New checkpoints in cancer immunotherapy

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Summary
Immune responses must be fine-tuned to allow effective clearance of invading pathogens, while maintain tolerance to self-antigens. T cells are the major effector cells for fighting and killing tumor cells. Immune checkpoints play a pivotal role in T cell activation, and determine the functional outcome of T cell receptor (TCR) signaling. The blockade of immune checkpoints CTLA-4 and PD-1 has already been one of the most successful cancer immunotherapies. In this review, we will focus on three novel inhibitory B7 family checkpoint molecules, B7-H3, B7S1 and VISTA. The aim of this article is to summarize their expressions in tumors as well as their roles in controlling and suppressing T cell immune responses and anti-tumor immunity. These pathways may be explored in future cancer immunotherapy.

KEYWORDS
B7-H3, B7S1, immune checkpoints, immunotherapy, VISTA

1 INTRODUCTION

Immune responses must be fine-tuned to allow effective clearance of invading pathogens, while maintain tolerance to self-antigens. T cells play a central role in cell-mediated immunity against cancers. The activation of T cells needs two signals, signal one is antigen-specific signal to the T-cell receptor (TCR) by specific antigens on major histocompatibility (MHC) molecules expressed on antigen-presenting cells (APCs), and signal two is costimulatory signal provided by B7 and other molecules. Co-inhibitory molecules function to restrain T-cell function. The balance between co-stimulatory and co-inhibitory signals determines T cell function or tolerance. third

Cancer cells exploit several mechanisms to escape from immune attack. Firstly, malignant cells coordinate an immunosuppressive micro-environment via secreting pro-inflammatory cytokines to recruit Tregs and myeloid-derived suppressor cells (MDSCs). Secondly, the myriad of genetic and epigenetic alternations that are characteristic of all cancers provide a diverse set of neo-antigens that the immune system can distinguish tumor cells from their normal counterparts. However, to escape immune-mediated elimination, cancer cells may lose their antigenicity, as a result of loss of MHC expression or dysregulation of antigen processing machinery. Thirdly, tumors may escape immune elimination by decreasing T cell-mediated killing. Expression of programmed cell death receptor ligand 1 (PD-L1) on tumor cells reduces their immunogenicity in human and murine cancers. PD-L1, one of inhibitory immune checkpoint molecules, is mainly expressed on immune cells as well as on cancer cells and functions as a co-inhibitory molecule for T cell activation. Its engagement on PD-1 was reported to cause impaired cytokine production and loss of cytotoxicity of activated T cells. Immune checkpoint blockade has recently become one successful cancer immunotherapies. Ipilimumab (MDX-010, Yervoy; Bristol-Myers Squibb, Princeton, NJ, USA), a fully human monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), was approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic melanoma in 2011 on the basis of survival benefit. The clinical success and US FDA approval of ipilimumab have brought tumor immunology to the forefront of cancer treatment. On September 4th, 2014, FDA granted accelerated approval to pembrolizumab, a monoclonal antibody formerly known as lambrolizumab that specifically targets PD-1, for use in patients with advanced or unresectable melanoma who fail to respond to other therapies. On December 22, 2014, the FDA approved nivolumab (Opdivo; Bristol-Myers Squibb, another antibody targets PD-1) for the treatment of patients with unresectable or metastatic melanoma and disease
progression after ipilimumab therapy and, if the patient is positive for a BRAF V600 mutation, after treatment with a BRAF inhibitor. This agent was approved under the accelerated approval program based on the surrogate end points, overall response rate, and duration of response. In 2015, FDA approved nivolumab for the treatment of squamous lung cancer. In 2016, nivolumab was also approved for treatment of Hodgkin's lymphoma and FDA approved PD-L1 inhibitor (Atezolizumab; Roche, Basel, Switzerland) to treat the patients with bladder carcinoma.

The immune checkpoint blockers represent a new, efficient alternative to the standard management of advanced cancers. However, the patient response rates was still low in most cases, suggesting targeting other or combinatorial targeting of immune checkpoints. The B7 family now comprises at least 10 members, which are CD80 (also known as B7.1), CD86 (also known as B7.2), B7-H1 (also known as PD-L1 or CD274), B7-DC (also known as PD-L2 or CD273), B7-H2 (also known as ICOSL), B7-H3 (also known as CD276), B7S1 (also known as B7-H4, B7x, or Vtcn1), B7-H5 (also known as VISTA, GI24 or PD-1H), B7-H6 and B7-H7 (also known as HHLA2). In this review, we will focus on three inhibitory B7 family checkpoint molecules, B7-H3, B7S1 and VISTA, as their study is emerging. The aim of this article is to address the biology of those three inhibitory molecules as well as their roles in controlling and suppressing T cell immune responses and anti-tumor immunity. These knowledge may help pave roads for novel checkpoint therapies.

2 | B7-H3 IN CANCER IMMUNOTHERAPY

2.1 | B7-H3 biology

B7-H3, also known CD276, is a type I membrane protein with its sequence similarity to the extracellular domain of other B7 family members. The B7-H3 gene is located on chromosome 15 in humans, while on chromosome 9 in mice. The gene consists of ten exons, among which exons 4 to 7 encode the extracellular IgV-IgC domains. Murine B7-H3 contains one IgV and one IgC domains, while human B7-H3 has an alternate isoform containing a tandem repeat of IgV and IgC domains (VCVC) and this isoform is the most common form expressed in multiple tissues. No functional difference has been observed between those two isoforms. B7-H3 mRNA is not detectable in peripheral blood mononuclear cells (PBMCs), although it is found in various normal tissues and in several tumor cell lines. Expression of B7-H3 protein, however, can be induced on dendritic cells (DCs) and monocytes by inflammatory cytokines, such as IFNγ, and a combination of phorbol myristate acetate (PMA) and ionomycin.

B7-H3 have different patterns of fucosylation. Recently, Chen et al. compared glycosylation patterns of B7-H3, before and after treatment with peptide-N-glycosidase and observed profound difference between a normal gingival epithelial cell line (SG) and a gingival squamous cell carcinoma cell line (Ca9-22). The overexpressed B7-H3 in Ca9-22 cells showed that a more complex and diverse pattern of glycosylation carrying more fucose residues, which can better interact with DC-SIGN and Langerin on immune cells compared to that from normal cells, indicating that the glycans on B7-H3 may also play an important role in the immune system. The receptor(s) for B7-H3 remains unknown. Both murine and human B7-H3 fusion proteins fail to bind to resting T cells. In contrast, this fusion protein can recognize T cells stimulated with PHA or ConA, indicating that B7-H3 receptor is induced upon T cell activation.

2.2 | B7-H3 expression in tumor cells

Recent studies found aberrant B7-H3 expression on a wide variety of cancers, including stomach, lung, prostate, kidney, ovary, endometrium, colorectum, pancreas, liver, oral, bladder and breast. In non-small cell lung carcinoma (NSCLC) patients, both surface and soluble B7-H3 act as a poor prognosis marker as described in Table 1. These data suggest the contribution of B7-H3 to immune dysfunction and tumor progression. Moreover, the expression of B7-H3 in/on sarcoma and carcinoma of prostate, mouth, pancreas, liver, kidney, breast, colorectum, ovary, head and neck, skin, esophageal and endometrium was also correlated with advanced disease and poor outcome (Table 1). Nuclear B7-H3 expression in colon cancerous cells was independently and significantly associated with reduced metastasis-free, disease-specific and overall survival, indicating that nuclear B7-H3 might be involved in colon cancer progression and metastasis and could become a useful prognostic marker in colon cancer. However, the same group in 2014 demonstrated that nuclear B7-H3 was not a strong prognostic biomarker in an independent colorectal cancer cohort. The discrepancy might be related to the use of single-core tissue microarrays for detection of the heterogeneously expressed B7-H3.

In contrast to the above findings, tumor-associated B7-H3 expression in patients with gastric carcinoma was positively related with the survival time, as shown in Table 1. Loos et al. also showed that expression of B7-H3 was associated with prolonged survival in human pancreatic cancer. The opposite results about the association of B7-H3 expression with clinical outcome could be due to different patterns of fucosylation and different isoform expression in cancer cells, since alternations in glycosylation play a role in a diverse set of biological phenomena such as tumor cell metastasis, intracellular communication and inflammation.

The cellular mechanisms that regulate B7-H3 expression in cancer cells are poorly understood. One report showed that B7-H3 in human renal cell carcinoma (RCC) was a direct target of miRNA-187. Overexpression of miR-187 decreased B7-H3 mRNA expression. Therefore, downregulation of miRNA-187 might play roles in RCC progression via deregulating cancer-related gene B7-H3 in RCC. In melanoma, miRNA-29c was shown to directly target B7-H3 3′ untranslated region (3′UTR), miRNA-29c has tumor suppressor function in that it is significantly downregulated during melanoma progression. The expression level of miRNA-29c was found inversely correlated with B7-H3 protein level. Therefore, transfection of miR-29c mimic significantly reduced B7-H3 expression. In addition, IL-10 regulates B7-H3 expression level. The discrepancy might be related to the use of single-core tissue microarrays for detection of the heterogeneously expressed B7-H3.
<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Year</th>
<th>% of B7-H3 expression in sample</th>
<th>Expression isoform</th>
<th>Clinical significance</th>
<th>Bad/good</th>
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<td>Non-small cell lung cancer</td>
<td>2006</td>
<td>37</td>
<td>Membrane and cytoplasm</td>
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<td>Induce tumor evading from host immune surveillance</td>
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<td>93</td>
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<td>Associated with disease spread and poor outcome</td>
<td>Bad</td>
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<td>Associated with cancer progression</td>
<td>Bad</td>
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<td>Correlates with biochemical failure and clinical relapse</td>
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<td>Membrane</td>
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<td>Associated with low histo-differentiation, lymph node migration and TNM stages</td>
<td>Bad</td>
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<td>Pancreatic cancer</td>
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<td>88</td>
<td>Membrane and cytoplasm</td>
<td>Co-expression of B7-H3 and B7-H4 is associated with poor prognosis</td>
<td>Bad</td>
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<td>Pancreatic cancer</td>
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<td>88</td>
<td>Membrane and cytoplasm</td>
<td>Correlates with better postoperative survival</td>
<td>Good</td>
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<td>Associated with cancer recurrence and patient death</td>
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<td>Correlated with cancer progression</td>
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<td>mRNA</td>
<td>Associated with extent of regional nodal metastasis</td>
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<td>Membrane</td>
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<td>72</td>
<td>Membrane, cytoplasm, and nuclei</td>
<td>Positive association between nuclear B7-H3 expression and vascular invasion in colon cancer patients</td>
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<td>Associated with increased recurrence and mortality</td>
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<td>Membrane and cytoplasm</td>
<td>Associated with distant metastasis</td>
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<td>Melanoma</td>
<td>2013</td>
<td>-</td>
<td>-</td>
<td>Related to migration and invasion</td>
<td>Bad</td>
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<td>90</td>
<td>Membrane and cytoplasm</td>
<td>Associated with tumor progression and metastasis</td>
<td>Bad</td>
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<td>Associated with tumor invasion depth</td>
<td>Bad</td>
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<td>Membrane and cytoplasm</td>
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<td>Gastric cancer</td>
<td>2007</td>
<td>39.50</td>
<td>-</td>
<td>Positively related to patient survival</td>
<td>Good</td>
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</table>
expression in cancerous cells. Tumor-associated macrophage (TAM)-derived IL-10 was shown to upregulate B7-H3 expression on murine lung cancerous cells.

2.3 | The role of B7-H3 in T cell responses

When first discovered, B7-H3 was reported to serve a co-stimulating effect on the proliferation of both CD4+ and CD8+ T cells, enhances the induction of cytotoxic T cells and selectively stimulates IFNγ production in the presence of TCR signaling, suggesting that B7-H3 is a costimulatory molecule for T cell activation,9 which was also confirmed by Zhang et al.68 They showed that the GST/hB7-H3 protein produced in bacteria had modest biological activities to induce T cell proliferation and enhance IFNγ as well as IL-10 secretion.

In contrast, other groups have proposed the opposite function of B7-H3. Soluble murine and human B7-H3 provide inhibitory signals to T cells.49-52 B7-H3-deficient mice developed experimental autoimmune encephalomyelitis (EAE) several days earlier than their wildtype littermates, and accumulated higher concentrations of autoantibodies.48 Moreover, B7-H3 negatively regulates Th2-mediated immune responses during the induction phase of murine experimental allergic conjunctivitis, since the injection of anti-B7-H3 Ab significantly augmented conjunctival eosinophil numbers and IL-5 production by splenocytes.51 Recently, Veenstra et al.53 provided strong evidence that B7-H3 might play an inhibitory role on T-cell proliferation. The inhibition may govern through nuclear factor of activated T cells (NFAT), NF-κB, and AP-1 factors, three major signaling pathways through which TCR regulates gene transcription.54

2.4 | The immunological role of B7-H3 in tumor immunity

B7-H3 functions in cancers are starting to be described. In EL4 lymphoma gene therapy, Sun et al.35 found that intratumoral injection of a mouse B7-H3 pCDNA3 expression plasmid led to complete regression of 50% tumors, or significantly slowed tumor growth. In colon cancer gene therapy, intratumoral injection of an adenovirus-expressing mouse B7-H3 (Ad-B7-H3-GFP) resulted in a reduction in tumor size compared to the controls. Moreover, Ad-B7-H3-GFP treated animals showed significantly higher frequencies of tumor-specific IFNγ-producing CD8+ T cells and higher IL-12 levels than control animals,56,57 as shown in Figure 1. Luo et al.58 also reported that expression of B7-H3 by transfection of the mouse P815 tumor line enhanced its immunogenicity, leading to the regression of tumors and amplification of a tumor-specific CD8+ CTL response in syngeneic mice. Similarly, B7-H3-transiently transfected melanoma cells could enhance induction of human primary CD8+ cytotoxic T cells. In addition, genetically modified oral squamous cell carcinoma (OSCC) cells encoding B7-H3 enhanced the induction of tumor specific immune response.59 By means of a highly physiologic, spontaneous prostate model in mice, Kreymborg et al.60 found that mice lacking B7-H3 showed dramatically increased tumor sizes. This is not due to non-immunologic tumor cell-autonomous function of B7-H3, but a cell-extrinsic mode of action for B7-H3 during tumor development. All these reports indicate that B7-H3 may exert an anti-tumor effect on tumor regression for multiple cancers and play a role in regulating cell-mediated immune responses against cancer.

In contrast, there are several papers indicate that B7-H3 exerts pro-tumor effect on tumor progression. In a murine lung cancer model, a fraction of macrophages in tumor stroma expressed surface B7-H3 and TAM-derived IL-10 could stimulate membrane B7-H3 expression on cancerous cells. Blockade of B7-H3 by incubation of either tumor macrophages or cancerous cells with B7-H3 monoclonal antibody (mAb) could markedly enhance anti-tumor immunity.61 In the patients with NSCLC, tumor-residing DCs produced less IL-12 and more IL-10 than lung-residing DCs. Those tumor-residing DCs expressed higher levels of surface B7-H3 and those B7-H3 molecules play a crucial role in suppressing T cell-mediated anti-tumor immunity.62 In human breast cancer tissues, Liu et al.35 also found that high B7-H3 expression played an important role in tumor progression and invasiveness. Moreover, this expression appeared to be correlated with the ability of B7-H3 to promote IL-10 secretion. B7-H3 can also exert its effect...
on the induction of TAM M2-polarization. In the context of hepatocellular carcinoma (HCC), B7-H3 promoted PMA-induced THP-1 cells to differentiate into the M2 phenotype, with increased expression of arginase 1 (Arg1), vascular endothelial cell growth factor (VEGF) and macrophage-derived chemokine (CCL22) mRNA following coculture with HepG2 cells. However, this phenomenon was abrogated through knockdown of B7-H3 by RNA interference or by blocking STAT3 signaling pathway. These results suggest that the B7-H3-mediated STAT3 signaling pathway is an important mechanism for inducing M2-type polarization of TAMs, which accelerates HCC development.

Thus, the role of B7-H3 in anti-tumor immunity has been controversial, too, with conflicting costimulatory and coinhibitory functions. It might be due to different patterns of B7-H3 fucosylation or different isoform expression in cancer cells. Moreover, B7-H3 might have more than one receptor on T cells.

### 2.5 The non-immunological role of B7-H3 in tumor immunity

Besides its immunological role of B7-H3 in tumor immunity, it also exerts a non-immunological function in tumor immunity. B7-H3 knockdown by RNA interference in pancreatic cancer decreased tumor cell migration and transwell invasion up to 50% in vitro, as described in Figure 1. In the context of oral carcinoma, esophageal carcinoma, breast cancer as well as acute monocytic leukemia, knockdown of B7-H3 suppressed tumor cell proliferation, while restoration of B7-H3 expression enhanced tumor growth. In addition, silencing of B7-H3, through lentivirus-mediated delivery of stable short hairpin RNA, was observed to increase the sensitivity of the human pancreatic carcinoma cell line Panc8988 to gemcitabine as a result of enhanced drug-induced apoptosis, indicating that B7-H3 induces gemcitabine resistance in pancreatic carcinoma cells. In HCC, B7-H3 was able to stimulate the wound healing, metastasis and invasion of hepatoma cells by targeting epithelial-to-mesenchymal transition (EMT) via JAK2/Stat3/Slug signaling pathway, while no obvious influence on cell growth and apoptosis. By using one mAb 376.96, which recognizes a B7-H3 epitope expressed on ovarian carcinoma cells (OCCs), Fauci et al. showed that mAb 376.96 inhibited the in vitro growth of chemosensitive and chemoresistant OCCs and reduced the content of cancer initiating cells when used with Sunitinib. B7-H3 could be a promising therapeutic target for anti-metastasis therapy.

### 2.6 Antibodies targeting B7-H3

Modak et al. immunized BALB/c mice with human neuroblastoma and generated mAb (8H9) in 2001 (Table 2). mAb 8H9 is broadly reactive with human solid tumors and displays favorable tumor uptake for both sarcoma and brain tumors in xenograft models. When conjugated to cobra venom factor, it induces efficient complement-mediated tumor lysis. In early phase human clinical trials, 131I-8H9 prolongs survival among high-risk patients with solid tumors suffering from central nervous system (CNS) metastasis. However, it is until
2009 that they described the identification of 4lg-B7-H3 as the target for mAb 8H9.\textsuperscript{79} It has been tested in several clinical trials and is a promising target for radioimmunotherapy of leptomeningeal metastases (clinicaltrials.gov identifier NCT00089245), diffuse intrinsinc pontine glioma (NCT01502917), and peritoneal metastases (NCT01099644).\textsuperscript{75}

In 2012, Loo et al.\textsuperscript{76} generated one mAb against B7-H3 (MGA271) and evaluated its anti-tumor activity. The data showed that MGA271 mediates potent antibody-dependent cellular cytotoxicity against a broad range of tumor cell types. Furthermore, in human CD16A-bearing transgenic mice, MGA271 exhibited potential anti-tumor activity in B7-H3-expressing xenograft models of renal cell and bladder carcinoma. Toxicology studies carried out in cynomolgus monkeys revealed no significant test article-related safety findings. Recently, MGA271 is being tested in several phase I/II clinical trials. NCT01391143 started in 2011 in order to evaluate the safety of MGA271 when given by intravenous (IV) infusion to patients with refractory cancer. NCT02381314 is another ongoing clinical trial and its purpose is to evaluate the safety of MGA271 in combination with ipilimumab when given to patients with B7-H3-expressing melanoma, squamous cell carcinoma of the head and neck (SCCHN), NSCLC and other B7H3 expressing cancers. The safety of MGA271 in combination with pembrolizumab is also in a clinical trial (NCT02475213), which is given to patients with B7-H3-expressing melanoma, SCCHN, NSCLC, and other B7-H3 expressing cancers.

Another mAb 376.96,\textsuperscript{77} which recognizes a B7-H3 epitope expressed on OCCs, was reported in 2014.\textsuperscript{49} The mAb-376.96-defined-B7-H3-epitope was found to be expressed on both differentiated ovarian cancer cells and cancer initiating cells (CICs) in chemosensitive and chemoresistant ovarian cancer cell lines. However, it is not evaluated in clinical trials. In 2016, one novel anti-CD3 x anti-B7-H3 bispecific antibody (B7-H3Bi-Ab) was reported by Ma et al.\textsuperscript{78} They tested the specific cytotoxic activity of activated T cell (ATC) armed with this bispecific antibody against tumor cell in vitro and in vivo. In contrast with unarmed ATC, an increase in cytotoxic activity of B7-H3Bi-armed ATC against tumor cells was observed at effector/target (E/T) ratios of 5:1, 10:1, and 20:1. Moreover, B7-H3Bi-armed ATC secreted more IFNγ, TNFα and IL-2 than unarmed ATC. Infusion of B7-H3Bi-armed ATC inhibited tumor growth in severe combined immunodeficiency (SCID) xenograft models, along with a significant survival benefit. Now this bispecific antibody is in a phase I clinical trial (NCT02628535).

All these data about mAb against B7-H3 show that B7-H3 is a promising target for cancer immunotherapy for B7-H3-expressing tumors although its function as immune modulator still remains to be explored.

3 | B7S1 IN CANCER IMMUNOTHERAPY

3.1 | B7S1 biology

B7S1, also known as B7-H4, B7x, or Vtnc1, is another B7 family member discover by three laboratories almost simultaneously.\textsuperscript{79–81} The genomic DNA of human B7S1 is mapped on chromosome 1 comprised of six exons and five introns spanning 66 kb, of which exon 6 is used for alternative splicing to generate two different transcripts.\textsuperscript{82} Similar genomic structure is also found on mouse on chromosome 3. B7S1 mRNA is widely distributed in mouse and human peripheral tissues, while cell surface expression of B7S1 protein is limited and shows an inducible pattern in hematopoietic cells.\textsuperscript{80} Fresh isolated human T cells, B cells, monocytes and DCs do not express B7S1 on cell surface. In contrast, B7S1 surface expression is induced on those human immune cell subsets by IFNγ, LPS, TNFα and PMA/ionomycin. Interestingly, Treg cells are also one of the stimuli for the induction of surface B7S1 expression. Kryczek et al.\textsuperscript{83} showed that Treg cells, but not conventional T cells, induced APCs to produce high levels of IL-10. These APC-derived, rather than Treg cell-derived, IL-10 is responsible for surface B7S1 induction on APCs in an autocrine loop, leading to APC immunosuppression. Therefore, Treg cells convey suppressive activity to APCs by stimulating B7S1 expression through IL-10.

The receptor for B7S1 is yet unidentified. The initial three B7S1 papers in 2003 all used either mouse or human B7S1 fusion proteins to stain both resting and activated T cells. It is agreed that putative receptor is expressed on activated T cells. In 2013, Jeon et al.\textsuperscript{84} showed that in addition to activated T cells, MDSCs also express B7S1 receptor. Moreover, B7S1 binds to MDSCs more potently than activated T cells, indicating that these two cell types may express different B7S1 receptors or the same receptor at the highly distinct levels.\textsuperscript{84}

3.2 | B7S1 expression in tumor cells

Recent studies found that aberrant B7S1 was expressed in a wide variety of tumors, including gastric carcinoma,\textsuperscript{85} RCC,\textsuperscript{86} ovarian carcinoma,\textsuperscript{87,88} lung carcinoma,\textsuperscript{89} uterus cancer,\textsuperscript{90} breast carcinoma,\textsuperscript{87,88,91} prostate carcinoma,\textsuperscript{15} glioma,\textsuperscript{92} pancreatic carcinoma,\textsuperscript{30} bladder carcinoma,\textsuperscript{93} cervical carcinoma\textsuperscript{94} and melanoma.\textsuperscript{95}

In 2006, Krambeck et al.\textsuperscript{96} found the expression of B7S1 in tumor cells from RCC patients and the association of intra-tumor B7S1 expression with poor prognosis. Patients with tumors expressing B7S1 were three times more likely to die from RCC compared with patients with tumor lacking B7S1. Additionally, 81.5% of the specimens exhibited tumor vasculature endothelial B7S1 expression, whereas only 6.5% of normal adjacent renal tissue vessels exhibited endothelial B7S1 expression. In addition to RCC, B7S1 expression by other tumor cells, including NSCLC,\textsuperscript{14} prostate,\textsuperscript{15} stomach,\textsuperscript{85,97,98} esophageal,\textsuperscript{99} cervix,\textsuperscript{94} kidney,\textsuperscript{100} skin,\textsuperscript{95} lung,\textsuperscript{83} pancreatic,\textsuperscript{102} thyroid,\textsuperscript{103} bladder,\textsuperscript{93} colorectal,\textsuperscript{104} osteosarcoma\textsuperscript{105} and ovarian,\textsuperscript{106} directly correlating with disease stage, progression or recurrence, and inversely with tumor-infiltrating lymphocytes and patient survival. These findings implied that B7S1 is a potential prognostic marker for patients with malignant tumors.

Median concentrations of soluble B7S1 (sB7S1) in the serum from patients with ovarian cancer,\textsuperscript{86} kidney cancer,\textsuperscript{107} gastric cancer,\textsuperscript{108} osteosarcoma\textsuperscript{105} and liver cancer\textsuperscript{109,110} were significantly higher than those in healthy volunteers. Median levels of sB7S1 were significantly correlated with tumor size, lymph node metastasis, the depth of tumor invasion and tumor-node-metastasis classification. Interestingly, the overall survival rate was significantly lower in patients with high sB7S1
levels when compared with low sB7S1 levels. The risk of death was significantly higher in patients with high sB7S1 levels than those with low sB7S1 levels, implying that the expression of sB7S1 is a valuable blood biomarker for predicting the progression and prognosis of patients in a wide variety of cancers.

The factors that induce tumor cell-associated B7S1 expression remain to be fully elucidated (Figure 2). In the context of ovarian cancer, IL-6 and IL-10 are detected in a high concentration in the tumor microenvironment (TME).\textsuperscript{111} Interestingly, these two cytokines stimulated TAM to express surface B7S1. The similar regulation is also detected in glioma.\textsuperscript{112} Glioma-initiating CD133\textsuperscript{+} cells produced IL-6 and IL-10 in TME. IL6-activated STAT3 bound to the promoter of B7S1 gene and enhanced B7S1 expression on TAMs. In addition, culture of lung cell carcinoma (LCC) cell line with TAM-derived IL-10 or TNF\textalpha resulted in the surface B7S1 expression on tumor cells.\textsuperscript{89} Hypoxia is another factor that upregulates B7S1 transcription in primary CD133\textsuperscript{+} multiple myeloma cells and cancer cell lines.\textsuperscript{113} Hypoxia-inducible factor-1alpha (HIF-1alpha) can bind to proximal hypoxia-response element sites within the B7S1 gene promoter. Discovery of the factors that induce B7S1 expression is of great significance, as knockdown or pharmacological inhibition of those factors results in downregulation of B7S1 expression.

3.3 | The role of B7S1 in T cell responses

B7S1 ligation of T cells exerts a profound inhibitory effect on the growth, cytokine secretion, and development of cytotoxicity function.\textsuperscript{83} The human B7S1 fusion protein produced in bacteria\textsuperscript{114} as well as in 293T cells\textsuperscript{115} had obvious biological activity to inhibit T cell proliferation. Moreover, B7S1.Ig fusion protein produced in 293T cells also arrested cell cycle progression of T cells in G0/G1 phase and induced T-cell apoptosis. Lee et al.\textsuperscript{116} investigated the effect of B7S1.Ig on differentiation of Th17 cells and they found that B7S1.Ig treatment inhibited the generation of Th17 cells, which subsequently decreased IL-17 production. Consistent with its inhibitory function in vitro, B7S1-deficient mice mounted mildly augmented Th1 cell responses.\textsuperscript{117} In contrast, a mAb that blocks binding of B7S1 to its receptor enhanced T cell proliferation in vitro and exacerbated EAE in vivo.\textsuperscript{79} All these implied that B7S1 plays an important role in restricting T-cell activation and function.

The mechanism by which B7S1 inhibits T cell immune response has also been investigated. Wang et al.\textsuperscript{118} found that culture of CD3\textsuperscript{+} T cells with a B7S1.Ig fusion protein showed reduced TCR signaling events, including phosphorylation of the MAP kinases, ERK, p38, and JNK. B7S1.Ig treatment also inhibited the phosphorylation of AKT kinase and impaired its kinase activity as assessed by the phosphorylation of its endogenous substrate GSK-3. In contrast, the phosphorylation state of the TCR proximal tyrosine kinases ZAP70 and lymphocyte-specific protein tyrosine kinase (LCK) are not affected by B7S1 ligation. These results indicate that B7S1 inhibits T-cell proliferation and IL-2 production through interfering with activation of ERK, JNK, and AKT, but not of ZAP70 or LCK.

3.4 | The role of B7S1 in tumor immunity

B7S1 is a negative regulator for T cell-mediated anti-tumor immune responses in vitro. In the context of cervical cancer, culture of T cells from the patients directly with B7S1 protein resulted in the arrest at G1/G2 phase of the patients’ activated T cells.\textsuperscript{100} B7S1 also inhibited the proliferation of CD4\textsuperscript{+} T and CD8\textsuperscript{+} T cells, but promoted the proliferation of Tregs and the secretion of IL-10 and TGF-beta1. In the setting of melanoma, overexpression of B7S1 on melanoma cells did not alter the cytotoxicity of different CD8\textsuperscript{+} effector cells, but drastically inhibited cytokine production.\textsuperscript{125} In NSCLC, B7S1 protein levels in A549 cells were knocked down by transfected with B7S1-specific shRNA-expressing plasmid, leading to enhanced Jurkat cell proliferation, decreased apoptosis, stimulated cell cycle progression and elevated production of IFN\gamma, IL-10 and IL-2.\textsuperscript{119} B7S1 is also a negative regulator of tumor immunity in vivo. Abadi et al.\textsuperscript{120} used the 4T1 metastatic breast cancer model and B7S1

FIGURE 2 Possible factors regulate B7S1 expression on TAM and tumor cells. IL-6/STAT3 signaling pathway induces B7S1 expression on TAMs as well as tumor cells in an autocrine loop and a paracrine action. HIF-1 and TAM-derived IL-10 are also factors that regulate B7S1 expression on tumor cells. B7-S1 plays a role in tumor invasion and metastasis

Tumor microenvironment

Tumor-associated macrophage

Tumor cells

FIGURE 2 Possible factors regulate B7S1 expression on TAM and tumor cells. IL-6/STAT3 signaling pathway induces B7S1 expression on TAMs as well as tumor cells in an autocrine loop and a paracrine action. HIF-1 and TAM-derived IL-10 are also factors that regulate B7S1 expression on tumor cells. B7-S1 plays a role in tumor invasion and metastasis

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knockout mice to investigate the effect of host tissue-expressing B7S1 on tumor progression, since 4T1 cells were B7S1 negative in vitro and in vivo. B7S1 (+/-) mice had significantly fewer lung 4T1 tumor nodules than did wildtype mice. Furthermore, B7S1 (+/-) mice showed significantly enhanced survival and a memory response to tumor re-challenge. The presence of B7S1 correlated with reduced overall and tumor-specific T cell cytokine responses, as well as with increased infiltration of immunosuppressive cells, including tumor-associated neutrophils, macrophages, and regulatory T cells, into tumor-bearing lungs. These results suggest that host B7S1 may enable metastasizing cancerous cells to escape local anti-tumor immune responses through interactions with the innate and adaptive immune systems. In ovarian cancer, B7S1-expressing APCs negatively regulate T-cell-mediated anti-tumor immunity. Zou et al. demonstrated that primary ovarian tumor cells express intracellular B7S1, whereas a fraction of tumor macrophages expresses surface B7S1. B7S1+ tumor macrophages, but not primary ovarian tumor cells, suppress tumor-associated antigen-specific T cell immunity. Blocking B7S1 restored the T cell stimulating capacity of the macrophages and contributes to tumor regression in vivo.

B7S1 not only dampens the anti-tumor Th1 responses, but also inhibits the pro-tumorigenic function of MDSCs. MDSCs derived from B7S1 KO mice suppressed T cell proliferation more potently than their WT counterparts. Although the primary growth of 4T1 tumors in B7S1 KO hosts was similar to that in WT mice, tumors that had grown in B7S1 KO hosts grew much more slowly than those from WT mice when subsequently transplanted into WT hosts. Importantly, this differential tumor growth during the secondary transplantation was abrogated when recipient mice lacked T cells, indicating that the immune environment in B7S1 KO hosts allowed outgrowth of 4T1 tumors with reduced immune-evasive capacities against T cells. Thus, B7S1 can inhibit both anti-tumor T cells and pro-tumor MDSCs, influencing the immune-evasive character of the outgrowing tumors.

In addition, B7S1 expression on APCs is another key mechanism responsible for immune evasion by tumors. Monocytes in patients with gastric carcinoma also showed immunosuppressive properties. Matsunaga et al. showed that B7S1 expression on circulating monocytes for patients with gastric carcinoma was upregulated. Co-culture of gastric cancer cell lines and monocytes led to upregulation of B7S1 expression on monocytes, which may benefit gastric cancer cells from immune evasion. Tumor environment may condition local DCs to become dysfunctional in the phenotype, and that the high expression of B7S1 may contribute to the tumor infiltrating DCs to mediate immune invasion.

3.5 | The non-immunological role of B7S1 in tumors

B7S1 has been proposed to promote epithelial cell transformation in ovarian, breast and esophageal carcinoma. In a human ovarian cancer cell line with little endogenous B7S1 expression, Salceda et al. found that overexpression of B7S1 increased tumor formation in SCID mice. Cheng et al. also found that B7S1 promoted tumor cell proliferation rate and increased cell adhesion, migration and invasion, indicating that B7S1 may directly promote malignant transformation of ovarian cancer cell line. In a breast cancer cell line, overexpression of B7S1 protected epithelial cells from anoikis, while siRNA-mediated knockdown of B7S1 mRNA and protein expression increased caspase activity and apoptosis. In the oesophageal squamous cell carcinoma (ESCC), B7S1 silence suppressed cell proliferation and colony formation of ESCC cell lines, in which B7S1 was highly expressed compared with normal esophageal tissue.

B7S1 facilitated ESCC cell proliferation through promoting IL-6/STAT3 positive loopback pathway activation. B7S1 silencing in ESCC cells reduced IL-6 secretion, STAT3 activation and p-STAT3 translocation from cytoplasm to nucleus. Moreover, IL-6 receptor antagonist Tocilizumab did not prevent p-JAK2 and p-STAT3 down-regulation induced by B7S1 silencing. Furthermore, ESCC cell proliferation and colony formation were downregulated by Tocilizumab, demonstrating that IL-6 upregulation induced by B7S1 was necessary for cells growth.

In pancreatic cancer, B7S1 induced Erk1/2 signaling pathway to promote tumor growth. The inhibition of B7S1 by siRNAs increased cell-cell adhesion and decreased the formation of pseudopodia. B7S1 siRNA inhibited cell proliferation, colony formation and migration of pancreatic cancer cells. Moreover, increased apoptosis in pancreatic cancer cells following B7S1 silencing was demonstrated in vitro and in a xenograft tumor model, which was associated with increased caspase activity and decreased Erk1/2 phosphorylation both in vitro and in vivo. Loss of B7S1 function thus prevents tumor growth through many processes, including the induction of apoptosis and inhibition of the Erk1/2 signaling pathway.

In cervical cancer, B7S1 depletion suppressed oxygen consumption rate, ATP production, and mitochondrial membrane potential and mass, but increased reactive oxygen species production. Electron transport complex III activity was significantly impaired in the cervical cancer cells following B7S1 silencing. Mitochondrial dysfunction in B7S1 siRNA-treated cells significantly augmented oxidative stress, which strongly activated the JNK/P38/caspase axis in the presence of doxorubicin, resulting in increased apoptotic cell death. This B7S1 silencing significantly downregulated the cAMP/cAMP response element-binding protein/PGC1-alpha signaling pathway. These findings suggested that B7S1 has a role in the regulation of mitochondrial function, which is closely related to cancer cell physiology and drug sensitivity. Taken all these together, B7S1 is a cancer promoter and might be a potentially important therapeutic target.

3.6 | Immune checkpoint inhibitor targeting B7S1

Although not yet targeted clinically, B7S1 is a promising target for cancer immunotherapy. In 2013, Dangaj et al. reported that they generated a yeast-display scFv library derived from tumor-associated B cells from patients with ovarian cancer to isolate and validate novel anti-B7S1 scFVs. In a humanized mouse model of ovarian cancer, tumor-bearing mice were treated with anti-B7S1 scFVs and the treatment delayed the growth of established tumors, indicating that antibody binding of B7S1 could restore anti-tumor immunity. Antibodies
targeting the B7S1 pathways may extend the survival of cancer patients by restoring T cell-mediated anti-tumor responses.

In 2014, Jeon et al. developed an in vivo system to screen therapeutic monoclonal antibodies against B7S1 and found that the clone 1H3 significantly inhibited growth of B7S1-expressing tumors in vivo via multiple mechanisms. Furthermore, the surviving mice given 1H3 treatment were resistant to tumor rechallenge, suggesting that targeting B7S1 on tumors is a promising cancer immunotherapy and humanized 1H3 may be efficacious for immunotherapy of human cancers. Zhang et al. generated one functional anti-human B7S1 mAb 5G3 through hybridoma method. MAb 5G3 specifically bound to B7S1 molecule and MAb 5G3 could block the inhibitory role of B7S1 molecule on A549 cells and reduce the apoptosis of Jurkat cells, suggesting that MAb 5G3 is an antagonistic antibody.

Although B7S1 function in tumor immunity has not been well studied and its receptor remains unknown, its restricted normal tissue distribution, its overexpression on a broad spectrum of cancer cells as well as TAM and its functional activity in transformation all suggest B7S1 as a new target for therapeutic intervention.

4 | VISTA IN CANCER IMMUNOTHERAPY

4.1 | VISTA biology

In 2011, two research groups independently identified murine B7-H5; one referred to it as V-domain Ig suppressor of T cell activation (VISTA). The location of this open reading frame is on murine chromosome 10 consisting of seven exons. The extracellular Ig domain of murine VISTA shares significant sequence homology with PD-L1 and PD-L2. It is primarily expressed in hematopoietic cells. Analysis of several hematopoietic cell types revealed expression of VISTA mRNA in peritoneal macrophages, splenic CD11b+ monocytes, CD11c+ DCs, CD4+ T cells, and CD8+ T cells, but at a lower expression level in B cells, while VISTA protein is highly up-regulated on APCs and Foxp3+ CD4+ regulatory T cells, but not on B cells, NK cells or granulocytes. Murine VISTA expression was quickly lost in several cell types upon in vitro culture, regardless of the activation status.

Similar to murine VISTA, human VISTA was predominantly, if not exclusively, expressed in hematopoietic tissues or in tissues that contain significant numbers of infiltrating leukocytes. Its gene locates on human chromosome 10. Interestingly, expression of VISTA was particularly high in human placenta, which may be indicative of a functional role of VISTA in allofetal tolerance. Human VISTA was not expressed by B cells or CD56+ NK cells, but highly expressed within myeloid compartment, including monocytes, mDCs, pDCs and neutrophils. It was reported that in human peripheral blood, VISTA expression correlated with CD11b expression.

The binding partner(s) of VISTA is currently unidentified. Both naïve and antigen-experienced T cells are sensitive to VISTA-induced suppression upon activation, which suggest that its receptor may be expressed on activated T cells. Our preliminary data showed that T cells from kidney cancer patients after re-activation expressed its receptor by using VISTA-Fc fusion protein (unpublished data).

4.2 | VISTA expression in tumor cells

So far there is only one report about VISTA expression in clinically resected tumors. Membranous VISTA protein was expressed on normal ductal epithelium within the pancreas. Other cell types from the normal pancreas, such as acinar cells and islet cells, did not express VISTA. In adenocarcinoma, VISTA staining was decreased or absent. Interestingly, VISTA expression in intraductal papillary mucinous neoplasms varied with grade. Normal ducts adjacent to tumors were highly positive, indicating that VISTA expression was restricted to ductal cells in the normal pancreas and the expression was down-regulated in pancreatic adenocarcinomas. The study suggests that loss of the VISTA signal may contribute to immune evasion of pancreatic adenocarcinoma. However, the mechanism by which loss of VISTA benefits pancreatic adenocarcinoma from immune evasion remains unknown. The expression of VISTA in other types of tumor and the correlation of its intratumor expression and disease progression as well as patient survival are highly warranted.

5 | THE ROLE OF VISTA IN T CELL RESPONSES

VISTA-Ig negatively regulates CD4+ T cell responses by suppressing early TCR activation and arresting cell division but with minimum direct impact on apoptosis. In addition, VISTA expressed on APCs can suppress antigen-specific T cell activation during cognate interactions between APCs and T cells. A VISTA-specific monoclonal antibody interferes with VISTA-induced suppression of T cell responses by VISTA-expressing APCs in vitro. All these findings suggest that VISTA is a negative immune checkpoint protein, which suppress T cell activation. In addition as a co-inhibitory ligand on APCs that suppress T cell responses, VISTA also functions as a co-inhibitory receptor for CD4+ T cells. CD4+ T cells in mice lacking VISTA exhibited a dramatically increased response to antigen stimulation. Furthermore, delivery of a VISTA-specific agonist mAb directly inhibited CD4+ T cell activation both in vitro and in vivo, validating a co-inhibitory function of VISTA directly on CD4+ T cells (Figure 3).

Despite the apparent normal hematopoietic development in young VISTA knockout mice, VISTA genetic deficiency led to a gradual accumulation of spontaneously activated T cells, accompanied by production of a spectrum of inflammatory cytokines and chemokines. Enhanced T-cell response was also observed upon immunization with neoantigen. The data further confirm that VISTA is a negative checkpoint regulator whose loss of function lowers the threshold for T-cell activation, allowing for an enhanced pro-inflammatory phenotype and an increase in the frequency and intensity of autoimmunity under susceptible conditions. VISTA/PD-1 double knockout mice exhibited significantly increased numbers of spontaneous activation of T cells than the single knockout mice. When bred onto the 2D2 TCR
transgenic mice, which are predisposed to development of inflammatory autoimmune disease in the CNS, the level of disease penetrance was significantly enhanced in the double knockout mice compared with in the single knockout mice. Consistently, the magnitude of T-cell response toward foreign antigens was synergistically higher in the double knockout mice, implying that VISTA and PD-1 non-redundantly regulate murine T-cell responses.

Flies et al. investigated the mechanism by which VISTA induced T cell tolerance in the setting of graft-versus-host disease (GVHD) and they found two distinct mechanisms. First, signaling via VISTA co-inhibitory receptor potently arrests alloreactive donor T cells from activation and expansion in the initiation phase. Second, donor regulatory T cells are subsequently expanded to maintain long-term tolerance and GVHD suppression, indicating the crucial function of VISTA as a co-inhibitory receptor on allo-reactive T cells and its function in the regulation of T cell tolerance. In a word, VISTA is a negative immune checkpoint protein expressed on APCs and CD4\(^+\) T cells.

**6 | THE ROLE OF VISTA IN TUMOR IMMUNITY**

Since VISTA is a negative immune checkpoint for T cell activation, its functions in tumor immunity are starting to be described. MCA105 (methylcholanthrene 105) fibrosarcoma does not express VISTA. VISTA-expressing MCA105 grew vigorously in vaccinated hosts, whereas the control tumors lacking VISTA expression failed to thrive. However, both MCA105-control and MCA105-VISTA tumors grew at an equivalent rate in vaccinated animals in which both CD4\(^+\) and CD8\(^+\) T cells were depleted using corresponding mAbs. This gain of function approach indicates that VISTA overexpression on tumor cells interferes with protective antitumor immunity in the host, which is dependent on T cells.

VISTA-deficient animals were highly resistant to tumor induction in a murine brain glioma model. In this model, brain tumor cells were directly inoculated into the left hemisphere of the brains of WT or VISTA knockout mice. VISTA knockout mice treated with ionizing radiation had significantly extended survival compared with WT mice, indicating that loss of VISTA confers resistance to brain tumor growth in a radiotherapy setting. Moreover, a significantly increased percentage of IFN-\(\gamma\) CD4\(^+\) T cells was found in the brains, but not in the spleens of tumor-bearing VISTA knockout mice; meanwhile, there were no significant differences in IFN-\(\gamma\)/CD8\(^+\) T cells in either brains or spleens. Importantly, depletion of CD4\(^+\) T cells in vivo using anti-CD4 mAbs resulted in the elimination of tumor resistance in VISTA knockout mice treated with radiotherapy, whereas depletion of CD8\(^+\) T cells by anti-CD8a mAb had no impact on tumor growth or on overall survival. Therefore, VISTA selectively suppresses CD4\(^+\) T cell-mediated tumor immunity in this mouse glioma model.

**7 | THE NON-IMMUNOLOGICAL ROLE OF VISTA IN TUMOR**

Although its immunological role in tumor immunity is emerging, its non-immunological role are poorly understood. In the context of fibrosarcoma, VISTA-expressing tumor cells grew vigorously in vaccinated hosts compared with the tumor cells lacking VISTA expression. However, the effect of VISTA expression on tumor cell proliferation, adhesion, apoptosis, migration and invasion are highly needed. Moreover, the same study on other tumor cells is urgently demanded.

**8 | IMMUNE CHECKPOINT INHIBITOR TARGETING VISTA**

Wang et al. generated one neutralizing monoclonal antibody against VISTA, 13F3. In the context of melanoma, 13F3 treatment increased the number of tumor-specific T cells in the periphery and enhanced the infiltration, proliferation, and effector function of tumor-reactive T cells within the TME. Anti-VISTA altered the suppressive feature of the TME by decreasing the presence of monocytic MDSCs and increasing the presence of activated DCs within the TME. In addition, VISTA blockade impaired the suppressive function and reduced the emergence of tumor-specific Foxp3\(^+\)CD4\(^+\) regulatory T cells. Consequently, targeting VISTA with a blocking antibody significantly suppressed tumor growth.

In the CT26 colon cancer model, targeting VISTA and PD-L1 simultaneously, on day 2 after tumor inoculation, led to tumor regression and long-term survival, whereas targeting each molecule alone was less effective. Analysis of tumor-specific T-cell activation showed synergistically enhanced cytokine production (IFN\(\gamma\) and TNF\(\alpha\)) and granzyme B production by tumor-specific CD8\(^+\) T cells from tumor-draining LN, indicating the combo therapy of VISTA inhibitor and PD-L1 inhibitor achieved optimal tumor-clearing therapeutic efficacy. Another combinatorial regimen using VISTA inhibitor and a peptide-based cancer vaccine with TLR agonists as adjuvants were tested. VISTA blockade synergized with the vaccine to effectively impair the
growth of established tumors. All these findings lay a foundation for designing VISTA-targeted approaches either as a monotherapy or in combination with additional immune-targeted strategies for cancer immunotherapy.

Now targeting VISTA has entered clinically trials. NCT02671955 is an open-label, first-in-human, phase 1 study of the safety, pharmacokinetics, and pharmacodynamics of JNJ-61610588, a fully human IgG1 Kappa anti-VISTA monoclonal antibody, in subjects with advanced cancer. CA-170 (referred to its clinicaltrials.gov identifier NCT02812875), a small molecule, is tested in another phase I clinical trial, which directly targets the PD-L1/PD-L2 as well as VISTA immune checkpoints, leading to activation of T cell proliferation and cytokine production. CA-170 is orally administered in adult patients with advanced solid tumors or lymphomas who have progressed or are non-responsive to available therapies and for which no standard therapy exists.

9 | CONCLUSION

The success of CTLA4 and PD-1 antagonists in clinics has validated the checkpoint inhibition as immunotherapy strategy. To increase the patient responses and the spectrum of cancers to be treated, there is a need to explore the role of novel checkpoint pathways in cancer immunity.

B7-H3, at least in mice, has a co-inhibitory function on T cells and NK cells. These results highlight the potential usage of this pathway for cancer immunotherapy. Antibodies targeting B7-H3 are being tested in a phase I/II clinical trials, indicating it represents a promising target for cancer immunotherapy.

B7S1 plays a significant role in the “immune escape” theory of tumors. Furthermore, multiple studies have identified B7S1 as a potential biomarker for multiple solid tumors. Although no anti-B7S1 targeting therapies has been investigated in any disease indication clinically, B7S1 remains a high priority candidate for targeted inhibition or elimination in a wide variety of cancers. Therefore, generation of a highly specific anti-human B7S1 antibody would open the door to robust preclinical studies.

VISTA is predominantly expressed by myeloid cells. VISTA expressed on APCs can suppress antigen-specific T cell activation during cognate interactions between APCs and T cells. VISTA-deficient animals were highly resistant to tumor induction in a murine brain glioma model. Furthermore, targeting VISTA with a blocking antibody significantly suppressed tumor growth. These offered insight into the functional role of VISTA in tumor biology and immune evasion.

Immune-based therapy offers a novel strategy to eliminate or kill tumor cells via activating the body’s immune system. Based on their potential, restricted expression and functional roles in cancer, B7-H3, B7S1 and VISTA appear to be promising new targets for immune-based therapies, alone or in combination with other therapies. However, more studies especially in primary human tumor specimens are needed. In addition, their receptors are seriously needed to be identified before we understand the actions of these pathways in T cells and other immune cells.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

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