**B7-H4 Expression by Nonhematopoietic Cells in the Tumor Microenvironment Promotes Antitumor Immunity**

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**Abstract**

The B7 family plays a critical role in both positive and negative regulation of immune responses by engaging a variety of receptors on lymphocytes. Importantly, blocking coinhibitory molecules using antibodies specific for CTLA-4 and PD-1 enhances tumor immunity in a subset of patients. Therefore, it is critical to understand the role of different B7 family members since they may be suitable therapeutic targets. B7-H4 is another member that inhibits T-cell function, and it is also upregulated on a variety of tumors and has been proposed to promote tumor growth. Here, we investigate the role of B7-H4 in tumor development and show that B7-H4 expression inhibits tumor growth in two mouse models. Furthermore, we show that B7-H4 expression is required for antitumor immune responses in a mouse model of mammary tumorigenesis. We found that the expression levels of B7-H4 correlate with MHC class I expression in both mouse and human samples. We show that IFNγ upregulates B7-H4 expression on mouse embryo fibroblasts and that the upregulation of B7-H4 on tumors is dependent on T cells. Notably, patients with breast cancer with increased B7-H4 expression show a prolonged time to recurrence. These studies demonstrate a positive role for B7-H4 in promoting antitumor immunity. Cancer Immunol Res; 3(2); 184–95. ©2014 AACR.

**Introduction**

Members of the CD28/B7 family of coregulatory molecules have been shown to play a critical role in the modulation of tumor-specific immune responses (1). A related family of molecules, B7-H (homolog), has been identified and consists of six members: B7-DC (PD-L2), B7-H1 (PD-L1), B7-H2, B7-H3, B7-H4 (B7x, B7S1, VTCN1, and DD-0110), and B7-H6 (reviewed in refs. 2, 3). B7-H4 was cloned and characterized in 2003 (4–6) and was reported to be expressed on cells of the nonlymphoid lineage, including the lung, ovary, prostate, and pancreas (5, 7). Although controversial, studies have also shown that B7-H4 is induced upon stimulation of T cells, B cells, monocytes, and dendritic cells (5, 7, 8).

B7-H4 binds to an unknown receptor on activated T cells and has been implicated as a negative regulator of T-cell proliferation, cell-cycle progression, and IL2 production (4–6). Studies using anti-B7-H4 or B7-H4Ig have shown that blocking B7-H4 interactions inhibits T-cell proliferation, cytokine secretion, and CTL function. B7-H4 knockout mice do not develop spontaneous autoimmunity and have a relatively mild phenotype compared with Cdl4−/− or Pd-1−/− mice (9). Mice with B7-H4 deficiency were shown to be more resistant to Streptococcus pneumoniae pulmonary infection. Notably, these mice had lower bacterial loads, which coincided with an increase in activated CD4+ or CD8+ T cells and fewer neutrophils in the lungs (10). B7-H4 was also shown to negatively regulate neutrophil sensitivity to Listeria monocytogenes infection via inhibiting the expansion of neutrophil progenitors in the bone marrow. Consequently, B7-H4−/− deficient mice were less susceptible to Listeria infection due to enhanced neutrophil-mediated innate immunity (11).

High expression of B7-H4 has been reported in tumor-cell lines as well as tumor tissues (5). Overexpression of B7-H4 has been shown in non–small-cell lung cancer (12), serous, endometrioid, and clear-cell ovarian cancer (13–16), breast cancer (13, 17), uterine cancer (18), prostate cancer (19), renal cancer (20), gastric cancer (21, 22), pancreatic ductal adenocarcinoma (23), and esophageal cancer (24). In ovarian cancer, high B7-H4 staining was observed in invasive carcinomas (16). Furthermore, high expression of B7-H4 was associated with cancer progression (20).
Although B7-H4 is upregulated in a variety of tumors, its role in cancer has not been examined extensively. Overexpression of B7-H4 was shown to promote transformation and tumor formation in epithelial cells, and was proposed to have a direct effect on tumorigenesis, independent of the immune system (13, 14). Notably, B7-H4 was suggested as a cellular morphogenetic factor that directly affects cell proliferation and apoptosis, indicating that it may be required for tumor progression (25).

Studies have shown that B7-H4 has a potential impact on immune surveillance in the tumor microenvironment. A series of studies by Kryczek and colleagues (26–28) have proposed that regulatory T cells (Treg), but not conventional T cells, trigger the production of IL6 and IL10 by macrophages, leading to the upregulation of B7-H4 and inhibition of proliferation of tumor-specific T cells. Collectively, results from these studies suggest that B7-H4 upregulation limits natural immune surveillance of tumors.

In this study, we investigated the role of B7-H4 in mammary tumor development, as well as its impact on antitumor immune responses. In sharp contrast to the negative modulatory role previously ascribed to B7-H4, results from our studies demonstrate that B7-H4 expression inhibits tumor growth and is required for antitumor immunity.

Materials and Methods

Animals and virus

Lymphocytic choriomeningitis virus glycoprotein (LCMV-gp) was cloned under the expression control of 2.5-kb whey acidic protein (WAP) promoter. WAP-gp transgenic mice were generated in the C57BL/6 background. WAP-gp mice were crossed with MMTV-PyMT (originally from Dr. William Muller, McGill University, Montreal, QC, Canada, but have been bred into the C57BL/6 background and kindly provided by Dr. Sandra Ghendler, Mayo Clinic, Scottsdale, AZ). WAP-gp/MMTV-PyMT double transgenic mice were then crossed with B7-H4+/− mice (9) to generate WAP-gp/MMTV-PyMT/B7-H4+/− mice. Rag2−/− mice were purchased from The Jackson Laboratory. Mice were housed in micro-isolator cages in the animal colony at the Ontario Cancer Institute (OCI; Toronto, ON, Canada). Mouse studies were performed in an animal biosafety level-two facility under a protocol approved by the OCI Animal Care Committee. The LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. Mice were infected intravenously with 200 plaque-forming units (PFU) LCMV-WE.

Histology

Histologic analysis was performed on tissue embedded in Histocryl (Fisher Scientific), submerged in ice-cold isopentane, and followed by snap freezing in liquid nitrogen. Sections were stained with anti-mouse CD8 (BD Biosciences). Images were acquired with the Zeiss imaging microscope (Carl Zeiss, Canada Ltd.) equipped with bright-field capabilities and a digital camera (Carl Zeiss, Canada Ltd.). Images were processed with Adobe Photoshop version 7.0 (Adobe Systems, Inc.). Images were quantified for CD8 positivity using Aperio ImageScope software (v11.2; Aperio). Percent positivity (total number of positive pixels/total number of pixels) of the whole sections was acquired with the software’s calculated value set using the recommended algorithm.

Flow cytometry

Tetramer and intracellular staining was performed as described previously (29). Briefly, single cells or peripheral blood lymphocytes (PBLs) were stained using phycoerythrin (PE)-labeled gp33/MHC class I tetramers for 15 minutes at 37°C, then stained with anti–CD8-PerCP (clone 53-6-7; BD Biosciences) for 30 minutes at 4°C followed by RBC lysis. Monomers were purchased from Baylor College of Medicine Tetracer facility (Houston, TX). For intracellular cytokine staining, single cells were stimulated with either gp33 or the negative control adenovirus (AV) peptide together with GolgiPlug for 6 hours. Cells were then stained for anti–CD8-PerCP (clone 53-6-7; BD Biosciences) for 30 minutes at 4°C followed by fixation and permeabilization using Cytofix solution (BD Biosciences) according to the manufacturer’s protocol. Cells were then stained with PE-conjugated anti-TNFα (clone TN3-19.12; eBiosciences) or allophycocyanin (APC)-conjugated anti-IFNγ antibodies (clone XM16.2; eBiosciences). Data are expressed as mean ± SEM. Differences between two different groups were analyzed using the Student t test. P values of <0.05 were considered as statistically significant. For granzyme B staining, cells were first stained for anti–CD8-PerCP for 30 minutes at 4°C, followed by fixation and permeabilization using Cytofix solution. Cells were then stained with PE-conjugated anti-granzyme B antibody (clone FGB12; Invitrogen). Data were analyzed using FlowJo software (TreeStar). Human breast tumors were obtained from the University Health Network (UHN) BioBank, as approved by the Research Ethics Board. For staining, tissues were weighed and then digested for 2 hours using digestion buffer [5% calf serum, 1 mg/mL of collagenase (Roche Diagnostics), and 30 μg/mL of DNase (Roche Diagnostics) in PBS]. Single cells were then stained for CD45 (clone 2D1; eBiosciences), B7-H4 (clone MH43; Abcam), and HLA-A, -B, -C (clone G46-2.6; BD Biosciences). Tumor supernatants were collected and cytokines identified using an immunoarray assay kit (eBiosciences). For human correlation studies, breast tumor samples from 20 patients were included. Median Fluorescence Intensity (MFI) for each staining was calculated using FlowJo software. Outcome variables were analyzed with the Student t test and the Spearman correlation coefficient.

Reverse transcription PCR

Tissues from different organs were homogenized. Cells were then lysed using the Qiagen QIAshredder columns, and RNA was isolated using the Qiagen RNaseq Mini Kit (per the manufacturer’s instructions). cDNA was synthesized by annealing 2 μg of RNA at 65°C for 5 minutes in the presence of oligo-dT and reverse transcription was performed at 25°C for 10 minutes, 37°C for 50 minutes, and 70°C for 15 minutes. PCR for gp was performed using following primer sets:

- (forward) 5'-caac gcc aga tta gac gtc tcg c-3'
- (reverse) 5'-ggc tgt ttt gga cat gaa ccc-3'

RNA extraction, cDNA synthesis, and real-time PCR

RNA for real-time PCR was harvested from normal or tumor mammary tissue using the Qiagen RNeasy Mini Kit according to the manufacturer’s protocol. cDNA was synthesized using an iScript cDNA Synthesis Kit as per the manufacturer’s instructions (Bio-Rad). Reaction components were obtained from the LightCycler FastStart DNA Master SYBR Green Kit (Roche Canada) and the LightCycler instrument (Roche), and corresponding software were used for all reactions. The PCR was performed in a final
volume of 20 μL, 0.5 μmol/L of each primer and 5 μL template cDNA (concentration, 100 ng/μL). Primer sets were designed for mouse B7-H4 as:

(forward) 5′-gcc gtt cag caa gtc aag ttt-3′
(reverse) 5′-ccc gtc ctc tcc aat gtt tc-3′

Standard curves were established for each primer set and both reference and target reactions were performed for each sample.

Bone marrow chimeras
Bone marrow from C57Bl/6 or B7-H4−− mice was obtained by flushing the cavities of freshly dissected femurs with PBS and further washed with PBS. Recipient WAP-gp/MMTV-PyMT and WAP-gp/MMTV-PyMT/B7-H4−− mice were irradiated with 900 cGy (137 Cs; Gammacell 40). Four hours later, mice received 3 × 10⁶ bone marrow–derived hematopoietic leukocytes via tail vein infusion. Three months after bone marrow transplantation, mice were bled and immune system reconstitution was confirmed by staining for CD4⁺ and CD8⁺ PBL. Mice were infected with 200 PFU of LCMV strain WE followed by weekly CT scans. Data are expressed as mean ± SEM. Differences between two different groups were analyzed using the Student t test. P values < 0.05 were considered as statistically significant.

Cytotoxicity assay
Designated mice were infected with LCMV. Eight days later, splenocytes were isolated and used as effector cells and incubated
B7-H4 Is a Positive Regulator of Antitumor Immunity

Results

B7-H4 plays an important role in limiting tumor growth

We examined the MMTV-PyMT mouse model to assess the role of B7-H4 on mammary tumor growth. These transgenic mice express the polyoma middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter, and spontaneously develop mammary tumors. Our initial characterization focused on determining whether tumors from this model upregulate B7-H4 expression. Normal mammary tissues and mammary tumors from MMTV-PyMT mice were excised and RNA extracted from multiple tissues from C57Bl/6, MMTV-PyMT, and WAP-gp/MMTV-PyMT mice. Data shown are representative of three independent experiments. T, thymus; S, spleen; K, kidney; O, ovary; L, lung; M, mammary. C, MMTV-PyMT mice. Data shown are representative of three independent experiments. D, MMTV-PyMT (n = 8) and WAP-gp/MMTV-PyMT (n = 13) mice were infected with LCMV and the CD8 response was evaluated in the peripheral blood by staining with a gp33-specific tetramer. E, MMTV-PyMT (n = 8) and WAP-gp/MMTV-PyMT (n = 13) mice were infected with LCMV and % gp33+CD8+/total CD8+ T cells in the peripheral blood were calculated. F, CFSE-labeled LCMV-gp-specific P14 T cells were transferred into WAP-gp/MMTV-PyMT or control MMTV-PyMT mice. Data shown are representative of three independent experiments. G, MMTV-PyMT (n = 12) and WAP-gp/MMTV-PyMT (n = 11) mice were infected with LCMV and the production of IFNγ and TNFα were evaluated in peripheral blood CD8+ T cells by intracellular flow cytometry, and the percentage of IFNγ+ CD8+ (H) or TNFα+ CD8+ (I)/total CD8+ T cells in the peripheral blood were calculated. Cells were also cocultured with antigen-presenting cells expressing a control AV peptide and cytokine production was below 1% for IFNγ and for TNFα (data not shown).

siRNA transfection

Small interfering RNA (siRNA) duplexes for B7-H4 were obtained from Origene. SKB-R3, MCF-7, and HCC-1143 cell lines were transfected with a mixture of 10 μg of a control, scrambled siRNA by HiPerFect Transfection Reagent (Qiagen). At 48 hours after transfection, cell-surface expression of B7-H4 and HLA-A, -B, -C was determined by flow cytometry.

Patient survival analysis

The Kaplan–Meier plot was based on B7-H4 microarray gene expression data from a set of 2,681 breast cancers. These data are publicly available through the survival analysis tool kmPlotter (30).

with 51Cr-labeled EL-4 target cells pulsed with the gp-specific peptide [KAVYNFAQATM] or a control AV peptide [SGPSNTPFEI] at defined effector to target ratios as indicated. Supernatants were harvested after 5 hours. Percentage specific lysis was calculated as (experimental lysis – spontaneous lysis)/total lysis – spontaneous lysis) × 100. Spontaneous lysis was below 25% of the total lysis.

Published OnlineFirst December 19, 2014; DOI: 10.1158/2326-6066.CIR-14-0113
Figure 3. Tumor immunity is impaired in the absence of B7-H4. A, WAP-gp/MMTV-PyMT (n = 10) and WAP-gp/MMTV-PyMT/B7-H4<sup>−/−</sup> (n = 10) mice were infected with LCMV and the CD8<sup>+</sup> T-cell response was evaluated in the peripheral blood by staining with a gp33-specific tetramer. B, % gp33<sup>+</sup>CD8<sup>+</sup>/total CD8<sup>+</sup> T cells in the peripheral blood were calculated. WAP-gp/MMTV-PyMT (n = 6) and WAP-gp/MMTV-PyMT/B7-H4<sup>−/−</sup> (n = 6) mice were infected with LCMV. C, IFNγ production was evaluated in CD8<sup>+</sup> T cells from peripheral blood along with (D) % IFNγ<sup>+</sup>CD8<sup>+</sup>/total CD8<sup>+</sup> T cells was calculated. E, TNFα production was also evaluated in CD8<sup>+</sup> T cells from peripheral blood. F, Percentages of TNFα<sup>+</sup>CD8<sup>+</sup>/total CD8<sup>+</sup> T cells in the peripheral blood were calculated. (Legend continued on the following page.)

Rahbar et al.
C57Bl/6 donor mice were isolated and transplanted into irradiated WAP-gp/MMTV-PyMT (n = 31). WAP-gp/MMTV-PyMT/B7-H4/C0 (n = 5) mice were infected with LCMV and monitored by weekly CT scans. Total tumor volume was quantified and expressed as fold-change compared with week 16. H, bone marrow cells from B7-H4/C0 or C57Bl/6 donor mice were isolated and transplanted into irradiated WAP-gp/MMTV-PyMT (n = 5; total of 32 tumors) and WAP-gp/MMTV-PyMT/B7-H4/C0 (n = 4; total of 30 tumors) mice, respectively. After reconstitution, chimeric mice were infected with LCMV, and monitored by weekly CT scans. I, bone marrow cells from C57Bl/6 or B7-H4/C0 donor mice were isolated and transplanted into irradiated WAP-gp/MMTV-PyMT (n = 8; total of 67 tumors) and WAP-gp/MMTV-PyMT/B7-H4/C0 (n = 4; total of 31 tumors) mice, respectively. After reconstitution, chimeric mice were infected with LCMV, and the tumor burden was monitored by weekly CT scans.

We evaluated the role of B7-H4 in mammary tumor development. Tumor growth was compared between MMTV-PyMT and MMTV-PyMT/B7-H4/C0 mice (Fig. 1D). By 20 weeks of age, larger tumors were observed in MMTV-PyMT/B7-H4/C0 mice (Fig. 1E), indicating that the primary tumors had already progressed to advanced or late carcinoma stage (31).

We next examined the role of B7-H4 in another tumor type using the RIP-Tag2 model. RIP-Tag2 transgenic mice express the SV40 large antigen (Tag) under the control of the rat insulin promoter (RIP), and the mice develop insulinomas (32). As β-cell islets become hyperplastic and eventually form tumors, more insulin is made leading to decreased blood glucose levels. In this model, tumor burden can be monitored by following blood glucose levels. RIP-Tag2 transgenic mice were bred onto the B7-H4/C0 background and the impact of B7-H4 on tumor growth was examined by monitoring blood glucose levels over time. Notably, RIP-Tag2 mice survive longer compared with RIP-Tag2/B7-H4/C0 mice (Supplementary Fig. S1). Blood glucose levels of RIP-Tag2/B7-H4/C0 mice were consistently lower than that of RIP-Tag2 mice, indicating that RIP-Tag2/B7-H4-deficient mice developed a greater tumor burden (Fig. 1F). These data reveal that the absence of B7-H4 results in enhanced tumor growth in two different oncogene-driven tumor models.

Expression of B7-H4 is necessary for antitumor immunity

To evaluate whether B7-H4 acts as a key negative regulatory molecule and inhibits antitumor immune responses, it was important to be able to evaluate T-cell immunity to a defined tumor antigen. Therefore, we generated WAP-gp transgenic mice, which express the LCMV-gp in mammary epithelial cells under the control of the WAP promoter (Fig. 2A; ref. 33). WAP is a major component of milk and is expressed late during pregnancy and throughout lactation (34). Reverse transcription PCR (RT-PCR) analysis from multiple tissues from lactating and non-lactating mice indicated that the LCMV-gp is detectable in the mammary gland from lactating WAP-gp mice, but not in non-lactating WAP-gp mice or C57Bl/6 control mice (Supplementary Fig. S2B). This indicates that the antigen is expressed at the appropriate time during lactation. In addition, previous studies have shown that WAP is induced in mammary tumors (35–38). WAP-gp mice were then bred with the MMTV-PyMT mice to produce WAP-gp/MMTV-PyMT mice. These mice spontaneously develop tumors with a defined tumor-specific marker, LCMV-gp, which enables us to study adaptive immune responses toward the model antigen. RT-PCR analysis of multiple tissues in MMTV-PyMT and WAP-gp/MMTV-PyMT mice indicated that the LCMV-gp was detected in the mammary tissues of WAP-gp/MMTV-PyMT but not in MMTV-PyMT or C57Bl/6 littermate control mice (Fig. 2B).

Therefore, using this model, the LCMV-gp is a well-defined antigen that is expressed upon mammary tumor development, similarly to lactation-specific genes.

It is possible that the expression of LCMV-gp on tumor cells could induce peripheral tolerance of CD8+ LCMV-gp-specific T cells. Therefore, we determined whether LCMV infection led to the induction of a normal antiviral response in the presence of the LCMV-gp+ tumor cells using WAP-gp/MMTV-PyMT and MMTV-PyMT mice. The induction of LCMV-specific CD8+ T-cell responses were normal as measured by gp-specific CTL activity and tetramer staining (Fig. 2C–E). Cytotoxic activity was specific for LCMV-gp peptide-pulsed target cells, because control AV peptide-pulsed targets showed less than 10% specific lysis (data not shown). Adoptively transferred CFSE-labeled P14 T cells recovered from the tumor draining lymph nodes underwent rounds of cell division in LCMV-gp/MMTV-PyMT mice but not in MMTV-PyMT mice (Fig. 2F), indicating that the tumor gp-antigen can be detected in vivo by gp-specific T cells.

In addition, we demonstrated that antigen-specific CD8+ T cells in peripheral blood of LCMV-infected WAP-gp/MMTV-PyMT and MMTV-PyMT mice produced comparable levels of IFNγ or TNFα (Fig. 2G–I). Therefore, LCMV-gp-specific T cells remained in the T-cell repertoire and could be activated after virus infection, despite the presence of tumors expressing LCMV-gp. To examine whether an antitumor immune response could be induced against the LCMV-gp transgenic self-antigen, tumor-bearing MMTV-PyMT and WAP-gp/MMTV-PyMT mice were infected with LCMV and the tumor burdens were monitored by weekly CT scans. As shown in Supplementary Fig. S3, LCMV infection impairs tumor growth in WAP-gp/MMTV-PyMT but not in MMTV-PyMT mice. Taken together, these data show that LCMV-gp is expressed in transgenic tumor-bearing WAP-gp/MMTV-PyMT mice and LCMV infection can induce an antitumor response that limits tumor growth.

To evaluate the potential impact of B7-H4 on antitumor immunity, we bred WAP-gp/MMTV-PyMT on the B7-h4/C0 background. RT-PCR analysis of multiple tissues in WAP-gp/MMTV-PyMT/B7-H4/C0 mice indicated that failure to express B7-H4 had no effect on the expression of the LCMV-gp in the...
mammary gland (Supplementary Fig. S4A). Notably, WAP-gp/MMTV-PyMT/B7-H4−/− mice developed tumors at a similar rate and frequency as MMTV-PyMT and WAP-gp/MMTV-PyMT control mice (Supplementary Fig. S4B). Consistent with data presented in Fig. 1E, increased tumor growth was observed in the absence of B7-H4 (Supplementary Fig. S4C). We then examined the LCMV response in WAP-gp/MMTV-PyMT/B7-H4−/− mice. In agreement with previous work (2, 4–6), an enhanced immune response was observed in the absence of B7-H4 (Supplementary Fig. S5). Accordingly, the LCMV-specific CD8+ T-cell response was enhanced in WAP-gp/MMTV-PyMT/B7-H4−/− mice that were deficient in B7-H4 compared with WAP-gp/MMTV-PyMT control mice (Fig. 3A and B) and increased IFNγ production (Fig. 3C and D) but not TNFα production (Fig. 3E and F). Therefore, these analyses support a negative regulatory role for B7-H4 in terms of T-cell function, as previously demonstrated.

To evaluate the impact of B7-H4 on LCMV vaccine-induced antitumor immunity, tumor-bearing MMTV-PyMT, WAP-gp/MMTV-PyMT, and WAP-gp/MMTV-PyMT/B7-H4−/− mice were infected with LCMV and monitored by weekly CT scans. Surprisingly, inhibition of tumor growth was not observed in LCMV-infected WAP-gp/MMTV-PyMT/B7-H4−/− mice compared with that in WAP-gp/MMTV-PyMT control mice (Fig. 3G).

Given this unexpected phenotype, we extended our analysis of B7-H4 to the RIP-gp/Tag2 insulinoma model. Both the SV40 large Tag and the LCMV-gp are expressed on the β-islet cells and driven by the RIP. These mice spontaneously develop tumors with a defined tissue-specific antigen, LCMV-gp. We generated RIP-gp/Tag2/B7-H4−/− mice and compared them with RIP-gp/Tag2 mice and control RIP-gp/Tag2 mice following LCMV infection, and tumor growth was monitored by blood glucose measurements. LCMV-vaccinated RIP-gp/Tag2 mice demonstrated a reduction in tumor burden as indicated...
by increased blood glucose levels, whereas the absence of B7-H4 limited this response (Supplementary Fig. S6). Therefore, in contrast with the negative regulatory roles in the tumor microenvironment and in T-cell function previously ascribed to B7-H4, our results demonstrate that B7-H4 is a crucial positive mediator of vaccine-induced antitumor immunity.

To evaluate whether B7-H4 expression by bone marrow–derived cells or stromal cells is required for an effective antitumor response, bone marrow chimeric mice were generated. WAP-gp/MMTV-PyMT and WAP-gp/MMTV-PyMT/B7-H4 mice were irradiated and reconstituted with bone marrow cells from B7-h4−/− or C57Bl/6 donor mice. Chimeric mice were infected with LCMV and tumor growth was monitored by weekly CT scans. Notably, reduced tumor growth correlated with the lack of B7-H4 expression by the host and not the bone marrow (Fig. 3H and I). Together, these data demonstrate a new role for B7-H4 expression by the tumor and/or stroma for promoting antitumor immunity.

Reduced expression of granzyme B in T cells from the B7-h4−/− tumor microenvironment

It is clear from studies with LCMV that this virus induces a strong cytotoxic T-cell response specific for the LCMV-gp, and that LCMV-specific tissue pathology is regulated by CD8 T cells (39–42). To understand why the immune response was impaired in the absence of B7-H4, we examined CD8 T cells in the tumor microenvironment. WAP-gp/MMTV-PyMT and WAP-gp/MMTV-PyMT/B7-H4−/− mice were infected with LCMV and the tumors were examined by immunohistochemistry for T-cell infiltration. A high degree of CD8 T-cell infiltration was found in tumors that expressed LCMV-gp, regardless of whether the tumors expressed B7-H4 (Fig. 4A and B). Interestingly, the levels of granzyme B in CD8+ T cells were strikingly reduced in T cells from the tumor (Fig. 4C) and the inguinal lymph nodes (Fig. 4D) from WAP-gp/MMTV-PyMT/B7-H4−/− mice compared with that in WAP-gp/MMTV-PyMT mice. Therefore, in the absence of B7-H4, vaccine-induced CTLs showed reduced granzyme B levels and this corresponded with the inability of the tumor-specific T cells to destroy the tumor.

Association between T cells, IFNγ, and increased B7-H4 in the tumor microenvironment

Previous work has shown that upregulation of coinhibitory family molecules is associated with IFNγ production and T-cell infiltration (43–45). To explore the potential link between IFNγ and B7-H4, we treated mouse embryonic fibroblast (MEF) cells, which do not express B7-H4, with IFNγ. As shown in Fig. 5A, IFNγ upregulated the expression of B7-H4 as well as MHC class I on these cells. To examine whether IFNγ production by T cells may have an impact on the expression of B7-H4 on the tumor, we generated MMTV-PyMT/RAG2−/− tumor-bearing mice that are deficient in T, B, and NKT cells. Several cell types produce IFNγ including Th1, Tc1, NK, and NKT cells (46, 47). MMTV-PyMT/RAG2−/− mice developed tumors 8 weeks earlier compared with...
B7-H4 expression correlates with MHC class I levels

When we analyzed the tumor microenvironment in WAP-gp/PyMT and WAP-gp/PyMT/B7-H4−/− mice, we found that the level of MHC-I expression was lower in tumors from WAP-gp/MMTV-PyMT/B7-H4−/− mice (Fig. 6A). We examined human breast cancer samples to determine whether the association between B7-H4 and MHC class I could be found in patients. Twenty tumor samples were dissociated by enzymatic digestion and single-cell suspensions were stained for CD45, B7-H4, and HLA-A, -B, -C (Supplementary Fig. S7). Our analyses show that HLA-A, -B, -C expression is highly correlated with B7-H4 expression on these human tumor samples (Fig. 6B; Spearman r = 0.8692).

We then studied the direct effect of B7-H4 on MHC class I expression in different human breast cancer cell lines using siRNA methodology. Notably, transfection of SK-BR-3, MCF-7, and HCC-1143 cell lines with specific siRNA against B7-H4, resulted in significant downregulation of HLA-A, -B, -C expression in these lines (Fig. 6C).

These data prompted us to examine whether the level of B7-H4 expression correlates with better prognosis in patients with breast cancer. We performed a meta-analysis of publicly available gene expression datasets. Notably, patient samples were divided into two groups based on their level of B7-H4 expression: higher or lower B7-H4-expressing compared with the median. This analysis showed that high expression of B7-H4 correlates with improved recurrence-free survival in patients with breast cancer (Fig. 6D).

Discussion

B7-H4 limits tumor growth

In this study, we have shown that B7-H4 is upregulated in mammary tumors from MMTV-PyMT mice (Fig. 1A–C). Although the absence of B7-H4 in the MMTV-PyMT model did not have a significant impact on early tumor development (Fig. 1D and Supplementary Fig. S4B), it played an important role in limiting tumor growth over time (Fig. 1E and F and Supplementary Fig. S1C). In the RIP-Tag2insulina model, the absence of
B7-H4 also led to a decrease in blood glucose levels, indicative of an increased tumor burden in RIP-Tag2/B7-H4−/− mice compared with RIP-Tag2/WT mice (Fig. 1F). As a corollary, our analysis showed that high expression of B7-H4 correlated with improved survival in patients with breast cancer (Fig. 6D). Previous studies suggested that the upregulation of B7-H4 was associated with poor prognosis and limited tumor immune surveillance (19–21, 24, 48, 49). Furthermore, high expression of B7-H4 by tumor cells and endothelial cells was associated with cancer progression and poor prognosis in renal cell cancer (20). In contrast with previous reports, results from our studies indicate that B7-H4 expression plays a positive role and limits tumor growth in a mouse insulinoma model and in a mouse model of mammary cancer.

Expression of B7-H4 by the tumor mass is required for antitumor immunity

Although our data support a negative regulatory role for B7-H4 on T cells during LCMV infection, the fact that tumor growth was not reduced after LCMV-vaccination in WAP-gp/MMTV-PyMT/B7-H4−/− mice was unexpected. Results from our studies showed that B7-H4 plays a novel role on nonhematopoietic cells and is essential for promoting efficient antitumor responses. The important role for B7-H4 was demonstrated by the impaired LCMV-induced antitumor response in bone marrow chimeric mice, in which the LCMV-gp+ tumor cells did not express B7-H4 (Fig. 4H). The absence of B7-H4 corresponded with decreased MHC class I expression in MMTV-PyMT/WAP-gp/B7-H4−/− mice (Fig. 6A). Analysis of human breast cancer samples from 20 patients recapitulated the preclinical findings (Fig. 6B). We also demonstrated that MHC class I expression was directly linked with that of B7-H4, as the knockdown of B7-H4 using siRNA in breast cancer cell lines led to decreased MHC class I expression (Fig. 6C). Importantly, CD8+ T cells from tumors or lymph nodes of B7-h4−/− tumor-bearing mice showed reduced levels of granzyme B, suggesting that the continued tumor growth observed in the absence of B7-H4 is related to the inability of CD8+ T cells to maintain high levels of granzyme B expression.

Previous work has shown that PD-L1 expression is induced by IFNγ (43, 44), and there is evidence suggesting that IFNγ-producing T cells upregulate PD-L1 at the site of the tumor (45). Our work demonstrates that IFNγ can induce the upregulation of B7-H4 (Fig. 5A), which is dependent upon the presence of T cells or NKT cells (Fig. 5C). These results support the concept that IFNγ is an important inducer of B7 family members in different tumor settings.

Impact of B7-H4 on tissue immunopathology

Our findings are in contrast with studies from other groups that show B7-H4 has a negative regulatory role in autoimmune models (7, 8). One possible explanation is that the autoimmune models are not dependent upon IFNγ and upregulation of MHC class I. In these models, diabetes occurs by transferring islet-specific transgenic T cells, whereas in the RIP-gp model, transfer of gp-specific transgenic T cells alone does not result in diabetes. Alternatively, these differences may be due to the potential binding of B7-H4 to different undefined receptors.

Blocking negative regulatory molecules in cancer immunotherapy

Blocking the negative signals from interactions of coinhibitory ligands and counter-receptors of the B7 family has shown considerable promise for cancer immunotherapy. The anti–CTLA-4 monoclonal antibody, ipilimumab, improved survival in patients with melanoma (50–52). Similarly, studies have demonstrated that blocking PD-1 interactions leads to clinical responses in a variety of cancers (53–56). Our data suggest that targeting B7-H4 may not be beneficial for immunotherapy, and that a considerable amount of work is needed to understand how the B7 family of molecules regulates immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

This work is funded in part by the Ontario Ministry of Health and Long-Term Care (OMOH LTC). The views expressed do not necessarily reflect those of the OMOH LTC.

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Acknowledgments

The authors thank Dr. Sandra Chendler for providing the MMTV-PyMT mice on a C57Bl/6 background. The authors also thank Dr. Küchi Murakami, Heather MacGregor, and Olivia Chan for critically reading the article. P.S. Ohashi holds a Canada Research Chair in Autoimmunity and Tumor Immunity. R. Rahbar is recipient of the Knudson PDF fellowship.

Grant Support

This work was supported by the Canadian Cancer Society and the Canadian Breast Cancer Foundation. This study was also supported, in part, by the Alexander von Humboldt Foundation (SFA2010) and the German Research Council (SFB974, LA2558/3-1). A. Schildknecht was supported by the Swiss National Fund.

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Received June 11, 2014; revised October 20, 2014; accepted November 11, 2014; published OnlineFirst December 19, 2014.

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B7-H4 Expression by Nonhematopoietic Cells in the Tumor Microenvironment Promotes Antitumor Immunity

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