for metastatic prostate cancer patients (NCT01303705).

10. Conclusion and future recommendations

Immunotherapy is considered a new light of hope to cure patients with hard-to-treat cancers as it demonstrated a durable positive response in patients with advanced metastatic malignancies that were refractory to conventional treatment protocols. Although the treatment with immune checkpoint inhibitors has demonstrated some intriguing outcomes in certain types of cancers such as metastatic melanoma and NSCLC (Sengupta and Honey, 2018). The efficacy and response rates are still low in other cancer types such as breast and prostate cancers (Tolba and Omar, 2018). Therefore, understanding the molecular basis behind tumor resistance to immune checkpoint inhibitors would be of great benefit to find novel ways to optimize its efficacy and extend its therapeutic benefit to a wider cohort of cancer patients. The up-to-date information regarding the extensive research efforts in this area both on the preclinical and the clinical levels was discussed in this review (Table 1). Several non-classical molecular immune targets were shown to be engaged as feedback resistance circuits to shut down the response to the classical immune checkpoint inhibitors. The resistance signaling targets encompass VISTA, CDK12, EZH2, and CD47.

Here we underpin some chief insights from the studies discussed in this review. VISTA was shown to be a negative immune checkpoint regulator that is boosted as a feedback mechanism to immune

Table 1
Summary of the current clinical studies of the novel immune-targeting agents to boost the patients' response to immunotherapy.

Immune targets	Investigated drugs	Cancer type	Clinical trial Phase	Clinical Trial ID/Reference
ICOS	ICOS agonist mAb (JTX-2011) alone or combined with immune checkpoint inhibitors (nivolumab, ipilimumab, or pembrolizumab)	Advanced or refractory solid malignancies	Phase 1/2	NCT02904226 (ICONIC)
	ICOS agonist mAb (GSK3359609) monotherapy or combined with pembrolizumab	Advanced solid malignancies	Phase 1	NCT02723955 (INDUCE-1)
CD40	CD40 agonist mAb (CP-870,893)	Advanced solid malignancies	Phase 1	(et al., 2007)
	CD40 agonist mAb with single metastasectomy	Metastatic melanoma	Phase 1	(Bajor et al., 2014)
	CD40 agonist mAb (SEA-CD40)	Advanced solid tumors	Phase 1	NCT02376699 (Grilley-Olson et al., 2018)
	CD40 agonist mAb combination with gemcitabine and nab-paclitaxel	Resectable pancreatic ductal adenocarcinoma	Phase 1	NCT02588443
	CD40 agonist mAb (ABB927) alone or combined with anti-PD-1 (ABBV-181)	Advanced solid malignancies	Phase 1/2	NCT02988960
	CD40 agonist mAb (APX005M) alone or combined with anti-PD-1 (nivolumab)	NSCLC or metastatic melanoma	Phase 1/2	NCT03123783
CD47	CD47 mAb (Hu5F9-G4)	Advanced solid tumors or acute myelogenous leukemia	Phase 1	NCT02216409
VISTA	CA-170 dual PD-L1/VISTA inhibitor	Lymphoma or advanced solid tumors such as TNBC, melanoma and NSCLC	Phase 1	NCT02812875
CDK12	Combined nivolumab (anti-PD-1) and ipilimumab (anti-CTLA4) immune checkpoint therapy	Metastatic PC or metastatic non-prostatic tumors with CDK12 loss mutations	Phase 2	NCT03570619 (IMPACT)
EZH2	EZH2 inhibitor (CPI-1205) combined with ipilimumab	Advanced solid tumors	Phase 1/2	NCT03525795 (ORIOn-E)
	EZH2 inhibitor (CPI-1205) with either enzalutamide or abiraterone/prednisone	Metastatic castration-resistant PC	Phase 1/2	NCT03480646 (ProSTAR) (Taplin et al., 2018)
	EZH2 inhibitor (CPI-1205)	B-Cell Lymphomas	Phase 1	NCT02395601
TLRs	TLR9 agonist SD-101 combined with pembrolizumab	Metastatic melanoma or metastatic head and neck cancers.	Phase 1b/2	NCT02521870 (SYNERGY-001) (Leung et al., 2017)
	Imiquimod (TLR7-agonist) and pembrolizumab	Advanced stage IIIB-IV melanoma	Phase 1	NCT03276832
	Imiquimod (TLR7-agonist) combined treatment of with cyclophosphamide and radiotherapy	Breast cancer with cutaneous metastasis	Phase 2	NCT01421017
OX40	9B12 (anti-OX40) monotherapy	Advanced stage cancers	Phase 1	NCT01644968 (et al., 2013)
	MEDI6469 (anti-OX40) in combination with stereotactic body radiation	Metastatic breast cancer	Phase 1/2	NCT01862900
	MEDI6469 (anti-OX40) in conjunction with surgery or radiotherapy	Metastatic colorectal carcinoma	Phase 1	NCT02559024
	MEDI6469 (anti-OX40) monotherapy	Advanced solid tumors	Phase 1	NCT02318394 (Bauer et al., 2015)
	MEDI6469 (anti-OX40) co-treatment with tremelimumab (anti-CTLA-4) or MEDI4736 (anti-PD-L1)	Advanced solid tumors	Phase 1b/2	NCT02205333
	MEDI6469 (anti-OX40) co-treatment with rituximab (anti-CD20)	Diffuse large B cell lymphoma	Phase 1b/2	NCT02205333
	Anti-OX40 in combination with cyclophosphamide and radiotherapy	Metastatic PC	Phase 1/2	NCT01303705
	MEDI6469 (anti-OX40) monotherapy or combined with MEDI4736 (anti-PD-L1)	Advanced solid tumors	Phase 1	NCT02221960 (Leidner et al., 2015)

checkpoint therapy in prostate cancer (Gao et al., 2017). Targeting VISTA represents a convenient strategy for tackling a wide spectrum of solid malignancies. Given that VISTA is expressed mainly by the tumor-infiltrating myeloid cells rather than being exclusively expressed by a certain subset of tumor cells (Wang et al., 2011). CA-170 is a recently developed oral dual inhibitor of VISTA/PD-L1, with an encouraging preclinical toxicity profile that qualified it for testing in clinical trials (Adusumilli et al., 2017). However, until now there are no clinical trials for this agent in prostate cancer, which is expected to benefit from it. Additional valid target to be considered is CDK12, which is involved in genetic stability (Blazek et al., 2011; Juan et al., 2016). Biallelic loss of CDK12 in a certain subset of metastatic prostate cancer led to genetic instability, increased neoantigen burden and better response to anti-PD-1 therapy (Wu et al., 2018). This finding offers two dimensions of benefit. First, CDK12 can be employed as a biomarker for the prediction of anti-PD-1 therapy success. Second, CDK12 inhibitors can be utilized as adjuvants in immune checkpoint therapy protocols. On a different ground, the inhibition of the chromatin-modifying enzyme EZH2 by CPI-1205 leads to an enhanced antitumor activity via halting Tregs activation with subsequent enhancement of T-cell mediated cytotoxicity (Goswami et al., 2018). The upregulated expression of EZH2 on peripheral CD4+ cells was observed as a compensatory resistance mechanism to anti-CTLA4 therapy in melanoma and prostate cancer patients (Subudhi et al., 2016; Goswami et al., 2018). These findings were extrapolated to the clinical setting and CPI-1205 combinations with anti-CTLA4 therapy or other endocrine therapies are being tested in advanced solid malignancies including prostate cancer (Taplin et al., 2018). The interaction between macrophages' protein SIRP-α and CD47 that is overexpressed on tumor cells leads to disruption of phagocytosis (Brown and Frazier, 2001; Jaiswal et al., 2010). The inhibition of CD47 resulted in the reactivation of phagocytosis and synergized the anti-tumor effects of rituximab (Chan et al., 2009; Majeti et al., 2009; Chao et al., 2010; Edris et al., 2012; Weiskopf et al., 2016; Zhang et al., 2016a). The humanized anti-CD47 mAb (Hu5F9-G4) showed an acceptable safety profile in non-human primates and moved forward to clinical trials in patients with leukemia or advanced solid tumors (Liu et al., 2015). Thus, there are opportunities to be tackled in this area including clinical testing of Hu5F9-G4 as an adjuvant with rituximab or similar FcR- activator antibodies as cetuximab and trastuzumab.

On the other hand, various immune costimulatory pathways were broadly investigated as possible targets to augment the antitumor immune response. Those targets include T-cell co-stimulatory molecular targets such as ICOS, CD40, OX40 in addition to TLRs that are expressed mainly by APCs and play a key role in innate immunity activation. A novel attractive noninvasive prognostic biomarker for the prediction of the effectiveness of anti-CTLA-4 therapy is the level of $ICOS^+$ T-cells in peripheral blood since it reflects the level of these effector cells within the tumor microenvironment (Liakou et al., 2008). Clinical studies on investigational agonistic ICOS mAbs as monotherapy or combined with immune checkpoint therapy for advanced solid tumors demonstrated promising preliminary outcomes with acceptable safety profile (Angevin et al., 2017; Yap et al., 2018). Furthermore, the activation of CD40 receptors resembles a double-edged sword as it contributes to T-cell priming along with the induction of cancer cells' apoptosis (Eliopoulos and Young, 2004). Being well-tolerated encouraged the extensive clinical investigations on anti-CD40 agonistic antibodies (Grilley-Olson et al., 2018). Ani-CD40 mAb is currently being tested clinically concurrently with either chemotherapeutics such as gemcitabine/nab-paclitaxel in pancreatic ductal adenocarcinoma or anti-PD-1 immune checkpoint therapy for NSCLC or metastatic melanoma or other advanced solid malignancies. Another T-cell costimulatory molecular target is OX40. While the activation of OX40 was shown to convey a strong costimulatory signal for T-cells, the monotherapy of activating anti-OX40 mAbs showed a very limited efficacy (Sugamura et al., 2004). Therefore, anti-OX40 is being tested clinically in combination with either immunotherapies (anti-PDL1, anti-CD20, anti-CTLA4) or conventional therapies including surgery, chemotherapy or radiation (Powderly et al., 2015). One of the resistance mechanisms to OX40 activation is the induction of IDO activity, which harbingers immune suppressive milieu (Spranger et al., 2013). Preclinical data reporting the synergy between anti-OX40 and indoximod (IDO inhibitor) should encourage the movement of this combination to the clinical setting (Berrong et al., 2018). Another perspective for overcoming tumor immune evasion is via invigorating TLRs, which are mainly expressed by APCs. The combined use of TLR agonists with immune checkpoint inhibitors is valid since each agent targets a different component in the tumor microenvironment, where TLRs agonists activate the innate immunity and checkpoint inhibitors (PD-1/PD-L1) activate the adaptive immune response. Preclinical studies indicated that concurrent administration of TLR7 or TLR9 agonists with PD-1 mAb produced promising results with augmented local and abscopal antitumor effects (Sato-Kaneko et al., 2017). The promising preliminary results generated from the clinical investigation of TLR9 agonist/pembrolizumab combination in metastatic melanoma and head and neck cancers should encourage further studies in other types of solid malignancies (Leung et al., 2017). The main limitation for the success of this approach is to confirm that the tumor cells lack TLRs expression since TLR7/9 agonists can enhance tumor growth in such phenotypes (Min et al., 2012; Kauppila et al., 2015).

Collectively, by underscoring the molecular mechanisms through which these innovative non-classical targets (Fig. 1) modulate the tumor microenvironment, novel immunotherapeutic protocols may be developed. Thus, the current review critically discussed the latest studies that have been concerned with understanding the underlying mechanisms for the negative clinical response to classical immunotherapies and the efforts to find ways for extending their benefit through proposing novel combinatorial strategies.

Conflict of interest statement

The authors declare that they have no competing interests.

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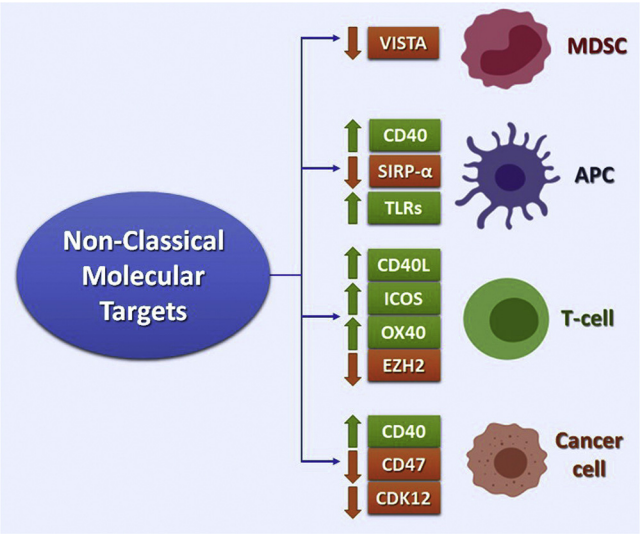

Fig. 1. Diagram depicting the non-classical immune targets beyond PD-1/PD-L1 immune checkpoints. **MSDC;** myeloid-derived suppressor cell, **APC;** antigen presenting cell, **VISTA;** V-domain Ig suppressor of T-cell activation, **SIRP-α;** signal regulatory protein alpha, **ICOS;** T-cell inducible co-stimulator, **EZH2;** enhancer of Zeste homolog 2, **Tregs;** regulatory T-cells, **CDK12;** cyclin-dependent kinase 12.

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