Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1

Manipulation of co-stimulatory or co-inhibitory checkpoint proteins allows for the reversal of tumor-induced T-cell anergy observed in cancer. The field has gained credence given success with CTLA-4 and PD-1 inhibitors. These molecules include immunoglobulin family members and the B7 subfamily as well as the TNF receptor family members. PD-L1 inhibitors and LAG-3 inhibitors have progressed through clinical trials. Other B7 family members have shown promise in preclinical models. TNFR superfamily members have shown variable success in preclinical and clinical studies. As clinical investigation in tumor immunology gains momentum, the next stage becomes learning how to combine checkpoint inhibitors and agonists with each other as well as with traditional chemotherapeutic agents.

Keywords: B7 family • checkpoint proteins • immunotherapy • TNFR superfamily • translational medicine

One of the hallmarks of cancer is the ability of the malignant cell to escape eradication by the immune system [1]. Proposed over a century ago, the concept of immune control of cancer continues to develop [2,3]. The existence of tumor antigens led Burnett and Thomas to form their hypothesis about cancer immune surveillance where the adaptive immune system was responsible for preventing the development of cancer in immunocompetent hosts. This hypothesis fell out of favor until the 1990s when improved mouse models of immunodeficiency were developed and particularly when the role of IFN- γ in promoting immune-mediated rejection of transplanted tumor in mice [4].

Tumors are variably infiltrated by cytotoxic T lymphocytes (CTLs), but a dense infiltration portends a better prognosis [5–7]. The T-cell response follows a complex interaction between an antigen-presenting cell (APC) and a T cell. TCR recognition of an antigen on MHC molecule is not sufficient, a second signal provided by a member of the B7 family is required [8]. CD28 provides the primary co-stimulatory signal for

the activation of T cells after it engages B7-1 (CD80) or B7-2 (CD86) [9]. CTLA-4 is a CD28 homologue that interacts with B7-1 and B7-2 and, in contrast to CD28, provides an inhibitory signal [10,11]. Newly identified members of the B7 family also provide inhibitory signals the roles of which continue to be explored [12]. Blocking CTLA-4 mediated inhibition of the T-cell effector response has been an attractive therapeutic target. Monoclonal antibodies (mAb) that block CTLA-4 are effective in mouse models of a variety of tumors [13–15]. Ipilimumab (Yervoy®) is US FDA approved for the treatment of metastatic malignant melanoma and represents the first success story of T-cell checkpoint inhibitor immunotherapy [16].

A more recent success story in cancer immunology is that of PD-1. PD-1 was first identified in lymphoid cells lines induced to undergo programmed cell death [17]. Later reports noted that PD-1 is expressed on activated T and B cells, dendritic cells (DCs) and monocytes upon stimulation where it is found to play an inhibitory role [18–20]. PD-1 is highly expressed on T cells and leads to

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T cell exhaustion [21,22]. PD-1 expression is also noted on CD4⁺ Foxp3⁺ regulatory T cells (Tregs) where it contributes to their inhibitory role [23]. Several mAbs targeting PD-1 have progressed through clinical trials. The first FDA approved mAb was pembrolizumab (Keytruda®), also known as lambrolizumab after showing response rates in melanoma patients who have progressed after first line therapy including immunotherapy with ipilimumab (KEYNOTE-001 trial) or in comparison to investigator-choice chemotherapy (KEYNOTE-002) [24,25]. The second PD-1 targeting mAb to receive FDA approval was nivolumab (Opdivo®). It was well tolerated in Phase I studies in solid tumors as well as lymphomas [26–30]. Similar to pembrolizumab, nivolumab was shown to be superior to chemotherapy in the second line setting in melanoma in the Phase III CheckMate-037 trial [31,32]. Nivolumab yielded better survival and higher response rates in comparison to docetaxel in the treatment of advanced squamous non-small-cell lung cancer [33]. Other PD-1 mAbs include pidilizumab (CT-011) which was the first one to reach clinical trials and remains in development (studies reviewed in [34]) and more recently MEDI0680 which is entering clinical trials [35,36].

Blocking CTLA-4 and PD-1 are not the exclusive path toward T-cell ‘dis-inhibition’. A variety of immunomodulatory pathways have been studied and exploited clinically with varying degrees of success and are at different stages of clinical development. Other members of the B7 family, part of the immunoglobulin superfamily, include B7x, HHLA2 and B7-H3 which play an inhibitory role. VISTA, Tim-3 and LAG-3 are members of the immunoglobulin superfamily have also been shown to play an inhibitory role. Immunomodulatory pathways include members of the TNF receptor family and their ligands which have been studied as targets for cancer immunotherapy. These inhibitory and stimulatory molecules that have been studied as therapeutic targets are depicted in **Figure 1A & B**, respectively. Finally, indoleamine 2,3-dioxygenase 1 inhibitors have also been studied as antitumor therapies as discussed below.

PD-L1 & PD-L2

The first reported ligand for PD-1 is PD-L1 (B7-H1) with wide expression at the mRNA level in lymphoid and nonlymphoid tissues [37]. It is a cell surface protein that is expressed on activated APC, T and B lymphocytes and other cells. It inhibits TCR mediated T-cell proliferation and cytokine production through the engagement of PD-1 [38]. The PD-1/PD-L1 interaction induce T-cell tolerance in lymphoid tissue before their exit to the periphery, and blockade of this interac-

tion can reverse T-cell anergy [39]. Additionally, PD-L1 expressed on tumor cells can also act as a ligand to deliver an anti-apoptotic signal that leads to resistance to cytolytic function of CTL as well as to Fas-induced and drug-induced apoptosis [40]. Another interesting fact is that B7-1 was also shown to interact with PD-L1 which results in inhibition of T cells [41,42]. A second ligand for PD-1 is PD-L2 (B7-DC), which inhibits TCR mediated T-cell proliferation and cytokine production [43,44]. It is mainly expressed on DCs and macrophages [44,45]. Recently, a novel binding partner for PD-L2 is identified named RGMb, which is important for the development of respiratory immune tolerance [46].

PD-L1 is expressed in a variety of human carcinoma specimens as well as hematological malignancies such as multiple myeloma, leukemia and peripheral T-cell lymphoma and has been correlated to poor prognosis [8,34]. Several mAbs that target PD-L1 have reached clinical trials. BMS-936559 is a fully human monoclonal IgG4 antibody that blocks PD-L1 [47]. Anti-PD-L1 antibodies inhibited tumor growth in murine syngeneic tumor models with a durable antitumor immunity. BMS-936559 can reverse *in vitro* Treg mediated suppression and does not cause antibody-dependent cytotoxicity or complement-dependent cytotoxicity [47]. The first clinical trial with BMS-936559 also demonstrated high tolerability and durable responses [47]. Other monoclonal anti-PD-L1 antibodies include MEDI4736 [48,49], atezolizumab (MPDL3280A) which demonstrated a 43% response rate in a Phase I clinical trial in metastatic urothelial bladder cancer patients resulting in an FDA breakthrough designation [50], and MSB0010718C which exhibits antitumor activity by blocking PD-L1 as well as antibody-dependent cell-mediated cytotoxicity [51,52].

B7x (B7-H4/B7- S1)

B7x is an inhibitory transmembrane protein that binds activated T cells and is a member of the B7 family [53–55]. It inhibits CD4 and CD8 cell proliferation and cytokine production [53]. It is hardly expressed on professional APC but is expressed on nonlymphoid tissues, mainly epithelial tissues where a role in immune tolerance is postulated [54,56–58]. It is expressed in the lung epithelium and is implicated in attenuating the immune response to bacterial infection in mice [56]. B7x is expressed in a variety of human cancers which include cancers of the brain, esophagus, lung, breast, pancreas, kidney, gut, skin, ovary and prostate [59]. Prostate cancer specimens from patients treated with radical prostatectomy had 15% prevalence of B7x expression and high expression was significantly associated with a higher risk of prostate cancer related death [60]. B7x expression in renal cell carcinoma is

Figure 1. Summary representation of T-cell molecules. (A) Summary representation of T-cell co-stimulatory molecules. **(B)** Summary representation of T-cell co-inhibitory molecules.

associated with adverse clinical and pathological features as well as poor survival [61]. Tumor expression of B7x in human gastric cancer predicts poor survival [62]. Similar findings were also reported in studies of ovarian cancer and lung cancer [63,64]. In a preclinical model, mouse colon carcinoma cells line CT26 transfected with murine or human B7x resulted in a higher number of lung metastasis and shorter survival [65]. Blockade of B7x with a mAb resulted in a reduction of number of lung metastasis in a CT26 as well as 4T1 based mouse models of lung metastasis [65]. B7x thus represents a very promising target for cancer immunotherapy.

HHLA2 (B7y/B7-H5/B7H7)

HHLA2 is another member of the B7 family that modulates T-cell function [66,67]. It is expressed on mono-

cytes and induced on CD19 positive B cells. HHLA2–Ig fusion protein bound resting and activated CD4 and CD8 T cells, as well as APC. It was shown to inhibit proliferation of CD4 and CD8 T cells in the presence of TCR signaling as well as T-cell cytokine production [66]. TMIGD2, also called CD28H or IGPR–1, is identified as one of the receptors for HHLA2 [67,68]. IGPR–1 was initially reported to be an adhesion molecule involved in angiogenesis [68]. HHLA2 expression in non-lymphoid tissues was limited to placenta, GI tract, kidney, gallbladder and breast, but its expression was more common in human tumor specimens including breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney and esophagus [68]. In a cohort of 50 patients with triple negative breast cancer, 56% of patients had HHLA2 expression

on their tumors, and high HHLA2 expression was significantly associated with regional lymph node metastasis and stage. Of interest, increase in HHLA2 expression was also due to an increase in gene copy number, and not just stimulation [68]. There is much to be discovered about HHLA2 and it represents a potential target for cancer immunotherapy.

B7-H3

B7-H3 was first identified as a molecule that binds a receptor on activated T lymphocytes [69]. Its expression was inducible on DCs and was initially thought to be costimulatory to T lymphocytes [69]. *In vivo* studies in mouse models showed that B7-H3 was an inhibitory to T lymphocytes and preferentially inhibits T helper cells type 1 response [70]. The receptor for this ligand is still unclear. It is expressed in some cancer cells and was associated with regional nodal metastasis [12,71]. Currently the majority of evidence suggests that this is a co-inhibitory ligand for T-cell response [72]. B7-H3 was found to be upregulated in graft-versus-host disease (GVHD) target organs and its absence in B7-H3^{-/-} mice resulted in augmented GVHD lethality and T-cell proliferation and function [73]. Increased B7-H3 expression in cancer specimens has been reported [74], and has been correlated to worse outcomes [60,75]. Therefore, B7-H3 is another potential target for cancer immunotherapy.

VISTA

VISTA is a recently discovered negative modulator of the immune system [76]. VISTA is primarily expressed on hematopoietic cells, including APCs and T cells [77]. It is a suppressor of CD4 and CD8 T cells. In addition, within the CD4 subset, both effector and memory T-cells are effectively suppressed. T cells cultured with soluble VISTA-Ig fusion protein show no shift in expression of CD45RA to CD45RO [77]. Another notable phenomenon is that, while it blocks proliferation, it does not induce apoptosis and thus the cells remain viable. Lastly, the suppressive effect on T cells appears to be long lasting, even after VISTA effect was removed. In addition to being immunosuppressive of T cells, it is also immunoregulatory. Under neutral conditions, VISTA-Ig is capable of suppressing naïve T-cells from forming Treg. This however was not the case when the culture conditions were changed, and in the presence of IL-2 and anti-CD28 VISTA-Ig was actually able to increase the proportion of Treg. Of note, VISTA does not appear to have any effect *in vitro* on B-cell proliferation or regulation. Its effect on cytokine production included reduced levels of IL-10, IFN- γ and TNF- α [77]. Anti-VISTA mAb exacerbate experimental auto-immune encephalomyelitis

as well as enhancing antitumor immune responses [76]. Anti-VISTA mAbs are able to increase the number of tumor specific T cells in the periphery and enhance the infiltration, proliferation and effector function of tumor infiltrating lymphocytes within the tumor microenvironment [76]. In a melanoma model, both transplantable and inducible cancers are suppressed effectively with anti-VISTA monotherapy [78]. VISTA's antitumor effect was also explored in concert with a peptide-based cancer vaccine where VISTA blockade synergistically impaired tumor growth.

CD27

CD27 is a T-cell differentiation antigen and member of the TNFR superfamily [79]. It has increased membrane expression on anti-CD3-activated T cells. Agonistic CD27 mAb resulted in enhanced proliferation of CD3 stimulated T cells. CD27 and its ligand CD70 are thought to have important effects on T-cell function [79]. Using intranasal influenza virus infection as a model system, CD27 has been shown to be a major determinant of CD8 T-cell priming at the site of infection. CD27 signaling, along with other signaling including CD28, is crucial for the generation of antigen specific CD8 T cells. Via cell survival stimulation, CD27 promotes accumulation of activated T cells thereby expanding the proportion of virus specific T cells [79]. The survival signal relies on IL-2R signaling and autocrine IL-2 production and CD27 is responsible for long-term survival of primed CD8 T cells, and hence memory. CD27 function has been extensively studied in mice and is transiently expressed during the germinal center reaction. CD27 expression is most abundant during the phase of expansion of primed B cells and is absent from memory B cells. It appears that CD27 T cells provide help to B cells to form small germinal centers. Additionally, CD27 signaling in B cells results in enhanced levels of plasma cell formation and increased IgG production. Interestingly, constitutive CD27 signaling could have alternate effects on cellular and humoral immunity [79]. Collectively, the data elucidated CD27 signaling as a determinant of germinal center kinetics. In mouse models, costimulatory effect of CD27 was necessary for anti-CD40 antitumor efficacy [80]. Antitumor efficacy was shown in a mouse model of lymphoma using an anti-CD27 mAb. Anti-CD27 demonstrated no effect in SCID mice suggesting the need for an intact adaptive immune response and that the response itself was not due to a direct effect on the lymphoma cells [80].

A fully human anti-CD27 mAb, IF5, increased survival in a mouse model of leukemia and lymphoma [81]. Its toxicity was assessed in a non-human primate model and has entered clinical development under the name

CDX-1127 (Varlilumab) and was assessed in a Phase I clinical trial [82]. The drug was well tolerated and responses included a complete response in a patient with stage IV Hodgkin's lymphoma who had previously failed stem cell transplant, chemotherapy and brentuximab-vedotin and three additional patients with stable disease.

OX40/OX40L

OX40 is a member of the TNFR superfamily and is expressed on activated CD4 and CD8 T cells as well as other lymphoid and nonlymphoid cells [83]. It is also expressed in natural killer (NK) cells, NKT cells and neutrophils. OX40L is also a member of the TNFR superfamily and its expression is inducible on APC. Nonlymphoid cells can also be induced to express OX40L which supports the role of the OX40/OX40L pathway in regulating the T-cell response. T cells themselves can express OX40L which represents an additional mechanism for T-cell response amplification. TCR signaling is sufficient to induce OX40 in activated CD4 and CD8 T cells, however this is augmented by CD28 and B7-1/B7-2 interaction and modulated by cytokines such as IL-1, IL-2 and TNF [83]. OX40L expression on the other hand is can be induced on APC upon activation by ligation of CD40 or by Toll-like receptors [83].

The OX40/OX40L pathway plays a large role in T cell expansion and survival, primarily by maintaining later proliferation and T-cell survival through the effector phase [83]. OX40 ligation can also directly inhibit naturally occurring Treg activity in mice providing another means to promoting effector T-cell proliferation and survival [83]. Foxp3 expression on naïve CD4 T cells is blocked by OX40/OX40L activity which supports a role in the suppression of naïve CD4 T-cells differentiation to become Treg. There have been conflicting reports however on the impact of OX40 signaling, which is expressed constitutively expressed on Treg, and may in fact promote Treg responses depending on the cytokine milieu [84].

The rationale for targeting OX40/OX40L signaling for cancer immunotherapy is supported by the expression of OX40 in tumor infiltrating lymphocytes. Pre-clinical models have shown that injection of agonist OX40L-Ig fusion proteins, OX40 mAb, RNA aptamers that bind OX40 and transfection of tumor cells or DCs with OX40L can all suppress tumor growth [83]. In mouse models of cancer including sarcoma, melanoma and glioma, among others, OX40 activity has been shown to decrease tumor growth [84]. The mechanism of tumor growth suppression is related to CD8 T-cell survival, and/or promotion of CD4 T-cell help for CD8 T cells. There may be an additional role via

augmentation of NK cell activity. Tumor infiltrating Tregs express high levels of OX40, and the signaling of OX40 within this environment suppressed their activity [85]. In animal models, stimulation of OX40 appears to both augment antitumor activity as well as suppress Treg activity. Human studies include a Phase I trial using anti-OX40 mAb 9B12 in patients with advanced cancers (NCT01644968 [86]) [87]. Therapy was well tolerated with no maximum tolerated dose reached. Most common grade 3 or 4 side effects included lymphopenia that was transient. Best response to therapy by response evaluation criteria in solid tumors (RECIST) only included stable disease with some tumor regression noted but less than 30% of overall tumor. One limitation of this agent was the induction of human antimouse antibodies, which precluded patients from receiving additional cycles. Nevertheless, this study provides evidence in humans that OX40 agonism can augment the immune system by stimulating CD4 and CD8 T-cell proliferation, CD8 IFN- γ production and increased antibody titers and T cell recall in response to tetanus immunization.

It seems that targeting OX40 alone may not be sufficient to elicit a robust antitumor response, and thus combination immunotherapy, particularly with antagonistic anti-CTLA-4 and anti-PD-1 antibodies, has been an area of study in preclinical models [84]. Combination therapies may stimulate complementary pathways that synergistically respond to poorly immunogenic or large tumors, which has been an area of weakness in immunotherapy. Naturally, synergism may also extend to worsening the toxicity profile of such therapy but the fact the anti-OX40 therapy was fairly well tolerated may be promising.

CD40/CD40L

CD40 and its ligand CD40L are members of the TNFR/TNF family [88]. CD40 is expressed on professional APC as well as other non-immune cells and tumors. Its ligand, CD40L is transiently expressed on T cells and other non-immune cells under inflammatory conditions [88]. Inherited lack of CD40L is responsible for x-linked hyper-IgM syndrome (H-XIM). It activates DCs and allows them to stimulate CD8 T-cell activation and proliferation [89].

Binding of CD40L to CD40 promotes CD40 clustering on the cell surface, as well as recruitment of adapter proteins known as TRAFs to the cytoplasmic domain of CD40 [88]. CD40 signaling is carried forward by different pathways which include MAPKs, NF kappa B, PLC and PI3K. Additionally, it is noted that JAK3 binds the cytoplasmic domain of CD40 and can mediate other cellular processes. CD40/CD40L can directly activate DCs [89]. This CD40/CD40L inter-

action is necessary for the maturation and survival of DCs and CD40-dependent maturation of DCs leads to sustained expansion and differentiation of antigen specific T cells. The increased life span of DCs is very important in driving cell-mediated immunity. Without the CD40 survival signal, passive apoptosis of T cell is induced [88].

CD40 signaling by B cells is required for the generation of high titers of isotype switched high affinity antibody as well as for the development of humoral immune memory [88]. The binding of CD40L on CD4 T cells to CD40 on activated B cells is an important step in initiation and progression of the humoral immune response. Once CD40 signaling is active, there are downstream effects including B-cell intracellular adhesion, sustained proliferation, differentiation and antibody isotype switching. This process is essential for memory B cells and long lived plasma cells. B-cell fate is heavily influenced by CD40 signaling [88]. This signaling via binding of CD40 and CD40L can help determine whether the maturing B cell becomes a plasmablast or seeds a germinal center. With the activated CD40 pathway, the B cell will go on to form a germinal center. The lack of CD40 signaling is sufficient to block germinal center formation. T helper cells are recruited to these germinal centers, some of which express CD40L on their cell surface, which serve to maintain the germinal center. The lack of CD40 signaling in germinal center B cells increase Fas dependent apoptosis.

CD40 is expressed in mouse and human models of melanoma, prostate and lung cancers, and carcinomas of the nasopharynx, bladder cervix and ovary [88]. Hematologic malignancies such as non-Hodgkin's lymphoma, Hodgkin's lymphoma, acute leukemias and multiple myeloma also express CD40. Anti-CD40L mAb treatment inhibits the generation of protective immune responses from potent tumor vaccines [90]. Additionally, using CD40 deficient mice, no protective antitumor immune response was induced following a protective vaccination regime. Early studies using a lymphoma model showed that agonistic anti-CD40 antibodies were able to eradicate tumor. Gene delivery of CD40L to DCs and tumor cells was sufficient to stimulate a long lasting systemic antitumor immune response in a murine model [90]. The approach of gene therapy with adenovirus expressing CD40L has been shown to be successful in colorectal carcinoma, lung carcinoma and melanoma murine models. CD40 agonism alone, however, was not sufficient for antitumor response likely due to the lack of TLR signaling.

Clinically, recombinant CD40L has been used in patients with solid tumors or non-Hodgkin's lymphoma given subcutaneously daily for 5 days in a

Phase I clinical trial [91]. Responses to therapy included 6% of patients with a partial response and one patient with a complete response. Humanized agonistic CD40 mAb include CP-870,893, SGN-40 and HCD 122. In a Phase I trial, CP-870,893 produced a partial response in 14% of patients (27% of melanoma patients). Dose-limiting toxicities were observed and included cytokine release syndrome [88]. Dacetuzumab (SGN-40) is a weak agonist and was studied in a Phase I trial in patients with refractory or recurrent B-cell non-Hodgkin's lymphoma [92]. Toxicity was shown to be acceptable and antitumor activity was seen with six objective responses, 13 patients with stable disease. The overall response rate for patients in this cohort was 18%. In a Phase II trial with this drug, 46 patients again with relapsed or refractory diffuse large B-cell lymphoma were treated and the overall response rate was 9% and disease control rate (stable disease or better) was 37% [92]. Lucatumumab (HCD122) is a fully human anti-CD40 antagonist and was tested in 28 patients with refractory or relapsed MM [93]. Responses included 12 patients with stable disease, and one patient maintaining a partial response for greater than 8 months. It was also well tolerated with a good safety profile.

CD137(4-1BB)/CD137L

CD137, also known as 4-1BB, is an induced T-cell costimulator molecule and a member of TNFR superfamily [94]. CD137 is induced on activated CD4 and CD8 T cells, NK cells and constitutively on DCs, Tregs, monocytes and myeloid cells [94,95]. Its ligand 4-1BBL is expressed on B cells, DCs, macrophages, activated T cells and endothelial cells [94-96]. Agonistic CD137 mAb stimulated the proliferation of CD4 and CD8 T cells, mainly CD8 CTL, increased cytokine production, prevent activation induced cell death [94,97] and in absence of cognate signals increases the memory T-cell expansion [98]. Large tumors in mice are eradicated with increased cytotoxic T-cell activity in poorly immunogenic Ag104A sarcoma and highly tumorigenic P815 mastocytoma using agonist CD137 mAb [99]. CD 137 agonist mAbs also increase adhesion molecules ICAM-1, VCAM 1 and E-selectin thus increasing the trafficking of activated T-cells into tumor and also prevent immune tolerance by preventing induction of CD8 CTL anergy to soluble tumor antigens [100].

Urelumab (BMS 663513) is humanized IgG4 mAb and PF 05082566 is humanized IgG2 mAbs for CD137 which are currently in clinical development [101,102]. BMS 663513 was tested in a Phase I/II trial with locally advanced and metastatic solid tumors. Initially patients with melanoma have been

enrolled but enrollment has been expanded to include renal cell and ovarian cancer patients [103]. Treatment is well tolerated and responses included three partial responses and four with stable disease [104]. A randomized Phase II trial (NCT00612664) in metastatic melanoma patients as a second-line therapy was terminated due to grade 4 hepatitis [95]. Urelumab is being tested with rituximab in B-cell non-Hodgkin's lymphoma or chronic lymphocytic leukemia as enhanced antibody-dependent cytotoxicity by rituximab was noted after activation of NK cells with CD137 [105]. Addition of anti-CD137 mAb to cetuximab improves efficiency of cetuximab in head and neck tumors as well as *KRAS* mutant and wild-type colorectal cancer, which provides further evidence for the use of immunotherapy, specifically anti-CD137, in combination with other agents [106]. PF-05082566 was tested in a Phase I study of 27 patients and is well tolerated with mostly grade 1 adverse effects with one grade 3 elevated alkaline phosphatase was noted [107].

GITR

GITR is a member of TNFR superfamily and is a co-stimulatory receptor. It has been originally discovered as up regulated in dexamethasone treated murine T-cell hybridomas [108]. It has very low expression in human T cells but constitutively expressed in human Treg [109]. Upon stimulation, naïve T cells and Treg upregulate GITR in a similar fashion to 4-1BB and OX40 suggesting their role in latter time points rather than early priming [110]. GITR-L is expressed in DCs, macrophages and B cells and is upregulated upon activation. GITR-L is also found in endothelial and activated T cells and may have role in leukocyte adhesion [110]. Its function is similar to OX40 and 4-1BB; it sends costimulatory signals inducing T-cell proliferation, effector function and protects T cells from activation induced cell death [110]. Combined anti-PD-1 blockade and GITR costimulation has potent anti-tumor activity in murine ID8 ovarian cancer model and is synergistic with chemotherapeutic agents. This combination promotes accumulation of CD4, CD8 T cells with decreased Treg and myeloid derived suppressive cells [111]. TRX518, an anti-GITR mAb, is being studied in a Phase I study in patients with stage III or stage IV melanoma or other solid tumor malignancies (NCT01239134 [86]). To circumvent autoimmune complications from immunomodulators mRNA transfected DCs are used locally to deliver anti CTLA-4 mAb and soluble GITR-L while increasing the anti-tumor immune responses [112]. Phase I clinical trial of a DC vaccine that entails intranodal injection of DCs transfected with mRNA encoding tumor antigens along with DCs transfected with mRNA encoding

soluble human GIRT-L and anti CTLA-4 mAb is in progress (NCT 01216436 [86]).

Tim-3

Tim-3 was first described in 2002 [113]. It is expressed on CD4 T cells and CD8 CTLs. Tim-3 binds several molecules including Gal-9, CEACAM1, HMGB1 as well as glycosylated molecules [114]. Binding of its ligand, Gal-9 induces cell death and thus illustrating its role as a negative regulatory molecule, with particular importance in Th1- and Tc1-driven responses. Tim-3 is expressed on all IFN- γ secreting Th1 cells as well as DCs [113]. Th1 immunity is regulated via binding of its ligand, Gal-9, directly triggering cell death. It has also been shown that Tim-3/Gal-9 binding also suppresses immune responses indirectly by expanding the population of myeloid derived suppressor cells [113]. Tim-3 has been implicated in inducing T-cell exhaustion in several scenarios including chronic viral infections such as hepatitis C and HIV, bacterial infections and cancer [113]. In murine models of colon adenocarcinoma, melanoma and mammary adenocarcinoma, Tim-3 can be co-expressed with PD-1 in tumor infiltrating CD4 and CD8 T cells. Tim-3⁺PD-1⁺ CD8 T cells were among the most impaired T cells with reduced proliferation and decreased production of IL-2, TNF and IFN- γ . *In vivo*, Tim-3 blockade in concert with PD-1 blockade produced a significantly higher antitumor effect than either one alone. Additionally, this combined blockade increased the frequency of proliferating antigen specific CD8 T cells.

LAG-3

LAG-3, also called CD223, is expressed on activated T cells, NK cells, B cells and tumor infiltrating lymphocytes [115]. It is closely related to CD4; it is a member of the immunoglobulin superfamily and its gene is located near CD4 on chromosome 12. LAG-3 is a negative regulator of T-cell activation and homeostasis [115]. LAG-3 binds to MHC class II molecules with high affinity [116]. LAG-3 cross-linking on activated human T cells induces T-cell functional unresponsiveness and inhibits TCR-induced calcium ion fluxes. Similar to CD4 and CD8, LAG-3 is considered to be a coreceptor to the CD3-TCR complex. Inhibition of cytokines, such as IL-2, is induced by LAG-3 *in vitro*. It decreases the pool of memory CD4 and CD8 T cells. It also increases the suppressive activity of Treg. For maximal suppressive activity of Treg, LAG-3 signaling is required [117]. It was not clear however, if the signal alone was sufficient. Within the tumor microenvironment LAG-3 promotes immune tolerance of the tumor by inhibiting APC and T-cell function [118].

Malignant mouse and human tissue has been shown to co-express PD-L1 and LAG-3 [119]. A significant percentage of tumor infiltrating CD4 and CD8 T cells from mouse tumor models of melanoma, colorectal adenocarcinoma and fibrosarcoma, express high levels of LAG-3 and PD-1. When using anti-LAG-3 immunotherapy, reduced growth of fibrosarcoma and colorectal adenocarcinoma is observed in some mice. This same effect is seen with anti-PD-1 monotherapy. Anti-LAG-3 produced a synergistic effect when combined with anti-PD-1 immunotherapy with 70% of the fibrosarcoma and 80% of colorectal adenocarcinoma mice noted to be tumor free [119]. This regimen, however, was shown to have no effect on the melanoma model. Interestingly, treating mice with anti-LAG-3/anti-PD-1 combined therapy is proposed to be less toxic given that LAG-3 and PD-1 co-expression is largely limited to tumor infiltrating lymphocytes.

IMP321 is a clinical grade LAG-3-Ig recombinant fusion protein that antagonizes normal LAG-3 functioning [120]. In 2009 the results of the first Phase I study involving IMP321 alone was released. Patients with advanced renal cell carcinoma were treated [121]. No significant adverse events occurred. Tumor growth reduction was seen and progression-free survival was better in those patients receiving higher doses (>6 mg). Out of eight patients treated with high dose IMP321, seven had stable disease at 3 months compared with only three of 11 in the lower dose group. Another Phase I trial of IMP321 and paclitaxel in metastatic breast cancer was conducted [122]. Patients treated with IMP321 were found to have a sustained increase in the number and activation of monocytes and DCs as well as an increase in the percentage of NK and long lived cytotoxic effector memory CD8 T cells, which correlates well with the preclinical data. Additionally 90% of patients had some clinical benefit, with only three of 12 patients progressing by 6 months. Objective tumor response rate was 50% compared with the historic control group of 25% [122]. IMP321 is well tolerated as well. There were several other Phase I trials combining IMP321 with other agents. In 18 patients with advanced pancreatic adenocarcinoma, IMP321 was combined with conventional gemcitabine and had a good safety profile [123]. Its clinical benefit was hard to evaluate likely due to suboptimal dosing of gemcitabine. IMP321 was combined with an anticancer vaccine MART-1 peptide and used in 12 patients with advanced melanoma [124]. Six patients received MART-1 alone and six in combination with IMP321. One patient experienced a partial response in the IMP321 group and none in the MART-1 alone group.

BTLA

BTLA (CD272) is a transmembrane protein that is expressed on Th1 cells as well as B cells and DCs [125–127]. BTLA, via interaction with HVEM (herpes virus entry mediator), inhibits cancer specific CD8 T cells [127]. HVEM is expressed in hematopoietic cells, including B and T cells, as well as in nonhematopoietic cells (parenchymal cells) [126]. HVEM is also expressed in melanoma cells and variety of solid tumors [126]. Hodgkin's lymphoma, B-cell non-Hodgkin's lymphoma and some T-cell non-Hodgkin's lymphomas use BTLA for immune evasion [127]. BTLA and HVEM are highly expressed in B-cell chronic lymphocytic leukemia suggesting their role in pathogenesis [126,128]. BTLA–HVEM is implicated in V γ 9V δ 2 T-cell proliferation, differentiation and has a critical role in their control of lymphogenesis [129]. T-cell responses against minor histocompatibility antigens on malignant cells play an important role for cure in hematological malignancies after allogeneic stem cell transplant via graft versus tumor effect. BTLA suppresses minor histocompatibility antigen-specific CD8 T cells after allogeneic stem cell transplant thus providing a rationale for its clinical utility in post transplantation therapies [130]. High BTLA expression is associated with shorter survival in gastric cancer [131]. BTLA expression is upregulated on cytotoxic CD8 T cells in peripheral blood of patients with hepatocellular carcinoma and correlates with disease progression and increased expression is associated with high recurrence rates [132]. Downregulation of BTLA *in vivo* can be attained by adding CpG oligonucleotides to vaccine formulation, which leads to increased resistance to BTLA/HVEM inhibition [133]. Because of the role of BTLA/HVEM in hematological as well as solid tumors, BTLA inhibition also represents a target for cancer immunotherapy.

IDO synthase

Tryptophan plays an important role in peripheral immune tolerance through its rate limiting tryptophan degradation along the kynurenine pathway, including IDO1 and tryptophan-2,3-dioxygenase (TDO). Shortage of tryptophan leads to cell cycle arrest, decreased proliferation through inactivation of mTOR pathway, while tryptophan metabolites can cause T-cell apoptosis, and induce differentiation of Tregs. Tumors can evade the immune system by hijacking tryptophan catabolizing enzymes IDO1 and TDO [134]. IDO1 protein is expressed in mature DCs in lymphoid tissues, some epithelial cells of female genital tract, placental and pulmonary endothelial cells [135]. IDO1 positive cells are scattered in human and tumoral lymphoid tissues particularly in cervical, colorectal and gastric carcinomas. It is highly present in vascular cells

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1.

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD27						
Varilumab (CDX-1127)	I	Dose escalation (0.1–10 mg/kg)	170	B-cell malignancies and solid tumors	December 2015	NCT01460134
	I	Stereotactic body radiation therapy randomized to before, after or with immunotherapy	21	Castrate resistant prostate cancer	March 2017	NCT02284971
	Ib	Two dose levels in combination with vaccine ONT-10	42	Breast and ovarian cancer	May 2016	NCT02270372
	I/II	Dose escalation of varilumab with nivolumab	190	Advanced solid tumors	December 2017	NCT02335918
	I/II	Dose escalation of varilumab with sunitinib	58	Metastatic clear cell carcinoma	March 2019	NCT02386111
	I/II	Dose escalation of varilumab with ipilimumab	100	Unresectable stage III or IV melanoma	December 2020	NCT02413827
OX40 & OX40L						
MEDI6469	Ib	Dose escalation study of drug given at different time intervals before surgery	55	Advanced head and neck squamous cancer	October 2019	NCT02274155
	I/II	In combination with radiation therapy and escalating doses of cyclophosphamide	37	Metastatic prostate cancer	October 2016	NCT01303705
	Ib/II	Alone or in combination with tremelimumab, MEDI4736 or rituximab	212	Advanced solid tumors or diffuse large B-cell lymphoma	October 2017	NCT02205333
MEDI0562	I	First in human	50	Selected solid tumors	November 2019	NCT02318394
MEDI6383	I	Alone or in combination with MEDI4736	212	Advanced solid tumors	April 2019	NCT02221960
Anti OX40 mab	I/II	Stereotactic body radiation therapy to metastatic lesions with anti OX49 antibody	40	Breast cancer patients with liver or lung metastasis	February 2023	NCT01862900
Anti OX40 mab 9B12	I	Dose escalation study	30	Advanced cancer	January 2015	NCT01644968
CD40 & CD40L						
Dacetuzumab (SGN-40)	I	Dose escalation	50	Relapsed or refractory non-Hodgkin's lymphoma	March 2007	NCT00103779
	I	Dose escalation	44	Recurrent or refractory multiple myeloma	November 2007	NCT00079716
	I	Dose escalation in combination with lenalidomide and dexamethasone	36	Relapsed multiple myeloma	February 2010	NCT00525447
	Ib	In combination with bortezomib	18	Multiple myeloma	April 2010	NCT00664898

Data taken from [86].

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1 (cont.).

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD40 & CD40L (cont.)						
CP-870,893	Ib	In combination with rituximab	22	Follicular and marginal zone lymphoma	November 2010	NCT00556699
	Ib	In combination with rituximab and gemcitabine	33	Diffuse large B-cell lymphoma	February 2010	NCT00655837
	II	Monotherapy	46	Relapsed diffuse large B-cell lymphoma	January 2009	NCT00435916
	I/II	Dose escalation	12	Chronic lymphocytic leukemia	October 2006	NCT00283101
	I	Dose escalation in combination with tremelimumab	32	Metastatic malignant melanoma	October 2014	NCT01103635
Lucatumumab (HCD122)	I	Dose escalation study in combination with gemcitabine	22	Unresectable pancreatic cancer	January 2011	NCT00711191
	I	Dose escalation	29	Advanced solid tumors	February 2006	NCT02225002
	I	Dose escalation in combination with carboplatin and paclitaxel	34	Metastatic solid tumors	July 2009	NCT00607048
	I	Dose escalation	28	Multiple myeloma	May 2009	NCT00231166
SEA-CD40	1b	In combination with bendamustine	?	Follicular lymphoma	May 2012	NCT01275209
	Ia/II	Dose escalation	111	Lymphomas	February 2013	NCT00670592
APX005M	I	Dose escalation	144	Advanced solid tumors	March 2019	NCT02376699
Recombinant CD40L	I	Dose escalation	32	Advanced solid tumors	December 2017	NCT02482168
Chi Lob 7/4	I	Dose escalation in combination with recombinant FLT3 ligand	?	Metastatic melanoma or kidney cancer	Completed	NCT00020540
ADC-1013	I	Dose escalation	29	Advanced cancer	October 2014	NCT01561911
AdCD40L	I/IIa	Alone or in combination with low dose cyclophosphamide with or without radiation	40	Advanced solid tumors	October 2017	NCT02379741
RO7009789	I/IIa	Alone or in combination with low dose cyclophosphamide with or without radiation	30	Advanced cancer	December 2017	NCT01455259
RO7009789	Ib	Dose escalation in combination with MPDL3280A	160	Advanced solid tumors	December 2017	NCT02304393
Data taken from [86].						

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1 (cont.).						
Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD137 & CD137L						
Urelumab (BMS663513)	I	Dose escalation	122	Advanced cancers	March 2019	NCT01471210
	Ib	In combination with cetuximab	104	Advanced colorectal carcinoma or head and neck squamous cell carcinoma	January 2017	NCT02110082
	Ib	Dose escalation in combination with rituximab	74	B-cell non-Hodgkin's lymphoma	December 2018	NCT01775631
	I/II	Dose escalation in combination with nivolumab	200	Advanced cancers	December 2018	NCT02253992
	II	In combination with rituximab	24	Advanced chronic lymphocytic leukemia	July 2020	NCT02420938
PF-05082566	I	Dose escalation and in combination with rituximab in B-cell lymphomas	126	Advanced solid tumors or B-cell non-Hodgkin's lymphoma	December 2017	NCT01307267
	Ib	Dose escalation in combination with mogamulizumab	70	Advanced cancers	June 2018	NCT02444793
	Ib	Dose escalation in combination with pembrolizumab	45	Advanced solid tumors	May 2017	NCT02179918
GITR						
TRX518	I	Dose escalation	40	Advanced solid tumors	December 2015	NCT01239134
LAG-3						
IMP321	I	Dose escalation	24	Advanced renal cell carcinoma	October 2008	NCT00351949
	I	Dose escalation in combination with paclitaxel	33	Metastatic breast cancer	January 2010	NCT00349934
	I	Dose escalation in combination with gemcitabine	18	Advanced pancreatic cancer	September 2012	NCT00732082
BMS-986016	I	Dose escalation in combination with nivolumab	198	Advanced solid tumors	May 2018	NCT01968109
	I	Dose escalation	88	Chronic lymphocytic leukemia, lymphomas and multiple myeloma	June 2018	NCT02061761
LAG525	I/II	Dose escalation in combination with PDR001	240	Advanced solid tumors	November 2017	NCT02460224
Data taken from [86].						

in renal cell cancer [135]. Expression of IDO1 is highest in endometrial and cervical cancers followed by kidney and lung [135]. IDO1 expression is associated with aggressive phenotype, poor prognosis, shorter survival and increased Tregs [136]. IDO1 inhibitors were shown to exhibit antitumor effect in mouse models alone as well as have synergistic effects with a variety of chemotherapeutic agents in preclinical models [137–140]. Indoximod is an IDO inhibitor that is being studied in a Phase II trial with taxane chemotherapy in metastatic breast cancer (NCT 01792050 [86]). IDO inhibitors can provide a synergistic effect when administered with vaccines and immunotherapy; IDO induction in response to inflammation can attenuate antitumor vaccine [139]. This provided the rationale for a randomized Phase II study of Indoximod with Sipuleucel-T (Provenge®) in the treatment of patients with asymptomatic or minimally symptomatic metastatic castration resistant prostate cancer (NCT01560923 [86]). Other studies include a Phase I study of NLG-919, an IDO inhibitor, for patients with advanced solid tumor malignancies and a Phase I/II trial of the indoximod in combination with ipilimumab for the treatment of unresectable advanced stage melanoma [141,142].

Conclusion & future perspective

Our understanding of immune dysfunction in cancer continues to develop. Growth and progression of cancer are made possible by the ability of the malignant cells to manipulate immune checkpoint pathways that prevent immune overstimulation. Significant progress has been made in the field with mAb against CTLA-4 and now PD-1 in clinical use. This gives credence to the efforts aimed at developing agents targeting other immune modulatory pathways. The success PD-1 inhibitors may very well translate to PD-L1 inhibitors being successful in the clinic. PD-L1 is widely expressed in a variety of tumors and PD-L1 inhibitors have shown impressive, albeit preliminary, results in areas of unmet need such as urothelial bladder cancer which has been resistant to conventional chemotherapy [34,50]. Other B7 family members are emerging as clinical target in preclinical models such as B7x, HHLA2 and B7-H3. Other immunoglobulin superfamily members such as Tim-3 and VISTA also show promise in preclinical models. LAG-3 targeting molecules have also reached clinical trials with good safety profile and evidence of antitumor efficacy.

TNFR superfamily member also hold promise in cancer immunotherapy. Preclinical and clinical data support targeting CD27 where a durable response was noted as well [81,82]. mAb targeting CD40L have reached clinical trials although the clinical experience in the CD40/CD40L pathway illustrates some of the

caution needed with immune disinhibition, particularly in unwanted side effects such as cytokine release syndrome [88,92,93]. OX40, CD137 and GITR antibodies have also reached clinical trials providing further evidence that TNFR family members are viable targets for cancer immunotherapy [101–104,112]. **Table 1** is the summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1.

The redundancy in inhibitory immune checkpoint molecules sheds light on the complexity of immune regulation. It also allows for the targeting of these molecules in succession or in combination. A new pathway may be targeted after the efficacy of an earlier immunotherapy is exhausted as evidenced by the success of PD-1 targeting mAbs in ipilimumab-refractory tumors indicating that tumors may recruit additional pathways when one is blocked, or several pathways may be in fact be recruited simultaneously by tumor cells [24,25,31]. Efficacy of ipilimumab in combination with nivolumab also illustrates that tumors may recruit more than one inhibitory pathway at the same time [143].

Another interesting dilemma that emerges as cancer immunotherapy gains momentum is integrating these novel agents with current regimens that mainly consist of conventional cytotoxic chemotherapy. On one hand, conventional wisdom may lead us to believe that chemotherapy may be synergistic. The immunosuppressive effect of chemotherapy however can have unpredictable effects on the immune system. Cyclophosphamide has been used as a Treg depleting agents in preclinical models with some success [144,145]. Combination regimens have also reached clinical trials [34,122,123]. Combination of immune checkpoint inhibitors have also been studied with traditional immunotherapies or targeted therapies such as rituximab and cetuximab where they show efficacy and tolerability [105,106,146]. With evidence pointing to better outcomes in second line setting or even first line setting of immunotherapies, cancer immunotherapy will play a vital role in the future of oncology [24,31,147].

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Executive summary**Cancer immunotherapy targets in clinical trials**

- PD L-1 blockade can restore cytotoxic T-lymphocyte antitumor activity.
- CD27 is a hematologic malignancy marker that has been targeted in Phase I trials.
- Lag-3 is a co-inhibitory molecule and blocking antibodies have reached clinical trials.
- CD40, CD137 and GITR agonism may play a role in cancer immunotherapy and have reached Phase I and Phase II trials.
- Indoleamine 2,3-dioxygenase is not a cell-surface protein and inhibitors have reached clinical trials.

Emerging targets for cancer immunotherapy still in preclinical study

- B7x, HHLA 2, B7-H3 are B7 family members that have inhibitory role and are expressed by tumor cells.
- Tim-3, VISTA and BTLA blockade are emerging targets in preclinical models.
- Agonism of stimulatory molecules OX40 and OX40L may also play a role in cancer immunotherapy.

Conclusion

- T-cell disinhibition is now a clinically effective approach.
- Targeting of several pathways may be done in succession or in concert.
- Immunotherapy can be more effective than chemotherapy especially in the second-line setting.

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