Title

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New combination strategies using PD-1/ L1 checkpoint inhibitors as a backbone

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Abstract

The discovery of immune checkpoints and subsequent clinical development of checkpoint inhibitors has revolutionized the field of oncology. The durability of the anti-tumor immune responses has raised the hope for long term patient survival and potential cure; however, currently only a minority of patients respond. Combination strategies to help increase antigen release and T cell priming, promote T cell activation and homing, improve the tumor immune microenvironment, all guided by predictive biomarkers, can help overcome the tumor immune-evasive mechanisms and maximize efficacy to ultimately benefit the majority of patients. Great challenges remain due to the complex underlying biology, unpredictable toxicity and accurate assessment of response. Carefully designed clinical trials guided by translational studies of paired biopsies will be key to develop reliable predictive biomarkers to choose which patients would most likely benefit from each strategy.

Keywords

Combination immunotherapy; checkpoint inhibitor; Radiotherapy; Chemotherapy; Cancer Vaccines; Oncolytic virus; immune-modulatory agent; tumor microenvironment; epigenetic modification; adoptive cell transfer

Introduction

Over the past century, generations of tumor immunologists and clinicians have been exploring the possibility of harnessing the patient's own immune system to fight cancer. Some success had been obtained with cancer vaccines and high dose cytokine therapy, but at a very low rate of response in a limited number of tumor types that are considered more immunogenic than others. Nonetheless, the durability of these responses, likely benefited from the memory of the adaptive immune system, inspired many to continue to study the

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mechanisms of immune escape by cancer. In the past decade, the clinical testing of immune checkpoint inhibition, especially the programmed cell death -1 (PD-1) checkpoint, has resulted in major breakthroughs in the development of modern immunotherapies with improved response rates in a variety of tumor types, most of which that were previously considered immunotherapy non-responsive.

PD-1 receptor is expressed on the surface of T cells when activated through T cell receptor (TCR) engagement [1]. PD-1 ligand (PD-L1), on the other hand, can be expressed by tumor cells constitutively or in response to interferon-induced signaling through the interferon receptor. When PD-L1 binds to PD-1, the T cell is deactivated and exhausted. This adaptive immune resistance appears to be a major immune evading mechanism by many cancers, as PD-L1 expression is frequently upregulated on the surface of tumor cells [2], and clinical development of PD-1/L1 inhibitors has led to consistent clinical benefit to patients with variety of cancers [3]. Currently three monoclonal antibodies blocking the PD-1/L1 checkpoint (pembrolizumab, nivolumab and atezolimumab) have been approved by regulatory bodies for the treatment of metastatic melanoma [4, 5], non-small cell lung cancer (NSCLC) [6, 7], renal cell carcinoma (RCC) [8], bladder cancer [9], Hodgkin's lymphoma [10], and head and neck squamous cell carcinoma (HNSCC), and have shown efficacy in many other different tumor types. Because the PD-1/L1 checkpoint occurs in the periphery at the effector phase of T cell activation, the toxicity profile is very favorable with less than 15% of patients experiencing severe side effects. More excitingly, a common feature of these checkpoint inhibitors is the plateau of the survival curves at the end of the tail, suggesting long term disease control and the potential of a cure, which has been a hallmark of immunotherapy.

Despite the unprecedented durable response rates observed with PD-1/L1 blockade, several common cancer types have shown very low frequency of response (breast, prostate, colon, etc) and even for the responding tumor types, only 10–40 percent of treated patients usually benefit. The great challenge that the immunotherapy field is facing is to develop biomarkers to predict response, identify patients less likely to respond, and develop rational combination therapies to improve the outcomes. Because the PD1/L1 checkpoint functions at the last step of effector T cell activation, a reasonable approach would be to use PD1/L1 inhibitors as the backbone of this combination. In this article, we reviewed the rationale and state of development of combination strategies to improve efficacy of anti-PD1/L1 therapy, including increase of tumor-specific antigen release and presentation, enhancement of T cell priming and homing to the tumors, augmentation of T cell effector function, suppression of immune suppressive cell populations (Tregs, MDSC, macrophages) and cytokine release in the tumor microenvironment.

1) Strategies to increase antigen release and T cell priming

It is important to understand that PD1 checkpoint inhibitors rely on the host's immune system to mount the tumor specific immune response that had been blocked at the last step of activation by the PD-1/L1 checkpoint. Therefore, strategies to increase tumor antigen release and presentation (chemotherapy, radiation therapy, oncolytic viruses, toll like receptor (TLR) agonists, cancer vaccines) and T cell priming (CTLA4 checkpoint inhibitors)

could rescue those patients who would otherwise not be able to mount this immune response and synergize with anti-PD1/L1 checkpoint inhibitors.

The first step of anti-tumor immune response is the processing of the dying cancer cells by antigen presentation cells (APC), including dendritic cells (DC). The maturation of APCs requires “danger” signals [11], such as damage associated molecular patterns (DAMPs) [12], recognized by innate pattern recognition receptors (PRR) including toll like receptors (TLR), RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors [13]. The activated APC then migrate to lymph nodes and present the tumor specific peptides via major histocompatibility complex (MHC) class I or II molecules to CD4 an CD8 T cells with the corresponding TCRs.

CTLA-4 checkpoint inhibitors—Tumor antigen presentation by APCs to naïve T cells and subsequent T cell activation in the regional lymph nodes not only require antigen presentation machinery and sequence-specific TCRs, but also binding of co-stimulatory molecules (CD80 or CD86 on APC and CD28 on T cells) [14]. This triggers CTLA-4 expression on the activated T cells, which competitively binds to the CD80/86 and attenuates T cell activation (Figure 1). Seminal work by Allison and colleagues [15] demonstrated the efficacy of anti-CTLA4 antibody therapy in eradicating tumor growth in mouse models, which lead to the clinical development and approval in 2011 of the first immune checkpoint inhibitor ipilimumab, a fully human IgG1 antibody against CTLA-4, for treatment of advanced melanoma. Phase III clinical trials observed a low but durable response rate that was translated into significant overall survival benefit when compared to gp100 vaccine or chemotherapy alone [16–18]. Translational studies indicated that CTLA-4 blockade therapy can increase T cell infiltration into the tumors regardless of clinical outcome [19], and broaden TCR repertoire in the peripheral blood [20]. However, it also induces tumor PD-L1 expression in the tumor microenviroment. This could explain why anti-CTLA4 treatment alone is not effective in clinical testing of other tumor types, including NSCLC, bladder cancer, prostate cancer, gastric cancer, mesothelioma, etc [21, 22] and provides rationale for the combination of anti-CTLA4 with anti-PD1/L1 inhibition.

The potential synergistic effect of combining inhibitors of the CTLA-4 and PD-1/L1 checkpoints is supported by testing in preclinical models [23]. The combination of ipilimumab and nivolumab was developed in patients with metastatic melanoma, and when compared to single agent ipilimumab or single agent nivolumab, it demonstrated high response rate (~60%), increased number of complete responses, and significantly improved progression free survival [24–26], which led to the approval of this combination therapy in treating advanced melanoma in 2015. It needs to be noted that the trial was only powered to compare combination *vs* ipilimumab and nivolumab *vs.* ipilimumab, but not combination vs nivolumab. Subgroup analysis suggest the benefit from combination therapy was mostly seen in the patients whose tumors were negative for PD-L1 staining. Nonetheless, the clinical benefit with the combination therapy is not without a cost, as more than half of the treated patients developed grade 3 or 4 treatment-related adverse events that are immune-mediated in nature. Short term follow-up studies suggested treatment of immune mediated adverse events with corticosteroids does not have impact on the outcome of the therapy [27] and any grade adverse events from nivolumab is associated with higher objective response

rate but not progression free survival [28]. However, longer term patient follow-up and prospective studies are needed to confirm these observations.

Combination of CTLA-4 and PD-1/L1 inhibitors has also been tested in NSCLC and other solid tumors, and different dose combinations and dosing schedules have been explored to improve tolerability and safety. An 39% objective response rate (and 39% stable disease) was observed with ipilimumab and nivolumab in metastatic renal cell carcinoma (Hammers 2014 ASCO, 4504). Early evidence of activity of ipilimumab plus nivolumab was also seen in patients with metastatic NSCLC (Antonia ASCO 2014, 8023). When different dosing schedules were explored to combine pembrolizumab and ipilimumab (10+3 vs 10+1 vs 2+1) for patients with advanced NSCLC (Patnaik, 2015 ASCO, 8011), 54% CR and PR rates were observed across the dosing cohorts, with no compromised efficacy at the low dose combinations. Another trial evaluated the combination of tremelimumab (anti-CTLA4) and durvalumab (anti-PDL1) for patient with NSCLC (Antonia, ASCO 2015, 3014). Increased dosing of tremelimumab but not durvalumab is associated with increased toxicity, and 26% of ORR was observed, including patients with PD-L1 negative tumors. Most recently, a phase I trial of frontline nivolumab monotherapy or combined with ipilimumab including decreased dose (1 mg/kg) and decreased dosing frequency (every 6 or 12 weeks) for patients with NSCLC (Hellmann, 2016 ASCO, 3001) showed manageable treatment-related adverse events and ORRs ranged from 13%–39%, and efficacy not affected by the decreased dose or frequency of ipilimumab. Responses were noted regardless of PD-L1 expression.

Radiation therapy—Local cytotoxic therapies, such as radiation therapy, can not only increase tumor antigen release, but also trigger the release of modulators of the innate immune response/DAMPs, such as type I interferon (IFN), calreticulin, ATP, etc, that can activate dendritic cells, and induce pro-inflammatory cytokine and chemokines, thus mediating a systemic anti-tumor immune response, the so-called abscopal effect [29–32]. Evidence supports this in situ vaccination function of radiation therapy includes enhanced peptide repertoire and MHC class I expression [33], increased tumor specific antigen expression [34] and T cell homing [35], or improving the tumor microenvironment [36], thus providing strong rationale to combine with immunotherapy.

Preclinical testing in immune competent mouse models indicates potential synergy of radiation therapy with both CTLA-4 [37] and anti-PD-1/L1 [38–40] checkpoint inhibitors, with efficacy demonstrated in both irradiated and non-irradiated tumors. Similar efficacy has been observed in case reports with concurrent radiotherapy and ipilimumab in patients with melanoma [32, 41] and NSCLC [42]. Although it was not clear whether the NSCLC case was a pure benefit of ipilimumab as the patient was naïve to ipilimumab before the combination therapy, in the melanoma case, the patient had demonstrated disease progression on ipilimumab before radiation therapy was given, and subsequently experienced significant tumor regression including the lesions not being irradiated. However, subsequent testing of this combination of local radiation therapy and systemic ipilimumab treatment for castration resistant prostate cancer patients did not show improved response when compared to ipilimumab alone in an early phase trial [43], nor survival benefit when compared to radiotherapy plus placebo in a phase III trial [44]. In a series of 22 advanced melanoma patients treated with radiation followed by 4 doses of systemic ipilimumab

demonstrated slightly improved response (18% partial response and 18% stable disease) than historical data of ipilimumab [45]. Subsequent correlative studies and relevant mouse modeling showed upregulation of PD-L1 in the resistant tumors, and addition of PD-1 blockade improved response in both treatment naïve tumors and the tumors that already demonstrated resistance to combination of radiotherapy and anti-CTLA-4 treatment [45]. It appeared that certain mode of radiotherapy, such as hypofractionated RT, is more effective than solitary dose RT to induce immune response [46].

Chemotherapy—The concept of combining chemotherapy with immunotherapy is seemingly counterintuitive, as chemotherapy is commonly associated with marrow suppression and low white blood cell counts, due to cytotoxicity to fast proliferating cells. However, preclinical and clinical data has suggested that there is rationale in support of this combination [47], including increasing the release of antigens and DAMPs, reduce the number of MDSCs (gemcitabine) [48], depletion of circulating regulatory T cells (cyclophosphamide) [49], etc. In genetically engineered and orthotropic lung adenocarcinoma models, oxaliplatin and cyclophosphamide could successfully sensitize host antitumor T cell immunity to immune checkpoint blockade by direct drug actions on tumor cells, as well as innate immune response through toll-like receptor 4 signaling, and increased tumor infiltration of anti-tumor $CD8^+$ T cells [50]. In a phase II randomized clinical trial of patients with advanced melanoma, combination of ipilimumab with dacarbazine showed higher response rate than treatment with ipilimumab alone [51]. Interestingly, the two partial responders (5.4%) in the ipilimumab monotherapy arm demonstrated durable response of more than 24 weeks, and were ongoing at the end of the study. Five responses occurred in the ipilimumab plus dacarbazine group (14.3%) including two patients who achieved complete response that were durable and ongoing at the end of study, but the other three partial responders subsequently experienced progressive disease. In a randomized phase II trial for treatment naïve NSCLC patients, there different regimens were compared, carboplatin/paclitaxel alone with either placebo (control) or ipilimumab concurrently (four doses of ipilimumab plus paclitaxel and carboplatin followed by two doses of placebo plus paclitaxel and carboplatin) or phased (two doses of placebo plus paclitaxel and carboplatin followed by four doses of ipilimumab plus paclitaxel and carboplatin) [52]. Interestingly, a small but significant improved immune related progression free survival (irPFS, primary endpoint) was observed with the phased ipilimumab regimen (delayed ipilimumab administration for two cycles) versus the control (5.7 vs 4.6 months), but this was not observed with the concurrent ipilimumab (starting ipilimumab concurrently with chemotherapy cycles) (5.5 vs 4.6 months). Similarly designed trials for treatment naïve extensive-stage SCLC patients showed similar irPFS benefit in the phased ipilimumab arm but not the concurrent arm [53]. These data suggest a potential benefit of having “induction chemotherapy” to initiate antigen release and immune response activation before combining with anti-CTLA4.

Clinical testing of combination of chemotherapy with anti-PD1/L1 therapy is ongoing in lung cancer and other tumor types that chemotherapy is standard of care. In NSCLC, frontline combinations of nivolumab with platinum-based chemotherapies demonstrated 43% of ORR but have been associated with increased toxicity of 47% grade 3–4 treatment-

related adverse events (Antonia, ASCO 2014, 8113). When pembrolizumab was combined with either carboplatin/paclitaxel or carboplatin/pemetrexed, 30% and 58% of ORR was observed while grade 3–4 toxicities was at 15% and 38% (Papadimitrakopoulou, ASCO 2015, 8031). Lastly, when atezolizumab (anti-PD-L1) was combined with nab-paclitaxel for patients with metastatic triple negative breast cancers (TNBC), a 71% ORR was seen but 56% of patients experienced grade 3–4 adverse events (Adams, 2015 San Antonio Breast Cancer Symposium, P2-11-06).

Cancer Vaccines—Cancer vaccines have been explored for decades with thousands of clinical trials being conducted with disappointing results, likely due to the subsequent immune checkpoints. Most solid tumors are poorly immunogenic and cancer vaccines can enhance tumor antigen presentation and recognition. Therefore, cancer vaccines are a rational combination partner with checkpoint inhibitors. Strategies that have been investigated include tumor-specific peptides with or without adjuvants, dendritic cell or engineered cellular vaccines, and live attenuated bacteria, etc.

Peptide vaccines have been shown to induce peptide-specific immune responses. A modified tumor antigen glycoprotein 100 (gp100) vaccine increased the frequency of melanoma-specific CD8 cells in patients with advanced melanoma [54]. However, in a phase III clinical trial of patients with advanced melanoma, combination of gp100 peptide with ipilimumab did not improve the overall survival when compared to ipilimumab alone [55], suggesting that checkpoints in the later phase of T cell activation, such as PD-1/L1 checkpoint, might be more important. The selection of tumor antigen and number of tumor antigens that can induce tumor specific immune response is still unclear. A phase I adjuvant trial for patients with advanced melanoma combining a multi-peptide vaccine (gp100, NY-ESO-1 and MART-1) with nivolumab showed significant increases in MART-1, NY-ESO+, and gp100+/CD8+ T-cell populations in peripheral blood after 12 and 24 weeks of treatment with nivolumab and vaccine [56]. Conversely, another study has shown that tumor-specific T cells might be sequestered at the vaccination site and anti-tumor immune response might be negatively influenced by certain vaccine preparations [57]. Therefore, selection of appropriate vaccine preparations is vital. Most recently, with the advancement of whole exome sequencing and prediction of “neo-antigens”, that is peptides unique to a particular mutations in the patient’s tumor vs normal tissue, combination of neo-antigen vaccine and checkpoint inhibitors presented a promising new strategy to trigger specific anti-tumor immune responses.

Dendritic cell (DC) vaccines have also been extensively studied. The first and only approved DC vaccine is Sipuleucel-T, which targets prostatic acid phosphatase and has been shown to improve OS in patients with metastatic CRPC but showed no impact on PFS or PSA levels [58]. Sipuleucel-T combined with the androgen receptor inhibitor enzalutamide concurrently or sequentially has been investigated in patients with CRPC and results in objective radiological and PSA tumor marker responses [59]. Combination of peptide-antigen loaded DCs or intratumoral injection of immature DCs with checkpoint inhibitors have been studied and efficacy was noted in preclinical mouse models [60–63]. When autologous DC were pulsed with MART-1(26–35) peptide and administered with a dose escalation of the CTLA-4 blocking antibody tremelimumab, of the 16 treated patients with advanced

melanoma, 2 partial response and 2 complete response were observed, all melanoma free between 2 and 4 years after study initiation, at the higher range of the expected response rate with either agent alone [64].

Another vaccination strategy is using genetically modified tumor cell vaccines, such as irradiated GM-CSF-producing allogeneic tumor cells (GVAX) that can provide tumor antigen, attract DC via GM-CSF, stimulate and amplify DC cell maturation [65]. In preclinical mouse models, combination of GVAX with CTLA4 [66–68] or PD-1 [69, 70] or both [71] checkpoint inhibitors promoted tumor eradication and survival of the treated animals, with increased CD8 cell infiltration and CD8/Treg ratio in these tumors [72]. Early phase clinical trial combining GVAX with ipilimumab for patients with castration resistant prostate cancer has shown safety and clinical benefit (PSA response or stabilization) [73]. Another study investigating the combination of GVAX with ipilimumab in 15 advanced pancreatic cancer patients also showed prolonged disease stabilization in 3 patients and declined tumor markers in 7 patients [74].

Bacterial vaccination is an alternative platform to induce anti-tumor immune responses, pioneered more than a century ago by surgeon William Coley (Coley's toxin) for patients with sarcoma [75]. Recently, live-attenuated *Listeria monocytogenes* vaccines encoding tumor-specific antigens have been developed, which naturally target DCs in vivo and stimulate both innate and adaptive cellular immunity and has shown efficacy in several animal models [76]. Combination therapy with checkpoint inhibitors is under clinical investigation, including a phase I trial for patients with HPV positive cervical or head and neck cancer combining live attenuated listeria encoding the HPV16 oncoprotein E7 (ADXS11-001) with durvalumab (anti-PD-L1) (NCT02291055), and another one combining live attenuated listeria encoding PSA (ADXS31-142) with pembrolizumab for patients with advanced prostate cancer (NCT02325557). Most recently, a phase 2 trial combined GVAX with or without a live-attenuated Listeria monocytogenes-expressing mesothelin (CRS207) for patients with advanced pancreatic cancer demonstrated significant overall survival benefit (HR 0.53, P=0.02) [77], and further combination of GVAX/CRS207 with nivolumab is ongoing (NCT02243371). Other bacterial vaccine platform are also being developed [78].

Oncolytic virus—Oncolytic viruses are genetically engineered virus constructs that can replicate in the tumor cells and elicit anti-viral immune response by the host in the local tumor microenvironment, and this interplay between the oncolytic virus and the immune system can be translated into virus-induced anti-tumor immune response [79]. The first approved agent of this class is talimogene laherperepvec (T-VEC), a modified oncolytic herpes simplex virus that encodes granulocyte-macrophage colony-stimulating factor (GM-CSF) with tumor specificity. Intratumoral injection of TVEC can promote tumor lysis and tumor antigen release and presentation, together with GM-CSF release, can attract DCs into the injected tumors and increase DC maturation and priming of T cells, thus stimulating a systemic tumor-specific immune response, providing a highly attractive combination approach with checkpoint inhibitors. OPTiM, a phase III trial of T-VEC vs GM-CSF in unresectable stage IIIB-IV melanoma improved the primary endpoint of durable response rate (DRR) in the T-VEC arm (16 vs 2%) [80]. Early phase study in patients with advanced melanoma combining TVEC with ipilimumab indicated safety and tolerability of this

combination with a seemingly improved response (50%) than would be expected with either drug alone [81]. A phase Ib/III study assessing the safety and efficacy of T-VEC plus pembrolizumab in unresected stage IIIB-IV melanoma is ongoing [82, 83] and the phase Ib result was reported at the 2016 ASCO annual meeting. Of the 21 enrolled patients, confirmed/not yet confirmed objective response rate (ORR) per immune related response rate (irRC) was 48%/57%; complete response rate was 14%/24%. A follow up randomized double-blinded phase 3 phase is under way.

Other oncolytic viruses that have been tested include intratumoral injection of coxsackievirus A21 in patients with unresectable Stage IIIC-IV M1c melanoma (Andtbacka, ASCO 2014, 3031), with reported 35% of irPFS at 6 months and best ORR per irRC of 24%. Combination of local injection of Newcastle Disease Virus (NDV) with systemic CTLA-4 checkpoint blockade has shown promising results in preclinical models of melanoma [84]. Additional modified viral vectors that have been tested in combination with CTLA-4 blockade or other immune-modulatory agents and shown safety and efficacy in preclinical models and phase I trials include attenuated poxvirus vaccine targeting mutated p53 [85], recombinant adenoviral vector expressing human Her-2/neu antigen [86], recombinant vaccinia and avipox viruses expressing carcinoembryonic antigen (CEA) and three T cell costimulatory molecules (B7.1, ICAM-1, and LFA-3) (CEA-TRICOM) [87] as well as poxviral-based vector encoding PSA and three T-cell co-stimulatory molecules (CD58, CD80, and ICAM1) (PSA-TRICOM) [88].

Toll like receptor agonists—Toll like receptors (TLR) are a part of the innate immune system and are expressed on a wide range of immune cells, including monocytes, dendritic cells, macrophages, etc [89, 90]. These innate immune cells have a critical role in the defense against infection and disease (including cancer) and TLRs are PRRs to detect pathogen-associated patterns and danger-associated patterns. Activation of TLRs on DC triggers maturation of the APC, induction of inflammatory cytokines and the subsequent priming of naive T cells for adaptive immunity. Therefore, it is rational to harness agonists of TLR signaling as vaccine adjuvants to enhance the induction of vaccine-specific responses against cancer. However, some TLRs, such as TLR 2, 4, 7, have been shown to promote tumor growth or chemotherapy resistance [91–93]. Therefore, careful selection of the subtype of TLR for activation and the specificity of activation are critical. In a mouse model of melanoma, TLR3 activation was shown to induce type I interferon and increase tumor infiltrating lymphocytes, and synergize with anti-PD-1 therapy [94]. TLR9 agonists could also increase T cell infiltration in the CT26 colon adenocarcinoma mouse model [95], indicating that this subtype of TLR agonists might be combined with checkpoint inhibitors. In a lymphoma mouse model, the combination of anti-OX40 and anti-CTLA-4 as well as intratumoral CpG (a TLR9 agonist) induced antitumor CD4 and CD8 T-cell immunity, and cured large and systemic lymphoma tumors without chemotherapy [96]. In another bladder cancer mouse model, intratumoral injection of CpG with aCTLA-4 or aPD-1 increased the survival of mice, with aPD-1 plus CpG being superior to either agent alone. The combination increased the number of circulating tumor-specific CD107a-expressing CD8 T cells and activated (CD25FoxP3-) CD4 splenocytes, as well as decreased numbers of Tregs in the tumors [97]. A phase I study combining TLR9 (PF-3512676) with tremelimumab

showed 2 (of 17) melanoma patients with partial responses, but with increased toxicity (2 dose limiting toxicities that required steroids) [66]. There are several phase I clinical trials ongoing testing the combination of TLR9 agonist with anti-PD1 therapy.

2) Strategies to promote T cell activation and homing

Following priming and activation in the lymph nodes, T cells migrate via the systemic vasculature to the tumors, facilitated by the adhesion molecules on the endothelium for extravasation, and recognize and eradicate the tumor targets via interaction of TCR and tumor antigen presented by the MHC molecules. There are multiple co-stimulatory or co-inhibitory receptors on activated T cells to regulate the activation, differentiation, function and survival of the T cells [98], which can be hijacked by the tumors to evade immune surveillance. But they also provide rationale for the development of effective immune-modulatory agents by targeting these receptors, the majority of which belong to either the immunoglobulin superfamily (CD28, ICOS, CTLA-4, PD-1, LAG3, TIM3, BTLA, VISTA, CD160, etc) or the tumor necrosis factor receptor superfamily (TNFRSF) such as GITR, OX40, 4-1BB/CD137, CD40, CD30, etc. [14]. Agonists of T cell co-stimulation (4-1BB, OX40, CD40, GITR, and ICOS) can amplify T cell activation and enhance anti-tumor immune responses, thus providing strong rationale to be combined with checkpoint inhibitors [99].

4-1BB/CD137 agonists—4-1BB/CD137 receptor is a co-stimulatory receptor found on both T cells and NK cells, as well as DCs and myeloid cells, and when activated, could improve T cell function and survival, as well as regulate Treg function. 4-1BB/CD137-deficient mice showed enhanced T cell proliferation but cytokine production and cytotoxic T cell activity were diminished. Interestingly, 4-1BB/CD137 deletion also led to an increase in myeloid progenitor cells in the periphery (blood, bone marrow, and spleen) [100]. Interestingly, tumor-reactive tumor-infiltrating lymphocytes (TIL) from freshly resected ovarian and melanoma tumors naturally express higher levels of CD137 than circulating T cells. CD137+ TILs also mediated superior antitumor effects in vivo, compared with CD137- TILs [101]. In mouse models, combination of 4-1BB/CD137 agonists with checkpoint inhibitors showed beneficial effects, including with CTLA-4 blockade in MC38 colon carcinoma [102] and GL261 glioblastoma [103] but not in B16 melanoma tumors, and with PD-1/L1 checkpoint inhibitors in colon carcinoma, B16F10 melanoma, and ID8 ovarian carcinoma [104–106], with the highest efficacy observed with triple therapy (CD137 agonist and blockade of both PD-1 and CTLA-4) [104]. A recent study in mouse models of colon carcinoma (MC38) and melanoma (B16F10) showed a critical need for BATF3-dependent DCs in cross-priming of tumor antigens to CTLs that subsequently upregulate PD-1 and CD137 and is crucial to the efficacy of immunostimulatory antibodies [107].

Two 4-1BB agonists are leading the clinical developments, urelumab and utomilumab, with single agent and combination therapies being evaluated. Urelumab was evaluated in a phase I study of 83 patients with advanced melanoma, renal cell carcinoma, ovarian, and prostate cancer (Sznol, ASCO 2008, 3007) with clinical activity seen across dose ranges and tumor types. A phase 2 trial of urelumab, however, was temporarily suspended due to high incidences of hepatotoxicity [108]. Phase I trial of utomilumab showed no significant

toxicity, with evidence of clinical activity seen in 9 of 24 patients (Segal, ASCO 2014, 3007). Multiple clinical trials are ongoing to evaluate the combination of 4-1BB/CD137 agonists with PD1/L1 checkpoint inhibitors (NCT02179918).

A study in mouse models also suggest potential synergistic effect of 4-1BB/CD137 agonists with antibodies that cause antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells and upregulate 4-1BB on the NK cells [109]. Activation of CD137 enhances NK cell degranulation and cytotoxicity. Combination of anti-CD137 with HER-2 [110] or CD20 [111] antibodies potentiated their antibody dependent cytotoxicity. Clinical trials are ongoing testing the combination of anti-CD137 with rituximab, cetuximab, elotuzumab (enhance NK cytotoxicity and ADCC) [112, 113].

OX40—OX40, also known as CD134, is found on T cell, NK cells and neutrophils, and is expressed transiently after T cell activation and important in the survival of activated T cells and T cell memory [114]. As OX40 is only expressed by activated T cells, it is rational to combine OX40 agonists with agents that increase T cell activation. Preclinical mouse model studies have provided evidence of synergy for combination with 4-IBB agonists [115], anti-PD-1 [116] and anti-CTLA4 [117] antibodies.

In a phase I study with advanced solid tumors, anti-OX40 antibody treatment showed an acceptable toxicity profile and regression of at least one metastatic lesion in 12 of 30 patients, with upregulated markers of immune activation in peripheral blood and increased antitumor reactivity of T and B cells [118]. Several anti-OX40 antibodies are currently being developed either as single agent or in combination with CD137 agonist, anti-CTLA4 and anti-PD1/L1 antibodies.

CD40—CD40 is constitutively expressed on APCs and B cells and its ligand CD40L is expressed on T cells. Activation of CD40 triggers APC maturation and expression of co-stimulatory molecules that promotes T cell activation [14]. In a genetically engineered mouse model of pancreatic cancer, an agonist CD40 antibody combined with gemcitabine chemotherapy demonstrated efficacy and a further mechanistic study showed that tumor regression required macrophages but not T cells or gemcitabine [119]. CD40-activated macrophages rapidly infiltrated tumors, became tumoricidal, and facilitated the depletion of tumor stroma. Suggesting a CD40-dependent mechanism for targeting tumor stroma. Phase I clinical trial of anti-CD40 (CP-870893) has been tested in combination with tremelimumab with 27.3% of ORR observed, but with significant toxicity including dose limiting colitis and uveitis [120]. Induced PD-L1 expression was found in acquired resistance to anti-CD40 treatment [121], providing rationale to combine with anti-PD1/L1 checkpoint inhibitors. Clinical trial testing combination of anti-CD40 and PD1/L1 blockage are active or underway (NCT02706353).

T cell exhaustion markers (TIM3, LAG3)—Other checkpoint / co-inhibitory molecules similar to PD-1 can be utilized by cancer cells to produce T cell exhaustion and dampen T cell activity. T cell immunoglobulin domain and mucin domain-3 (TIM3) is a receptor expressed on PD-1+ CD8+ exhausted tumor infiltrating lymphocytes. TIM3 knock out mice do not develop overt autoimmune disease but blockade of TIM3 can accelerate the

development of autoimmunity [122]. Preclinical evidence of synergy of anti-TIM3 antibody with PD-1 blockade and 4-1BB agonists have been observed [122, 123]. Lymphocyte activating gene 3 (LAG3) is expressed on both CD4 and CD8 T cells and is important in regulating Treg function. Studies in preclinical models support the combinatorial effect of blocking LAG3 and PD-1 [124]. Clinical trials of antibodies targeting TIM3 and LAG3 as single or combination therapy with checkpoint inhibitors are ongoing.

Targeted therapy—Molecularly targeted therapies are small molecule drugs that target tumor-specific driver mutations or tumor-dependent growth factors. Although drug resistance is a frequent occurrence with targeted inhibitors, a subset of treated patients who are long term responders [125, 126]. There is increasing evidence that at least certain targeted agents exert anti-tumor function through immune modulation. This topic has been extensively discussed elsewhere [127, 128]. Briefly, targeted therapy can have "immunesensitization" effects on the different components of the immune system, including increased antigen release, presentation and MHC expression, enhanced T cell function and homing, and improved tumor microenvironment, suggesting a potentially synergistic benefit of combining targeted therapy and immunotherapy beyond the expected additive effect of two effective treatments [129]. The first phase 1 trial of the *BRAF* inhibitor vemurafenib and ipilimumab in advanced melanoma was closed early due to dose limiting hepatotoxicity [130]. A separate trial involving ipilimumab and another BRAF inhibitor, dabrafenib, did not encounter hepatotoxicity (NCT01767454), suggesting a drug-specific process. However, the triple combination arm of dabrafenib, trametinib (MEK inhibitor) and ipilimumab was discontinued due to colon perforations [131]. Combination of BRAF plus / minus MEK inhibitors with PD-1/L1 inhibitors are better tolerated and currently there are several clinical trials are testing this combination and have shown encouraging results in treating metastatic melanoma.

3) Strategies to improve the tumor microenvironment

The concept of cancer immune editing introduced by Schreiber and colleagues in 2001 hypothesized the dual role of the host immune system as both suppressor and facilitator of tumor growth and progression, supported by a study using carcinogen-induced sarcomas generated from both wild type and RAG2−/− mice (deficient of T, B and NK cells), and subsequently implanted in wild type or RAG2−/− hosts. The tumor cells generated from wild type mice grew progressively when implanted in both wild-type and RAG2−/− hosts, and the tumors generated from RAG2−/− mice also grew progressively in RAG2−/− hosts, but nearly half of the tumors implanted in the immune-competent wild type mice were rejected. These results indicated that tumors arise from immune-competent hosts are less sensitive to immune attack, and highlighted that the tumor microenvironment might play a role in this immune escape [132]. Multiple components of the tumor microenvironment have been reported to be involved in the development of immune resistance and immune editing, and can serve as targets to improve the local immune environment and increase tumor immunogenicity, therefore are good combination partners of the checkpoint inhibitors.

IDO inhibitor—Indoleamine 2, 3-dioxygenase (IDO) is a cytosolic enzyme that catalyzes the breakdown of tryptophan to its metabolites [133]. IDO is widely overexpressed in tumor

cells and myeloid lineage cells, which has been associated with poor prognosis. IDO is produced by tumor cells and myeloid-derived suppressor cells (MDSCs) in response to inflammatory signals including interferon-gamma [134]. Seminal work done by Munn, Mellor and colleagues in 1998 suggest IDO might mediate immunosuppression based on the preferential sensitivity of T cells to tryptophan deprivation [135]. Subsequent studies provided evidence that IDO activity could suppress T cells and NK cells [136, 137], and was critical to support activity of FoxP3+ Tregs [138] and MDSCs [139]. Upregulation of IDO in the tumor microenvironment was a possible mechanism of resistance to anti-CTLA-4 immunotherapy, and when an IDO inhibitor was combined with anti-CTLA4 antibodies, it significantly enhanced the therapeutic efficacy in different animal tumor models, and was associated with increased tumor-infiltrating lymphocytes [140, 141].

There are several IDO inhibitors in clinical development. The most advanced is epacadostat (INCB024360), a selective oral inhibitor of the IDO1 enzyme. Preliminary data from a phase I trial combining epacadostat and ipilimumab (anti-CTLA4) in patients with metastatic melanoma (Gibney, ASCO 2014, 3010) was well tolerated with 23% of patients experiencing grade 3 adverse events, and a disease control rate (DCR) of 60% in immunotherapy-naive patients and 30% in patients who had received prior immunotherapy treatments, and ORR of 30% [142]. In a phase 1 trial of patients with advanced solid tumors, combination of epacadostat with pembrolizumab (anti-PD1) was well tolerated and also showed promising activity (Gangadhar, ESMO 2016,). Eighteen percent of patients had grade ≥3 treatment related adverse events, mostly rash (8%) and increased lipase (3%). Of 19 patients with treatment-naïve advanced melanoma, a disease control rate (DCR) of 74% and an ORR of 58% were observed. With a median follow up of 42 weeks, all responses were confirmed and ongoing and median PFS has not been reached. A randomized phase III trial testing the combination of pembrolizumab and epacadostat vs placebo is ongoing for treatment-naïve advanced melanoma. Indoximod, a tryptophan analogue, is another IDO inhibitor under development. Phase I testing of indoximod in 48 patients did not reach MTD at 2000 mg twice/day and 5 patients showed stable disease >6 months [143]. Combination with ipilimumab in patients with advanced melanoma was safe with no DLTs (Zakharia, ESMO 2015, 514) and a phase 2 study combining indoximod with ipilimumab or anti-PD1 (pembrolizumab or nivolumab) is currently ongoing (NCT02073123). GDC-0919 is also an IDO1 inhibitor and preliminary results from a phase I trial showed tolerable toxicity up to 800mg BID with a 21 day on/7 day off schedule and 44% of patients prolonged stable disease for more than 4 months (Nayak, ESMO 2015, 346).

CSF-1R inhibitor—Tumor represents a chronic inflammatory microenvironment that can skew microphages to an anti-inflammatory phenotype. Tumor-associated macrophages (TAM) play a crucial role in promoting tumor progression and resistance to chemotherapy in several mouse models [144, 145]. High density of macrophages is associated with poor prognosis in patients of with different cancer types. However, conflicting data exists for others with both positive and negative associations reported [146, 147], possibly due to the heterogeneity of the analyzed tumor stages, analyses performed and macrophage markers utilized (CD68 vs CD163 vs CD206, for example). Interestingly, macrophages in human colorectal cancer have been found to be functionally and phenotypically anti-tumor [148].

Production of the C-C chemokine ligand 2 (CCL2) and/or colony-stimulating factor 1 (CSF-1) are necessary to recruit macrophages to the tumor site, sustain their numbers [149], and there is growing interest in therapeutics targeting these ligands and/or their respective receptors in an effort to ablate the pro-tumorigenic properties of macrophages. Indeed, antibodies targeting CSF-1 receptor (CSF-1R) have been shown in some preclinical models (pancreatic cancer, cervical cancer and glioblastoma) to deplete immunosuppressive macrophages and increase the CD8/CD4 ratio in the tumors that led to improved outcome, and synergy with chemotherapy, radiation therapy, anti-angiogenic agents, adoptive cell transfer and checkpoint inhibitors [144]. The immune suppression by macrophages in animal models relies on arginase and NOS activity, but in humans this dependence is lacking [150]. Macrophages could directly suppress T cell responses through PD-L1 in patients with hepatocellular carcinoma [151], which provides additional rationale to combine CSF-1R inhibitors with PD-1/L1 checkpoint inhibitors [152]. Currently, there are several clinical trials ongoing to test this concept. Because macrophages are important in the homeostasis in the liver, hepatotoxicity can be a concern with single agent CSF-1R inhibition or combination therapies. In addition, questions remain whether to deplete macrophages or promote anti-tumor polarization would be more relevant and effective in human cancers. Translational studies using patient-derived samples would be key to answer these questions and guide the clinical design and management of toxicity.

TGFβ inhibitor—Transforming growth factor-β (TGFβ) is a cytokine that plays important roles in the tumor microenvironment including angiogenesis and immunosuppression by stimulating Tregs [153]. Increased level of TGFβ is associated with poor prognosis in multiple different tumor types [154, 155]. Preclinical models have shown synergy combining TGF-β receptor kinase inhibitor I with anti-CTLA-4 and inhibited tumor growth in a melanoma model (BRAFV600EPTEN−/−) [156] or fractionated radiation therapy by enhance T cell priming [36]. Clinical trials testing the combination of the TGF-β inhibitor (galunisertib) [157] and PD-1/L1 checkpoint blockade (durvalumab or nivolumab) are currently ongoing in patients with metastatic pancreatic cancer (NCT02734160) and NSCLC, hepatocellular carcinoma, or glioblastoma (NCT02423343).

Adenosine receptor antagonist—Adenosine was shown to inhibit T cell proliferation and cytotoxic function via the A2A receptor on T cells [158] as well as promote metastasis via the A2B receptor on tumor cells [159]. In addition, CD73 is the enzyme that dephosphorylates adenosine monophosphate (AMP) to form adenosine, thus also suppressing immune function and promoting tumor cell metastasis [160], as well as stimulates angiogenesis [161]. High expression of CD73 is associated with poor prognosis in different cancer types [162–164]. CD73 is also a potential biomarker for anti-PD-1 therapy, with high expression limiting anti-PD-1 efficacy, which can be rescued by concomitant A2A blockade [165]. Both A2A receptor antagonists and anti-CD73 antibodies serve as attractive targets to improve the immune microenvironment. In preclinical models, the combination of an A2A receptor antagonist with anti-CTLA-4 or anti-PD-1 synergistically inhibited tumor growth in breast cancer (4T1) and melanoma (B16F10) [166–168], and the combination of anti-CD73 and anti-CTLA-4 or anti-PD-1 enhanced the antitumor activity in colon (MC38), prostate (RM-1), and breast cancer (4T1) models [169]. Currently, clinical trials are ongoing

to test the safety and tolerability of combined A2A receptor antagonist (CPI-444) and anti-PD-L1 (atezolizumab) (NCT02655822), and the combination of anti-CD73 (MEDI9447) plus anti-PD-L1 (durvalumab) (NCT02503774) in patients with advanced solid cancer.

Chemokine receptor inhibitors—MDSCs and Tregs traffic to the tumor using specific chemokine and chemokine receptors. For example, tumors secret ligands CCL5, CCL7, and CXCL8, bind to their receptors CCR1 or CXCR2 expressed on subtypes of MDSCs [170], and attract MDSCs in the tumor microenvironment. Inhibitors of these chemokine receptors could abrogate immune evasion and improve antitumor T cell responses. In a mouse model of breast cancer, combination of a CCR1 inhibitor (CCX9588) and PD-L1 inhibitor synergistically reduced the tumor burden [171], and anti-CXCR2 plus anti-PD-1 improved survival in a rhabdomyosarcoma model [170].

CCR4 is highly expressed by Tregs in the blood and tumors [172] and anti-CCR4 inhibits Treg recruitment as well as promotes antibody-dependent cell-mediated cytotoxicity (ADCC), further reducing the Treg population [173]. Therefore, anti-CCR4 represents an attractive target to combine with immune-checkpoint blockade. Currently anti-CCR4 (mogamulizumab) in combination with nivolumab (NCT02705105), durvalumab (NCT02301130) and tremelimumab (NCT02301130) is being tested in the clinic in patients with advanced solid tumors.

CXCR4 is a receptor for the chemokine CXCL12 has been shown to promote an immunosuppressive tumor microenvironment through several mechanisms including Treg localization [174]. CXCR4 inhibitors have shown antitumor synergy with anti-PD-1 therapies in preclinical models [175] and are in clinical development. The most advanced is ulocuplumab, being tested in combination with nivolumab (NCT02472977).

Epigenetic modulation—Epigenetic modification changes gene expression or cellular phenotype without changing the DNA sequence, including DNA methylation, chromatin remodeling, etc., which could lead to changed expressions of tumor suppressor genes and/or proto-oncogenes, as well as immune related genes [176, 177]. Epigenetic silencing of immune-related genes is a feature of the cancer genome that impacts antigen processing and presentation by tumor cells, facilitates immune evasion, and modulates the tumor microenvironment, making it a promising therapeutic target and a candidate to combine with checkpoint inhibitors [178]. In preclinical models, histone deacetylase (HDAC) inhibitors can synergize with adoptive cell transfer therapy to treat B16 murine melanoma by increased MHC and tumor-associated antigen expression by tumor cells, a proliferative advantage and improved function of the adoptively transferred cells [179]. In a lymphoma model, hypomethylating agents have shown to restore gene expression and promote CD8+ T cell infiltration into the tumor attributed to demethylation-induced CD80 expression on tumor cells [180]. When combined with anti-CTLA-4, synergistic effect was seen in a murine mammary carcinoma and mesothelioma models with high CD8 and CD4 T cell tumor infiltration [181]. Both hypomethylating agents (AZA) and HDAC inhibitors (entinostat) could improve treatment outcome when combined anti–PD-1 and anti–CTLA-4 antibodies to eradicate modestly immunogenic CT26 colon adenocarcinoma or metastatic 4T1 mammary carcinoma by eliminating circulating and tumor-infiltrating granulocytic MDSCs

[182]. Based on these results, clinical studies are ongoing to investigate the safety and efficacy of combination approaches with epigenetic modulation.

4) Strategies to activate NK cells

Natural killer (NK) cells are part of the innate immune response system and can produce pro-inflammatory cytokines and kill cancer cells following nonspecific activation with cytokines (IL-12, IL-15, and IL-18), antigens or cytomegalovirus (CMV). In the past decade it was recognized that NK cells can have immunological memory and their antitumor responses may be enhanced long term [183]. Activated NK cells express killer-cell immunoglobulin-like receptors (KIRs), which serves as a checkpoint and inhibit the cytotoxic activity of NK cells after interaction with MHC-I on tumor cells [184]. It can be beneficial to block both PD-1 or CTLA-4 and KIR for the activation of both T- and NK cells, or for those patients with acquired intrinsic mutation in the interferon response pathway that renders the tumor cells resistant to T cell attack [185]. Clinical trials are ongoing to test the combination of anti-KIR (lirilumab) and nivolumab (NCT01714739) or ipilimumab (NCT01750580) in patients with advanced solid tumors.

5) Engineered T cells

For patients whose immune system has not been able to mount an effective anti-tumor response, adoptive T cell transfer (ACT) therapy is another promising strategy to combine with checkpoint inhibitors. For the treatment of melanoma, *ex vivo* culture and expansion of tumor infiltrating lymphocytes (TIL) and reinfusion of the tumor-reactive cells into the patient resulted in 50% of objective response rates and 22% of complete tumor regression [186]. Strategies to engineer peripheral T cells include to express chimeric antigen receptors (CARs) to target surface tumor antigens (e.g. CD19) or full T cell receptor (TCR) to target cytoplasmic tumor antigens (such as MART-1 or NY-ESO-1) [187]. However, there might be a lack of sufficient T cell infiltration into the tumor and an immunosuppressive tumor-microenvironment may limit the T cell function [188], and combination with checkpoint inhibitors can help maximize effector function. Indeed, anti-PD-1/L1 or anti-CTLA-4 when used together with ACT could synergistically reduce tumor growth and increased long-term survival in the MC38 colon carcinoma and B16 melanoma mouse models, as well as in transgenic Her-2 mice treated by ACT of Her2-specific CAR T cells and systemic anti-PD-1 [189–191]. Anti-PD-1 promoted the proliferation of T cells and their cytotoxic activity with increased IFN-γ production and chemokine upregulation (e.g., CXCL10) that result in increased T cell infiltration. Currently, the combination of ACT and anti-CTLA-4 or anti-PD1/L1 is studied in early phase clinical trials.

Conclusions and future directions

After more than a century of persistent exploration to harness the immune system to fight cancer, great strikes have been made in the past decade in the field of cancer immunotherapy. Immune-checkpoint blockade and combinations with other immune-modulatory agents has shown durable responses with improved survival in an excitingly long list of tumor types. Not all patients nor all tumor types would benefit from the treatment, and heterogeneous response have been seen even for the same patient, calling for

individualized immune-checkpoint combination approaches guided by predictive biomarkers for the optimal outcome for a larger number of patients with a broader spectrum of tumor types. The available approved and experimental therapeutic options discussed in this review creates therapeutic possibilities, but needs to be guided by sound scientific rationale and preclinical studies, as well as carefully designed clinical trials for effective treatment and confirm safety, as some of the toxicities may not be apparent in pre-clinical studies. The endpoints to evaluate effectiveness of immunotherapy should also be carefully chosen, as the durability of the clinical benefit, the hallmark of immunotherapy, needs to be evaluated in addition to the traditional response rate per RECIST. Besides the pharmacokinetics, confirmation of pharmacodynamics by paired tumor biopsies is critical in early phase clinical testing of combination immunotherapies given the underlying complicated biology, to be able to elucidate the available data and make informed decisions for greatest effectiveness in the most suitable population of patients, with minimal toxicities. With further advancement of our understanding of tumor immune-biology and accumulation of clinical experience, it is a matter of time before the power of the immune system can be fully harnessed to eradicate cancer.

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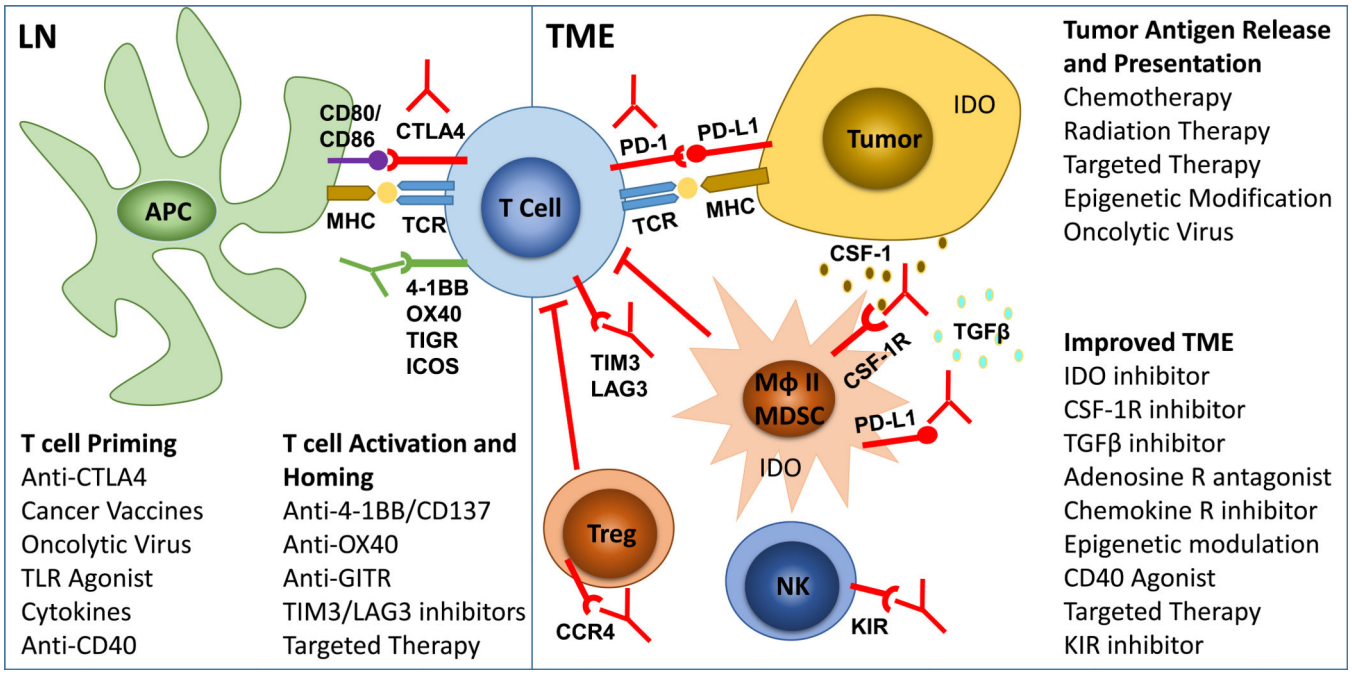

Figure 1.

Combination strategies to improve the anti-tumor effects of PD-1/L1 blockade. This includes to increase tumor-specific antigen release and presentation, enhance T cell priming and homing to the tumors, augment T cell effector function, suppress immune suppressive cell populations (Tregs, MDSC, type II macrophages), cytokine and metabolite release in the tumor microenvironment. LN: lymph node; TME: tumor microenvironment; APC: antigen presenting cells; MHC: major histocompatibility complex; TCR: T cell receptor; TLR: toll like receptor; Treg: regulatory T cell; MDSC: myeloid-derived suppressor cell; Mϕ II: type II macrophage.