Clinical Cancer Research

Molecular Pathways: Evaluating the Potential for B7-H4 as an Immunoregulatory Target

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Abstract

With the clinical success of CTLA-4 and PD-1 blockade in treating malignancies, there is tremendous interest in finding new ways to augment antitumor responses by targeting other inhibitory molecules. In this review, we describe one such molecule. B7-H4, a member of the B7 family of immunoregulatory proteins, inhibits T cell proliferation and cytokine production through ligation of an unknown receptor expressed by activated T cells. Notably, B7-H4 protein expression is observed in a high proportion of patients' tumors across a wide variety of malignancies. This high expression by tumors in combination with its low or absent protein expression in normal tissues makes B7-H4 an attractive immunotherapeutic target. Preclinical investigation into B7-H4–specific chimeric antigen receptor (CAR) T cells, antibody-mediated blockade of B7-H4, and anti–B7-H4 drug conjugates has shown antitumor efficacy in mouse models. The first clinical trials have been completed to assess the safety and efficacy of a B7-H4 fusion protein in ameliorating rheumatoid arthritis. *Clin Cancer Res; 23(12); 2934–41. ©2017 AACR.*

Background

The B7 family of immunoregulatory proteins is composed of 10 members—B7-1 (CD80), B7-2 (CD86), PD-L1 (B7-H1, CD279), PD-L2 (B7-DC, CD273), B7-H2 (ICOSL, CD275), B7-H3 (CD276), B7-H4, B7-H5 (VISTA), B7-H6, and B7-H7 (HHLA2)—all of which are membrane-anchored proteins with extracellular immunoglobulin-like domains important in the binding of their ligands (1). Members of this family can be expressed on various cell types, such as antigen-presenting cells (APC), T cells, and tumor cells (Fig. 1). *B7-h4* (*B7x, B7s1, Dd-0110, Vtcn1*) was first identified in 2003 through sequence similarities with other B7 family members (2–4). It encodes a heavily glycosylated, glycosylphosphatidylinositol (GPI)-linked protein (3, 4), and plays an inhibitory role in T cell function by limiting proliferation, cytokine production, and cytotoxicity (2–4).

B7-H4 shows a high degree of conservation of core amino acid residues with other B7 family members, but the residues involved in ligand-binding for B7-1 (CD80) or B7-2 (CD86) are not conserved (3). Experiments have shown that B7-H4 does not share binding partners with other B7 family members, as interactions have not been observed between B7-H4 and CD28 (2, 4), CTLA-4 (3, 4), ICOS, or PD-1 (2–4). Although BTLA was proposed to be the binding partner of B7-H4 (5), subsequent experiments were unable to demonstrate the binding of a B7-H4–Fc fusion protein to cells stably expressing BTLA (6). As yet, the B7-H4 receptor has not been identified.

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Expression and function of B7-H4

B7-H4 transcripts have been detected in a wide variety of tissues (2, 7, 8), whereas B7-H4 protein expression is tightly regulated and shows limited expression in most normal tissues (3, 7, 9–14). However, it has been observed in certain tissues including thymus, spleen (3), kidney, placenta (9, 10), female genital tract, lung, and pancreas (10).

Alterations in B7-H4 expression in various *in vivo* models demonstrate its immunosuppressive role. Transgenic overexpression of B7-H4 in pancreatic islets did not prevent islet inflammation (8) but protected mice from developing diabetes in the presence of T cells expressing either of the islet-specific transgenic T cell receptors (TCR) BDC2.5 (8) or AI4αβ (15). On the nonobese diabetic (NOD) background, a mouse strain prone to developing diabetes, the loss of B7-H4 expression on islet cells coincides with diabetes progression, and treatment that inhibits B7-H4 loss delays diabetes onset (16). Loss of B7-H4 in these models accelerated diabetes onset and severity (8, 15, 16). Detection of B7-H4 in human pancreatic tissue (10) and in the sera of patients with type 1 diabetes suggests it may also have an important role in the progression of human disease (17).

Other *in vivo* models also support an immunosuppressive role for B7-H4. Overexpression of B7-H4 in transplanted islets prolonged transplant survival and reduced proinflammatory infiltrates (18), and delivery of a B7-H4–Ig fusion protein protected mice against concanavalin A–induced hepatic injury (19). In addition, host deficiency in B7-H4 conferred resistance to a lethal pulmonary infection with *Streptococcus pneumoniae* (20). In this model, disease amelioration occurred in conjunction with an increase in infiltrating CD4 helper and CD8 cytotoxic T cells, and a concurrent decrease in neutrophils (20). Collectively, these findings indicate that B7-H4 plays an immunosuppressive and tissue-protective role in multiple mouse models.

B7-H4 in immunity

Controversy exists as to which immune populations express B7-H4. In mice, Prasad and colleagues observed B7-H4 on a minor population of T cells, the majority of B cells (defined as

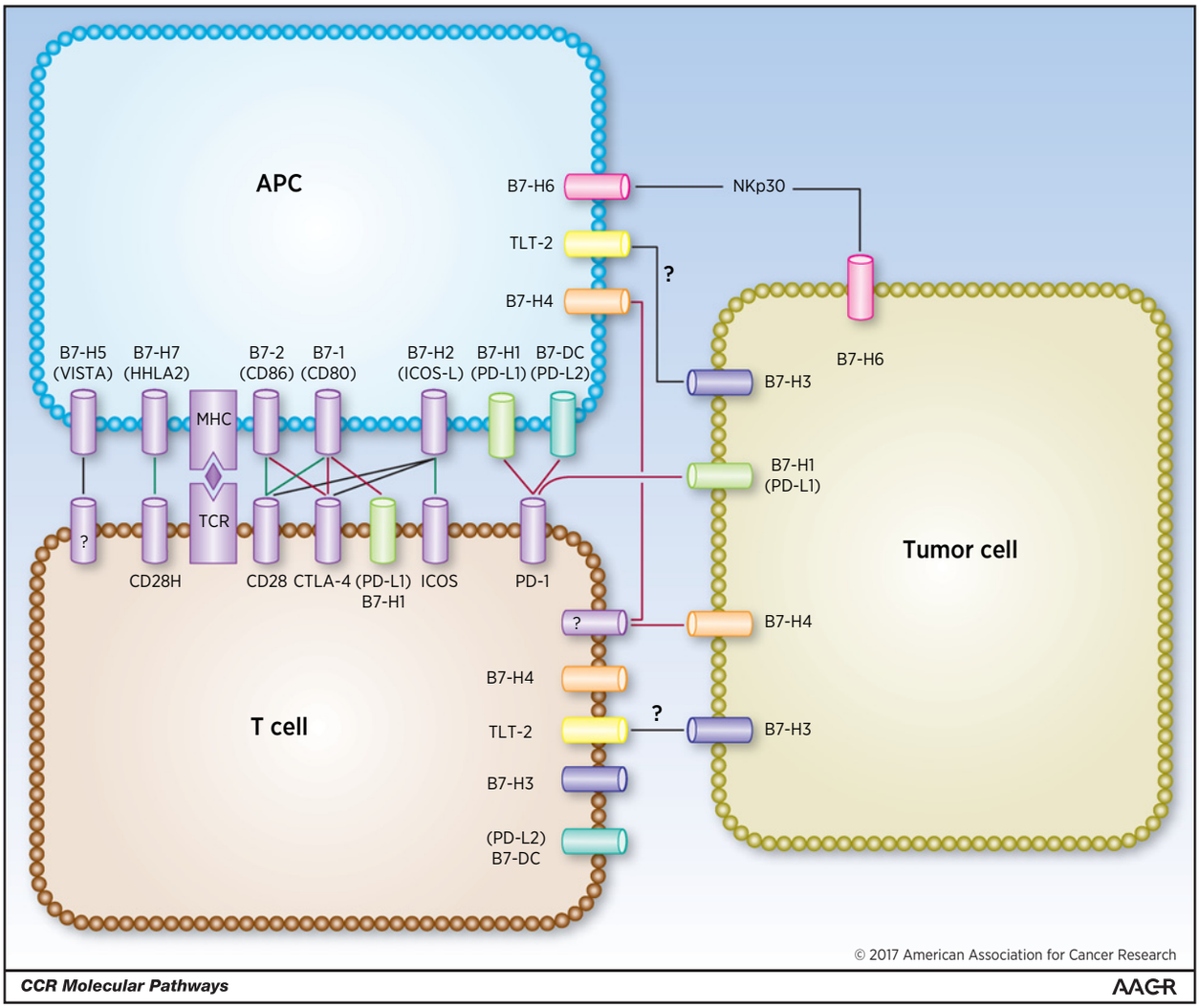

Figure 1.
Expression pattern of B7 family members and their co-receptors. Members of the B7 family are important mediators of both stimulatory (green lines) and inhibitory (red lines) interactions. MHC, major histocompatibility complex; TCR, T cell receptor.

B220$^+$), and thioglycolate-stimulated macrophages (2). Although Sica and colleagues did not observe B7-H4 on resting human T cells, B cells, or monocytes, its expression could be induced by phytohemagglutinin (PHA), lipopolysaccharide (LPS), and phorbol 12-myristate 13-acetate (PMA)/ionomycin, respectively (3); however, Prasad and colleagues found that stimulation of mouse B cells with LPS, IL4, anti-IgM, or anti-CD40 all downregulated B7-H4 (2). Lee and colleagues reported that B7-H4 expression was not observed on human or mouse dendritic cells, monocytes, macrophages, B cells, or T cells, regardless of stimulation (15). These differences may be due to the use of different antibodies for B7-H4 recognition or differences between mouse and human biology.

B7-H4 in innate immunity

B7-H4 expression has been reported on a highly immunosuppressive subset of tumor-infiltrating macrophages in ovarian cancer and can be induced on macrophages by stimulation with IL10 and IL6 (21). Immunosuppressive B7-H4$^+$ tumor-associated macrophages have also been observed in patients with glioma (22). *Ex vivo* stimulation of mouse macrophages with IL10 and TGFβ increased B7-H4 expression, and transfusion of these macrophages protected mice from adriamycin nephrosis (23). B7-H4 expression was STAT3-dependent (22) and blockade of both IL10 and IL6 (22, 24) or stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL4 (21) reduced B7-H4 expression. These data indicate that a number of stimuli can regulate B7-H4 expression (Fig. 2).

Macrophages isolated from ovarian tumors or malignant ascites expressed significantly higher B7-H4 levels than monocytes from normal blood or macrophages from nonmalignant ascites (21). B7-H4$^+$ macrophages were 2- to 6-fold more potent than B7-H4$^-$ macrophages at suppressing T cell

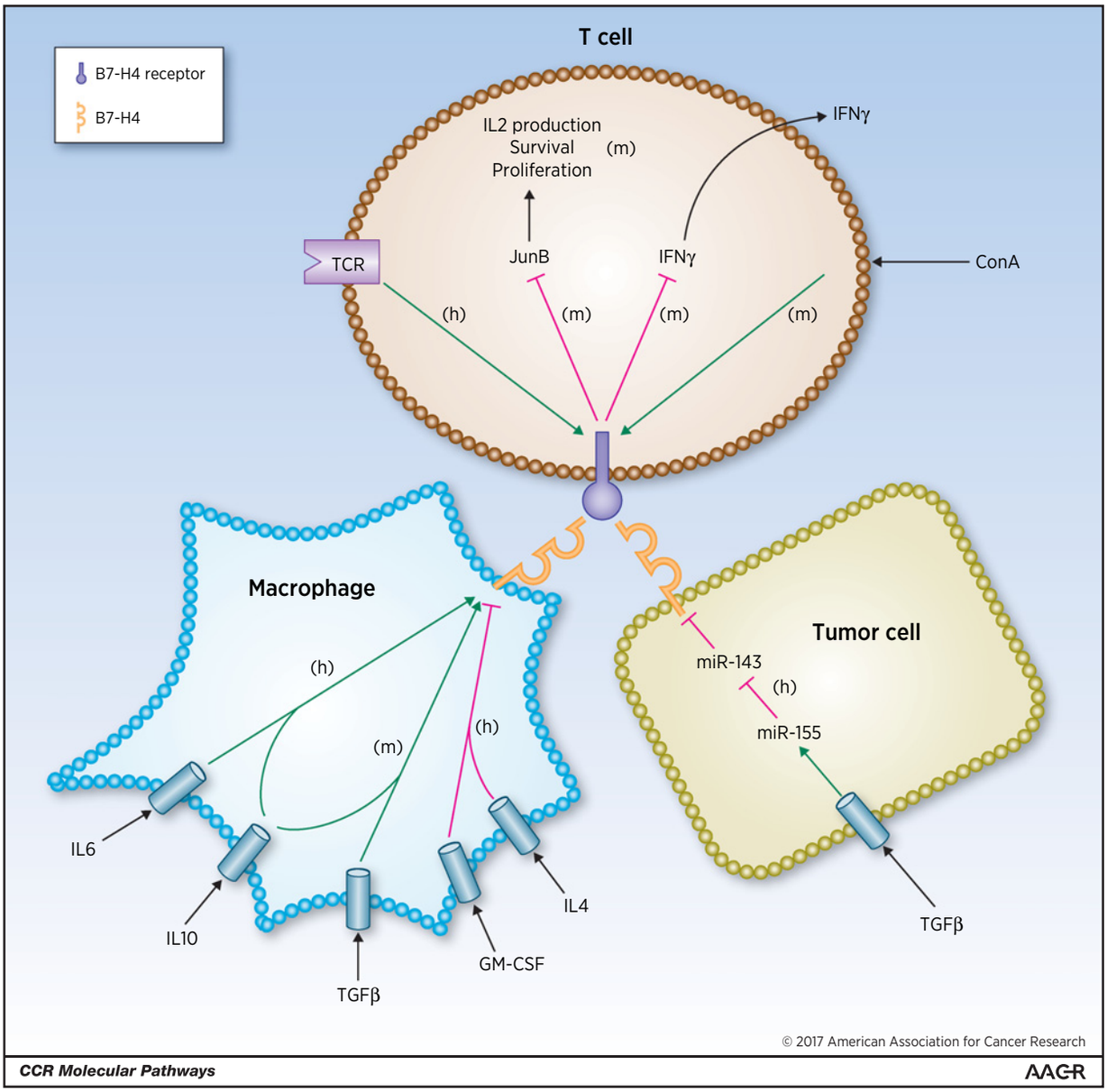

Figure 2.

Factors controlling B7-H4 and B7-H4 receptor expression. Stimulation of macrophages with IL6 + IL10 (21, 22, 24) or IL10 + TGFβ (23) has been shown to upregulate B7-H4 expression, whereas stimulation with GM-CSF + IL4 (21) reduces B7-H4 expression on macrophages. Stimulation of tumor cells with TGFβ1 (42) has been reported to upregulate B7-H4 protein expression. The receptor for B7-H4 can be upregulated on T cells following treatment with concanavalin A (2) or TCR stimulation (3). Engagement of the B7-H4 receptor inhibits survival, proliferation, IL2 production (2–4), and IFNγ production (28). Data acquired from experiments on mouse or human cells are denoted with "(m)" and " (h)", respectively.

proliferation despite similar levels of activation marker expression and cytokine production (21). Blockade (21, 23, 25) or knockdown (22) of B7-H4 neutralized the suppressive capacity of these macrophages. This immunosuppression mediated by B7-H4$^+$ macrophages was independent of PD-L1, arginase, and inducible nitric oxide synthase (iNOS; ref. 21). However, Leung and Suh found that myeloid-derived suppressor cells (MDSC) generated from B7-H4 knockout mice had enhanced suppressive activity and higher levels of iNOS (26).

Studies have examined the role of B7-H4 in neutrophils. B7-H4–deficient mice have reduced production of inflammatory cytokines (IL6, TNFα) and monocyte/neutrophil chemoattractants (MCP-1, KC, MIP-1α, MIP-1β) in lung homogenate after *S. pneumoniae* infection (20). However, loss of B7-H4 enhanced neutrophil-mediated resistance to *Listeria monocytogenes* infection, with an observable decrease in bacterial burden by day 3 post-infection (27). Similar increased numbers of neutrophils were seen in peripheral blood after the introduction of soluble decoy

B7-H4 molecules, suggesting that neutrophils receive inhibitory signals through B7-H4 (27). This neutrophil-mediated innate resistance was independent of adaptive immunity, demonstrating the effects of B7-H4 on multiple arms of the immune response (27). It is unclear how these effects on innate responses might impact therapeutic targeting of adaptive responses by B7-H4 blockade.

B7-H4 in adaptive immunity

Although T cell expression of B7-H4 is uncertain, the expression pattern of the B7-H4 receptor is more definitive. Studies have used B7-H4 fusion proteins to detect the expression of its receptor. Naïve T cells do not express the B7-H4 receptor, but its expression can be induced by stimulation with concanavalin A (2) or anti-CD3 (3). Ligation of the B7-H4 receptor by B7-H4 inhibits T cell proliferation and activation, in part, through a reduction in JunB expression (2), a component of the AP-1 family that binds the IL2 promoter and drives its transcription (Fig. 2). Interestingly, strong TCR stimulation or co-stimulation can overcome the inhibitory effects of B7-H4 (2). This suggests that the suppressive effects of B7-H4 may be more relevant in the context of low-affinity antigens, such as those associated with an antitumor response.

B7-H4 knockout mice support normal lymphocyte development without noticeable differences in the numbers, proportions, or level of activation in naïve mice (28). However, loss of B7-H4 plays a role in T cell polarization. Studies have shown that helper T cells can differentiate into many different subsets (T_H1, T_H2, T_H9, T_H17, T_H22, Treg, T_{FH}) defined by the cytokines they produce (29). T cells from B7-H4 knockout mice showed increased production of the prototypic T_H1 cytokine, IFNγ, in response to infection with *Leishmania major* (ref. 28; Fig. 2). Since T_H1 T cell responses improve the development of a cytotoxic T lymphocyte response, which in turn can promote tumor regression (30), blocking B7-H4 function may prove beneficial in improving antitumor immunity.

B7-H4 in tumor cell biology

Unlike in normal tissues, high levels of B7-H4 protein expression have been reported on a variety of human tumors (Table 1) including ovarian carcinoma (7, 9, 25, 31), breast cancer (9, 10), endometrial adenocarcinoma (11), bladder cancer (12), esophageal and oral squamous cell carcinoma (32, 33), glioma (22), prostate cancer (34), pancreatic cancer (35), cervical cancer (13), melanoma (36), lung cancer (7, 14, 37), gastric cancer (38, 39), and renal cell carcinoma (40, 41). *In vitro* assays using the colorectal carcinoma cell line HCT-116 show B7-H4 protein upregulation after culture with TGFβ1 through an increase in miR-155 levels, which in turn lead to a decrease in the B7-H4–inhibitory miR-143 (42). In renal cell carcinoma, higher expression of B7-H4 has also been reported on the tumor vasculature (81.5% of cases) compared with the expression on

Table 1. Correlations of clinicopathologic characteristics with high levels of B7-H4 expression on tumor cells as assessed by immunohistochemical staining

Tumor type	Antibody clone/supplier	Overall survival	Immune cell infiltration	Lymph node involvement	Late stage	Comments	Reference
Breast cancer	A57.1	ns	NR	ns	ns (AJCC)		(10)
Cervical cancer	bs-0673R	NR	Negative (CD8); ns ($FoxP3^+$)	NR	NR		(13)
Esophageal squamous cell carcinoma	3C8	Negative	Negative (CD3 in tumor nest, CD8 in stroma)	ns	Positive (AJCC)		(32)
Gastric cancer	Abbiotec	Negative	Negative (CD3)	ns	Positive (UICC)		(38)
Gastric cancer	USCN Life Science	Negative	Positive ($FoxP3^+$)	Positive	Positive (AJCC)		(39)
Melanoma	H74	Negative	Positive ($CD68^+$)	NR	NR		(36)
Non–small cell lung cancer	EP1165	Negative	NR	NR	NR		(37)
Oral squamous cell carcinoma	Cell Signaling Technology	Negative	NR	Positive	NR	Positive correlation with higher grade tumors	(33)
Ovarian cancer	A57.1	ns	NR	NR	ns (AJCC)	ns correlations with recurrence or higher grade tumors	(31)
Uterine endometrioid adenocarcinoma	A57.1	ns (DFS, DNS)	Negative (CD3 and CD8 TAL); ns (CD3 and CD8 TIL)	NR	NR	Positive correlation with high-risk tumors; positive correlation with immune infiltration only when B7-H4 expressed on cellular membrane and in cytoplasm	(11)
Pancreatic cancer	3C8	Negative	Negative (CD3); ns (CD8)	Positive	NR		(35)
Prostate cancer	R&D Systems, catalog number: AF2154	Negative (PFS)	NR	ns	NR	Positive correlation with recurrence	(34)
Renal cell carcinoma	hH4.1	Negative (CSS)	Positive (lymphocytic infiltration)	Positive	Positive		(41)
Urothelial cell carcinoma	USCNLIFE, USA	Negative (RFR)	NR	NR	Positive (UICC)	Positive correlations with recurrence and higher grade tumors	(12)

Abbreviations: AJCC, American Joint Committee on Cancer; CSS, cancer-specific survival; DFS, disease-free survival; DNS, data not shown; NR, not reported; ns, not significant; PFS, progression-free survival; RFR, recurrence-free rate; TAL, tumor-associated lymphocytes; TIL, tumor-infiltrating lymphocytes; UICC, Union for International Cancer Control.

tumor-adjacent vessels (6.5% of cases; ref. 41), although the functional significance remains unclear.

B7-H4 protein can be membrane bound or expressed in the cytoplasm (11). Human B7-H4 has a nuclear localization sequence (NLS) allowing it to shuttle between the cytoplasm and the nucleus, whereas an NLS has not been identified in mouse B7-H4 (40).

Membrane-bound B7-H4 can be cleaved by nardilysin, a metaloendopeptidase (17). Radichev and colleagues observed a decrease in the level of B7-H4 on the membrane of APCs from diabetic mice and patients that coincided with an increase in serum levels of soluble B7-H4 (17). They proposed liberation from the membrane as the mechanism resulting in increased levels of soluble B7-H4 in the serum of patients with more advanced ovarian cancer (43).

In tumor cell lines, reports suggest that B7-H4 has an intrinsic role in augmenting proliferation and decreasing susceptibility to apoptosis (14, 40, 44). Knockdown of B7-H4 in A549 cells, a lung adenocarcinoma cell line, inhibited cell proliferation, invasion, and migration. In addition, knockdown cells had lower expression of the antiapoptotic protein Bcl-2 and higher expression of proapoptotic protein Bax as well as caspase-3 and caspase-8 (14). B7-H4 overexpression in renal cell carcinoma cell lines Caki-1 and ACHN increased survival in response to doxorubicin or docetaxel chemotherapy (40). In esophageal squamous cell carcinoma cell lines, B7-H4 knockdown reduced proliferation and increased apoptosis, possibly through augmentation of IL6 production via the JAK2/STAT3 pathway (44).

Clinical–Translational Advances

B7-H4 and disease

The impact of B7-H4 expression has been evaluated in the context of cancer and autoimmunity. Although some reports have found correlations between high B7-H4 expression and increased lymphocytic infiltration (41), reduced levels of tumor invasion (45), and improved survival for patients with breast cancer (46), B7-H4 has generally been reported to correlate with lower survival rates, advanced clinical stage, increased lymph node involvement, decreased T cell infiltration, and increased macrophage infiltration (Table 1). Collectively, these studies suggest that B7-H4 expression contributes to, or is associated with, protumorigenic factors.

A study examining the influence of B7-H4 polymorphisms on breast cancer development in a Chinese population found that certain polymorphisms were associated with risk of breast cancer development and other parameters such as tumor size, progesterone or estrogen receptor positivity, or lymph node metastasis (47). Given that B7-H4 can be expressed by many different cell types and, in humans, can shuttle between the cytoplasmic and nuclear compartments, it is possible that it has many roles depending on the cellular context.

Levels of soluble B7-H4 vary with subtype of ovarian cancer (48) and are increased in patients with advanced stage ovarian cancer (43). Its presence in the serum is also a negative prognostic indicator for multiple diseases including glioma (22), renal cell carcinoma (49), type 1 diabetes (17), and rheumatoid arthritis (50).

B7-H4—a novel target for therapy?

Given the importance of B7-H4 in inhibiting T cell proliferation and effector function (2–4), efforts are underway to develop antibodies to block B7-H4–mediated T cell inhibition (51). Upregulation of B7-H4 with disease progression (Table 1) and its retention on metastases (31, 36) make it an attractive target for treating late-stage, refractory malignancies.

Studies support an inhibitory role for B7-H4 in tumor immunity. B7-H4 knockout mice developed fewer metastatic lung nodules after intravenous infusion of 4T1 breast cancer cells, a murine mammary tumor cell line, compared with control mice. Knockout mice also had higher survival rates, developed resistance to tumor rechallenge, and had an immune infiltrate characteristic of an improved antitumor response (52). Similarly, transplantation of the 4T1-12B breast tumor cell line, a 4T1 derivative that elicits a strong CD8 T cell response, showed reduced tumor growth in B7-H4–deficient hosts as a result of increased antitumor T cell activity (53). High levels of B7-H4 expression have been associated with lower levels of T cell infiltration (refs. 11, 18, 35, 38; Table 1), consistent with the idea that blocking B7-H4 interactions may promote T cell infiltration and enhance immunotherapy.

Antibodies against immune inhibitory molecules such as CTLA-4 and PD-1/PD-L1 have met with remarkable clinical success (54), and, therefore, it is important to evaluate the potential for B7-H4 blockade in enhancing antitumor responses. In addition, high B7-H4 expression has been linked with a higher incidence of PD-L1 co-staining (41), raising the possibility that dual blockade of PD-L1 and B7-H4 could increase the efficacy of treatment.

Treatment of mice with monoclonal antibodies (clones 1H3 and 12D11) that block the interaction of B7-H4 with its receptor significantly suppressed primary tumor growth, reduced metastatic nodules, prolonged survival, increased T and natural killer (NK) cell infiltration, and decreased numbers of infiltrating MDSCs. Jeon and colleagues found that half of the mice that received a B7-H4–blocking antibody survived tumor challenge, whereas none of the mice receiving the isotype control survived. Furthermore, mice that survived were protected against rechallenge, demonstrating the successful formation of immunologic memory. In addition, mice treated with a B7-H4–blocking antibody (clone 1H3) had a lower proportion of CD4 helper T cells with an exhausted phenotype. *In vitro* evaluation of the activity of 1H3 revealed that it promoted cell death through antibody-dependent cell cytotoxicity and blocked B7-H4–mediated co-inhibition of T cells (55). Similarly, Dangaj and colleagues found that an anti–B7-H4 single-chain antibody could restore antigen-specific T cell activation *in vitro* and delay the growth of established tumors *in vivo* (25).

Despite the evidence that B7-H4 may be an attractive negative regulatory target for intervention, it is important to note a distinction between CTLA-4, PD-1, and B7-H4 knockout mice. Unlike the profound (56, 57) and moderate (58, 59) autoimmune phenotypes seen in CTLA-4 and PD-1 knockout mice, respectively, the study of B7-H4 knockout mice has not reported a similar phenotype (27, 28). One interpretation could be that B7-H4 blockade will have less severe autoimmune consequences; however, the other possibility is that it may not be as efficacious.

The low expression of B7-H4 on normal tissue and upregulation on a wide variety of tumors raises the possibility that T cells expressing a chimeric antigen receptor (CAR) specific for B7-H4 could selectively target T cells to the tumor. A recent study by Smith and colleagues found that while T cells expressing a CAR specific for B7-H4 exhibit antitumor activity in a mouse xenograft

transplant model, mice reproducibly developed delayed, lethal toxicity 6 to 8 weeks after treatment. Postmortem analysis of mice showed B7-H4 expression by normal ductal and mucosal epithelial tissue coincided with severe histologic damage, demonstrating that B7-H4 expression by normal tissues was not low enough to avoid off-tumor effects (60). Although Leong and colleagues did not observe off-target activity after administration of anti–B7-H4 antibody–drug conjugate, they did not confirm recognition of mouse B7-H4 by the human antibody used in the drug conjugate. In addition, toxicity studies may have been too short to see delayed effects (61).

Although many reports have found that B7-H4 is a negative prognostic marker (Table 1), other studies suggest that B7-H4 plays a positive role in immune responses. Studies have noted higher levels of immune infiltration correlating with B7-H4 positivity in renal cell carcinoma (41) or when B7-H4 is expressed on both the membrane and cytoplasm in uterine endometrioid adenocarcinoma cells (11). One unexpected finding using mouse models showed that B7-H4 expression by non-hematopoietic cells was necessary to promote vaccine-induced tumor-specific immune responses using transgene-driven tumors (46). In this study, an improved antitumor T cell response was not observed in B7-H4–deficient mice. However, work by Jeon and colleagues in a transplantable tumor model showed reduced tumor growth, prolonged survival, and increased T and NK cell infiltration in response to treatment with anti–B7-H4 antibodies, suggesting that this phenotype may not arise in a situation where B7-H4 was blocked with an antagonistic antibody (55).

Progress from translational immunology to clinical applications has begun, with MedImmune LLC (in collaboration with Daiichi Sankyo Co., Ltd.) completing the first clinical phase I trial (NCT01878123) of AMP-110, an Fc fusion to the extracellular domain of B7-H4. The trial will be monitoring participant health to assess dose-limiting toxicities and adverse events in response to treatment with a single dose of AMP-110. This study also aims to determine the pharmacokinetics of AMP-110 and potential biomarkers of response to treatment. A second phase Ib trial of AMP-110 (NCT02277574) has also recently been completed by MedImmune LLC and collaborators to assess the safety, tolerability, pharmacokinetic, and clinical activity of multiple doses of the drug. Although both studies have been completed, results have not yet been reported.

Concluding remarks

Key outstanding questions remain related to the biology of B7-H4. More research is needed to identify the receptor(s) for B7-H4, the role of B7-H4 in neutrophil biology, as well as its unexpected role in vaccine-induced antitumor responses. There are, however, many properties associated with B7-H4 that make it an attractive target for antibody-based therapies. Studies suggest that blockade of B7-H4 should result in increased tumor-specific T cell responses (2–4, 8, 15, 21, 22, 55), and promising preclinical results show efficacy of B7-H4 antagonistic antibodies in reducing tumor growth and augmenting antitumor immunity (25, 55). In addition, the upregulation of B7-H4 on tumors has been used to selectively target drugs (61) and CAR T cells (60) to tumor tissues in preclinical mouse models. However, lethal on-target off-tumor activity of CAR T cells indicates that further work into targeting therapeutics to tumors expressing B7-H4 is needed. The momentum in immunotherapy will undoubtedly provide further impetus to explore the potential role for B7-H4 in the therapeutic setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H.L. MacGregor, P.S. Ohashi
Writing, review, and/or revision of the manuscript: H.L. MacGregor, P.S. Ohashi

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