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Fine tuning the immune response through B7-H3 and B7-H4

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Abstract

Summary—B7-H3 and B7-H4 belong to a new class of immune regulatory molecules, which primarily execute their functions in peripheral tissues to fine tune immune responses in target organs. In normal circumstances, while the mRNA for both molecules is broadly distributed, tight control at the post-transcriptional level is imposed. Under a pathogenic environment, such as inflammation and cancer, the control is often aberrant. Upon engaging their receptors, these molecules regulate the immune response in positive or negative ways depending on the expression and type of cells bearing the receptors. Thus, manipulation of the expression of these molecules and/or their receptors may represent a realistic opportunity to fine tune immune responses and to design new immunotherapeutic approaches.

Keywords

cell activation; costimulation; transgenic/knockout mice; immunotherapies; tumor immunity

Introduction

A major function for the molecules of the ‘classic’ B7 family, including CD80, CD86, and their receptors CD28 and cytotoxic T lymphocyte antigen (CTLA-4) is to provide positive and/or negative cosignaling at the initiation stage of T-cell responses, in conjunction with a T-cell receptor (TCR)-mediated antigenic signal. More recently identified B7-homologue molecules; including B7-H1, B7-DC, B7-H2, B7-H3 and B7-H4, however, represent a class of molecules with much more diverse functions in the adaptive immune system. Moreover, these molecules also play critical roles in the control of immune responses beyond the adaptive immune system. For example, B7-H4 is broadly expressed in many tissues and cells and is found to regulate host innate responses by suppressing growth of neutrophil progenitors in addition to T-cell responses. B7-H3 has also been demonstrated to regulate osteopoiesis in addition to its immunoregulatory function. Therefore, the finding of these molecules represents a new frontier of investigation for understanding the molecular regulation of the immune system involving much broader systems. This review focuses on the immune function and new developments regarding B7-H3 and B7-H4 molecules.

B7-H3 and its receptor TREM-like transcript 2 (TLT-2, TREML2)

B7-H3 (CD276) was identified as a homologue of the B7 family molecule in our laboratory by searching human genome databases for DNA sequences with homology to previously identified B7 molecules (1). B7-H3 is a type I transmembrane protein and shares 20-27% amino acid identity with other B7 family members. While this homology is rather low, its

putative secondary and even tertiary structures, which are predicted from its primary sequence, are shown to be highly homologous to other B7 family molecules. While murine B7-H3 has a single extracellular variable-type immunoglobulin (IgV)-IgC domain and a signature intracellular domain, human B7-H3 is found to possess an additional isoform, known as 4Ig-B7-H3, containing nearly exact tandem duplication of the IgV-IgC domain and most likely caused by exon duplication (2).

B7-H3 mRNA is broadly expressed in normal tissues (1,2), whereas its protein expression is relatively rare, suggesting that a tight post-transcriptional control is a major regulatory mechanism on its expression. Nevertheless, B7-H3 protein has been identified in human liver, lung, bladder, testis, prostate, breast, placenta, and lymphoid organs, albeit in low levels. In spite of this low basal-level expression, B7-H3 expression is induced on T cells, natural killer (NK) cells, and antigen-presenting cells (APCs), including dendritic cells (DCs) and macrophages (1,3,4). B7-H3 can be upregulated during the maturation from monocytes to DCs, or during the interaction between DCs and regulatory T cells. B7-H3 is found on fibroblasts, fibroblast-like synoviocytes, and epithelial cells and may potentially play a diverse role in the regulation of growth and differentiation of non-hematopoietic tissues (5-7). The broad expression pattern of B7-H3 mRNA and its inducibility at the protein level suggest diverse functions for B7-H3. For example, B7-H3 is found to be expressed in high levels in developing bones during embryogenesis, and its expression increases as osteoblast-precursor cells differentiate into mature osteoblasts (6).

B7-H3 has recently been identified to bind TLT-2, a molecule belonging to the triggering receptor expressed on myeloid cells (TREM) receptor family. Generally, the TREM family molecules function as modulators of the cellular response and play roles in both innate and adaptive immunity (8,9). TREM proteins are highly conserved across several species, including mice, humans, chickens, pigs, and cows (10,11). TLT-2 is a type I transmembrane protein and has a single IgV domain with two potential *N*-glycosylation sites, a serine/threonine-rich region with numerous predicted *O*-linked glycosylation sites, a single membrane-spanning region, and a short cytoplasmic tail (8,12). The human and mouse TLT-2 proteins are 60% homologous with Ig domains that are 80% similar (8).

Initially, human TLT-2 mRNA was found to be expressed in B-cell lines (FERN, IND, Raji, RJ.225), a T-cell line (Jurkat), and monocytic-cell lines (THP1, U937) (12). Expression of murine TLT-2 protein was determined by flow-cytometry analysis throughout B-cell development, especially at the early stages, and on B1 B cells within the peritoneal cavity, but not on T cells (8). TLT-2 expression is upregulated during emigration of monocytes from the blood into the peritoneal cavity and lung, where they differentiate into tissue macrophages. These macrophages can further upregulate TLT-2 when activated (8). Gr1⁺CD11b⁺ neutrophils, which constitutively express TLT-2, also markedly upregulate TLT-2 when activated by inflammatory stimulation (8). Hashiguchi *et al.* (13) recently determined that TLT-2 protein is expressed constitutively on CD8⁺ T cells and is induced on activated CD4⁺ T cells. Other cell lines with high cell-surface expression of TLT-2 are a T-cell line (MBL-2), B-cell lines (A20.2J, BAL17), and a monocytic/macrophage line (WEHI3B) (13).

Unlike other TREM family members except TLT-1, TLT-2 is not associated with DAP12 for signaling, as was predicted by the absence of charged amino acids in the transmembrane domain (12). The cytoplasmic tail of human TLT-2, however, does contain a potential consensus +xxPxxP Src homology 3 (SH3)-binding motif (+ represents a positively charged arginine) (12), with a similar +xPxxP sequence in the mouse. TLT-2 may bind with one or more SH3 or WW domain-containing effector proteins, although the specific proteins have not been determined thus far (8).

The cytoplasmic tail of human TLT-2 also contains two tyrosines, which can form motifs for endocytosis (YxxV) or for recruiting and activating signal transducers and activators of transcription 3 (STAT3) (YxxV or YxxC) (8). The endocytosis motif may mediate internalization of the receptor upon ligand recognition or monoclonal antibodies (mAb)-induced crosslinking and needs to be functionally tested for human TLT-2; however, neither motif is conserved in the mouse TLT-2 cytoplasmic domain (9). In contrast to other molecules encoded within the TREM gene cluster, TLT2 does not contain either an immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibitory motif (ITIM) associated with phosphotyrosine-based signaling.

B7-H3, a costimulator for T cell response

Using anti-CD3 antibody to mimic the T-cell receptor (TCR) signal, B7-H3Ig fusion protein is able to increase proliferation of both CD4⁺ and CD8⁺ T cells and selectively stimulates interferon- γ (IFN- γ) production *in vitro* (1). Inclusion of anti-sense B7-H3 oligonucleotides decreases the expression of B7-H3 on DCs and inhibits IFN- γ production by DC-stimulated allogeneic T cells (1). In addition, stimulation with B7-H3 transfectants preferentially upregulated the proliferation and IFN- γ production of CD8⁺ T cells. The contribution of B7-H3 to the costimulation of CD8⁺ T-cell responses has been confirmed by our lab and others through the successful induction of cytotoxic lymphocytes (CTLs) and anti-tumor immunity by B7-H3-transfected tumor cells (14-17).

TLT-2 has been shown to be a costimulatory TCR for B7-H3 (13). Transduction of TLT-2 into T cells resulted in enhanced IL-2 and IFN- γ production via interactions with B7-H3. Blockade of the interaction between B7-H3 and TLT-2 with mAb against B7-H3 or TLT-2 efficiently inhibited contact hypersensitivity responses mediated by CD8⁺ T cells (13).

Studies using B7-H3 knockout (KO) mice also support a costimulatory role of B7-H3. In an allogeneic transplantation model, treatment with the immunosuppressant rapamycin alone does not increase survival of grafts. In sharp contrast, cardiac and islet allografts survived indefinitely in B7-H3 KO mice with rapamycin treatment (18). Survived allografts showed markedly decreased production of major cytokine, chemokine, and chemokine receptor mRNA transcripts as compared to wildtype mice. Chronic rejection in two different cardiac allograft models was also inhibited in B7-H3 KO in conjunction with rapamycin treatment. These results indicate that B7-H3 functions to promote T-cell responses that mediate acute and chronic allograft rejection. Therefore, these data support that B7-H3 could promote T-cell function through TLT-2 by costimulation.

Is B7-H3 also a coinhibitor?

While B7-H3 has been shown to be a costimulatory molecule in our laboratory and others, several studies suggest that B7-H3 could also inhibit T-cell responses. Recombinant mouse and human B7-H3 proteins were shown to inhibit anti-CD3 mAb-induced T-cell proliferation, cytokine production, and activation of transcription factors, such as nuclear factor for activated T cells (NFAT), nuclear factor κ B (NF- κ B), and activator protein-1 (AP-1) (3,19,20). In B7-H3 KO mice, T helper 1 (Th1)-mediated hypersensitivity and the onset of experimental autoimmune encephalomyelitis (EAE) are augmented, and treatment with an anti-B7-H3 mAb exacerbated EAE (3,20). B7-H3 was also implicated in inhibiting Th2-mediated immune reactions when administration of blocking anti-B7-H3 mAb during the induction phase augmented the severity of Th2-mediated experimental allergic conjunctivitis (21). In a separate study, contact between CD4⁺CD25⁺ regulatory T cells (Tregs) and DCs leads to upregulation of B7-H3 on DCs, which when blocked with anti-B7-H3 showed increased CD4⁺ T-cell proliferation in a mixed lymphocyte reaction. The data

thus suggest that DC-associated B7-H3 induced by Tregs impairs T-cell stimulatory function (22).

Whereas these results suggest that B7-H3 is a negative regulator, it is unclear at the present time what factors are behind these contradicting observations. It is unknown if these inhibitory effects are mediated through the TLT-2 receptor. It is also worrisome that many so-called 'neutralizing antibodies' may not be just blocking antibodies but have other effects such as triggering the B7-H3 signal. Until these relatively simple issues are addressed, we may then have to entertain the idea that an additional receptor mediating inhibitory responses for B7-H3 may be present.

B7-H3 and cancer immunity

Expression of B7-H3 on the murine P815 mastocytoma line by transfection increases the immunogenicity of tumor cells (14). After inoculation of B7-H3-transfected tumor cells into syngeneic DBA/2 mice, approximately 50% of the mice became resistant to an otherwise lethal challenge of mock-transfected tumor cells. By transfer of TCR transgenic T cells reacting to endogenous tumor antigen P1A, regression of B7-H3+tumor cells is demonstrated to be accompanied with rapid expansion of tumor-antigen-specific CTLs *in vivo* (14). In this model, CD4⁺ T cells are not required for the induction of CD8⁺ CTL as CD4 depletion did not decrease tumor immunity to B7-H3-transfected P815. Similarly, intratumoral injection of an expression plasmid encoding murine B7-H3 led to complete regression of 50% of EL-4 lymphomas and significantly slowed tumor growth, which was mediated by CD8⁺ T cells and NK cells, and not CD4⁺ T cells (17).

Studies in other cancer models in mice have also shown that expression of B7-H3 leads to activation of tumor-specific CTLs that can slow tumor growth or eradicate tumors (14,15,23). In a hepatocellular carcinoma model, intratumoral administration of B7-H3-expressing plasmid and arsenic trioxide synergized to completely eradicate an established tumor (23), although administration of a single agent did not. In addition, mice with B7-H3-transfected colon cancer had significantly prolonged survival times (15). Moreover, when colon cancer cells established in the cecum of BALB/c mice were treated by intratumoral injection of an adenovirus expressing mouse B7-H3, results showed primary tumor regression as well as reduction of secondary metastasis *in vivo* (24). These results indicate that a high level of expression of B7-H3 may be a beneficial factor for immune responses against tumor antigens.

Many human cancers are also found to naturally express B7-H3. One early study in our laboratory showed that commonly used human cancer lines in the laboratory, such as HL-60 promyelocytic leukemia, K562 myelogenous leukemia, SW480 colon adenocarcinoma, A549 epithelial lung adenocarcinoma, and G361 melanoma, were found to express high level of B7-H3 mRNA (1). Subsequent studies demonstrate that prostate cancer, non-small-cell lung cancer, gastric carcinoma, ovarian cancer, renal cell carcinoma, urothelial cell carcinoma, and neuroblastoma, also express B7-H3 (1,5,7,25-28). Therefore, expression of B7-H3 on human cancers appears to be a fairly general phenomenon.

Several retrospective studies were performed attempting to correlate the expression of B7-H3 and cancer progression. A study in gastric carcinoma correlates elevated B7-H3 expression by cancer with an increased survival rate in patients (29). However, several other studies in other cancers do not concur with this finding. B7-H3 expression by either clear cell renal cell carcinoma (RCC) or the tumor vasculature was found to significantly associate with an increased risk of death from RCC (26). Likewise, a marked increase in B7-H3 expression was observed in the majority of prostate cancers, and statistical analysis

indicated that this expression is associated with poor prognosis. (30). It is noted that polyclonal antibodies to human B7-H3 were employed for this study.

There are several possible interpretations for these seemingly contradictory data. B7-H3 may use another receptor besides TLT-2 for inhibitory function. Therefore, depending on the affinity of differential receptors, tumor-associated B7-H3 may have distinct functional effects on receptor-bearing cells. It has been shown that natural expression of low level CD80 on murine colon cancer lines are inhibitory, presumably through the dominant binding of the CTLA-4 inhibitory receptor on T cells (31). This result is different from many studies showing that over-expression of CD80 by transfection is consistently costimulatory for T-cell-mediated tumor immunity by engaging the CD28 costimulatory receptor (31). High level expression of B7-H3 by transfection thus may engage TLT-2 to costimulate T-cell responses while other cancers which express low levels of B7-H3 may engage an inhibitory receptor. This hypothesis is yet to be tested in future studies. Another possibility is that cancers may express aberrant forms of B7-H3 on the cancer cell surface. In our own study, mAb from different hybridomas, although they all appear to be specific for human B7-H3, interact with tumor cells in a highly diverse pattern, even to the same tumor (Luo *et al.*, unpublished data). B7-H3 is heavily glycosylated, and differential glycosylation patterns may contribute to this diversity.

In addition to its effect on T cells, B7-H3 could also affect other immune cells. 5B14, a mAb which was generated against the ACN neuroblastoma cell line, reacted with all neuroblastoma cell lines as well as tumor cell infiltrates in bone marrow aspirates from neuroblastoma patients. This mAb was shown to enhance NK cell-mediated lysis of B7-H3⁺ tumor cells on either transfected or freshly isolated neuroblastoma cells (7). This result suggests that B7-H3 molecules expressed at the tumor cell surface exert a protective role from NK-mediated lysis by interacting with a still undefined inhibitory receptor expressed on NK cells.

B7-H4 as a co-inhibitor of T-cell response

B7-H4 (also called B7x or B7S1) was also identified by DNA sequence homology to other B7 molecules in our laboratory and others (32-34). Similar to B7-H3, mRNA encoding B7-H4 is widely distributed in murine and human peripheral tissues (33,35,36). In contrast, the expression of B7-H4 cell surface protein is generally absent in most human normal somatic tissues, except in normal human epithelial cells of the female genital tract, kidney, lung, and pancreas (35). In mice, one study observed broad cell surface expression of B7-H4 protein on mouse hematopoietic cells (32). The reason for the interspecies difference in B7-H4 expression is unclear.

The regulation of B7-H4 expression has only been studied in the human system. IL-6 and IL-10 can stimulate monocytes, macrophages, and myeloid DCs to express B7-H4, which can be downregulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (37-39). Interferons, on the other hand, seem to have little effect on the induction of B7-H4 expression.

The receptor for B7-H4 has not been discovered, although evidence indicates that a receptor can be induced and could function on T cells (32,33). Although B- and T-lymphocyte attenuator (BTLA) was initially proposed to be the receptor for B7-H4 (34), further studies show that this is not the case (40-42).

In vitro anti-CD3-mediated T-cell activation assays with plate-bound B7-H4Ig have indicated that B7-H4 inhibits CD4⁺ and CD8⁺ T-cell proliferation, cytokine production, and generation of alloreactive CTLs, by arresting the cell cycle (32-34). B7-H4 expressed on the

surface of surrogate APCs also inhibits T-cell proliferation (33,34). Likewise, *in vivo* blockade of endogenous B7-H4 by specific mAb promotes T-cell responses, indicating an inhibitory role for B7-H4 (33). B7-H4 KO mice in a BALB/c background (backcrossed for 5 generations) mounted mildly augmented Th1 responses and displayed slightly lowered parasite burdens upon *Leishmania major* infection compared to wildtype mice, suggesting an inhibitory role for B7-H4 in Th1 responses (43). With mice in a C57BL/6 $\times$ 129/Ola mixed genetic background, B7-H4 KO mice did not show any signs of autoimmunity or disruption of immune-cell homeostasis (43). In addition, B7-H4 KO mice of a mixed background mounted normal CTL responses against viral infections and normal hypersensitive inflammatory responses mediated by Th1 or Th2 cells (43). These results suggest that endogenous B7-H4 may be a fine tuner of the adaptive immune response.

B7-H4 as an inhibitor of innate immunity

By analysis of an independently created B7-H4 KO strain in our laboratory, we found an unexpected function of B7-H4 in the control of neutrophil growth (44). Initial studies showed that B7-H4 KO mice are more resistant to challenge of an otherwise lethal dose of *Listeria monocytogenes* (LM) than their littermates. Neutrophil numbers are increased in the peripheral organs of B7-H4 KO mice more so than their littermates, and neutrophils, through anti-Gr1 antibody depletion, are shown to be crucial for the elimination of bacterial infection. In contrast, other cell components remain unchanged. The resistance to LM is still observed in B7-H4 KO mice without the recombining activating gene-1 (RAG-1) gene, which have developmental deficiencies in T cells, B cells, γ - δ T cells, and NKT cells, supporting that innate immunity, and not adaptive immunity, is important in early control of LM infection. These data indicate a new function of B7-H4 as an important negative regulator of innate immunity through growth inhibition of neutrophils.

Interestingly, mice deficient in both B7-H4 and RAG-1 (double KO) have significantly more neutrophils than single B7-H4 KO mice in virtually all organs examined thus far, even in the absence of an infection (44). Upon lethal dose LM challenge, however, B7-H4 KO mice have more long-term survivors than double KO mice, suggesting that in addition to innate immunity, adaptive immunity is important in completely controlling LM and that B7-H4 KO mice may have enhanced adaptive immunity.

In addition, neutrophil-progenitor cell proliferation in wildtype bone marrow is significantly slower than those from B7-H4 KO mice (44). Moreover, the addition of recombinant B7-H4 *in vitro* inhibited the proliferation and division of Gr⁺CD11b⁺ neutrophil progenitors from bone marrow. The results indicate B7-H4 can inhibit cell-cycle progression of neutrophils. Cell apoptosis was also tested as a possible mechanism of B7-H4 inhibition. However, there was no increase in apoptosis in the bone marrow cultures from B7-H4 KO mice. Because neutrophil progenitors require granulocyte-colony stimulating factor (G-CSF) and stem cell growth factor to proliferate in culture, the role of B7-H4 could be simply interpreted as its antagonistic effect on these growth factors.

B7-H4 in cancer immunity

B7-H4 is found to be expressed in many human cancers. In our initial study, the majority of ovarian carcinoma (22 out of 26) was found to express high levels of B7-H4 (35). A follow-up study with large samples demonstrated that B7-H4 is overexpressed in ovarian papillary serous adenocarcinoma (88%), while mucinous and low malignant potential ovarian cancers and normal tissues were negative for B7-H4 (45). B7-H4 is also overexpressed at both the mRNA and protein levels in ductal (100%) and lobular (100%) adenocarcinoma of the breast and uterine endometrial cancers (46). By immunohistochemistry 95% of primary breast cancers and 98% of metastatic breast cancers (invasive lobular and invasive ductal)

are positive for B7-H4, independent of tumor grade or stage (36). Significant association was found between a high proportion of B7-H4 positive cells in invasive ductal carcinomas and a decreased number of tumor-infiltrating lymphocytes (47). In addition, B7-H4 expression was observed in lung cancer with lymph-node metastasis (35), renal cell carcinoma associated with poor survival (48), and prostate cancer associated with disease spread, recurrence, and death (28). Five out of 16 lung carcinomas were also found to express B7-H4 (35), whereas all 17 melanoma specimens were found negative. B7-H4 is preferentially expressed in non-dividing brain tumor cells and in a subset of brain tumor stem-like cells (49). Expression of B7-H4 in human tumors is most likely due to aberrant regulation of post-transcription in tumors, since in normal human tissue cell surface protein expression is rare, even though abundant B7-H4 mRNA is detected.

The role of B7-H4 in immune evasion in the cancer microenvironment is yet to be elucidated. It has been shown *in vitro* that B7-H4 dominantly inhibits T-cell responses to costimulation through arrest of cell-cycle progression, resulting in inhibition of proliferation and cytokine secretion (33). Other studies show that increased B7-H4 expression on tumor cells correlated with both decreased apoptosis and enhanced outgrowth of tumors in several models, including a severe combined immunodeficiency (SCID)/Beige xenograft outgrowth model (45). B7-H4 has also been shown to be extensively and variably N-glycosylated, which may serve as a 'barrier' mechanism to evade immunosurveillance, similar to the tumor shielding observed with B7-H1 (45). Therefore, the role of B7-H4 in tumor progression may be to transform pre-cancerous cells and then protect them from immunosurveillance. Further studies of the regulatory mechanisms and signaling pathways leading to B7-H4 expression will aid in understanding tumor immune evasion and provide additional targets for treating human cancer.

In addition to tumor cells, tumor-infiltrating macrophages (37,38) and endothelial cells of small blood vessels (48) in the cancer microenvironment are also found to constitutively express B7-H4. B7-H4 was found to be highly expressed on tumor-associated macrophages in the ascites of ovarian cancer patients and may contribute to tumor progression (37). B7-H4 blockade by anti-sense oligonucleotides restored the function of macrophages to stimulate T cells and lead to tumor regression *in vivo*. Further studies showed that tumor-associated CD4⁺CD25⁺forkhead box p3(Foxp3)⁺ Treg cells can trigger APCs including macrophages to produce IL-6 and IL-10 (38). These cytokines stimulate macrophage expression of B7-H4, whereas GM-CSF and IL-4 inhibit expression (37). Interestingly, since IL-4, IL-6, IL-10, and GM-CSF have not been found to regulate B7-H4 expression on tumor cells, B7-H4 on tumor cells may be functionally distinct from those on APCs and may be differentially regulated (38,48). In agreement with this, both Treg cells and macrophage B7-H4, but not tumor B7-H4, were negatively associated with patient outcome (38). B7-H4⁺ tumor macrophages may be a novel suppressor cell population in ovarian cancer that can be targeted therapeutically (37).

Summary

Recent data indicate that B7-H3 and B7-H4 primarily function in peripheral tissues to fine tune immune responses in target organs. While broad tissue distribution is observed for the mRNA of both molecules, limited protein expressions suggest that tight control is imposed at the post-transcriptional level. The recent identification of the new costimulatory receptor for B7-H3 will advance the understanding of the role for B7-H3 in immune regulation; however, more study is needed to understand the mechanism behind the observed inhibitory function of B7-H3. Further study also is needed to identify the inhibitory receptor for B7-H4. Under pathogenic conditions, such as inflammation and cancer, increased protein expression of B7-H3 and/or B7-H4 represent a realistic opportunity to design new

immunotherapeutic approaches and to fine tune the immune response through the manipulation of the expression of these molecules and/or their receptors.

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