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Host B7-H4 Regulates Antitumor T Cell Responses through Inhibition of Myeloid-DerivedSuppressor Cells in a 4T1 Tumor Transplantation Model

Joanne Leung*† and Woong-Kyung Suh*†‡

B7-H4, a member of the B7 family of T cell immunomodulatory proteins, has been shown to inhibit T cell responses and neutrophil expansion during bacterial infections. However, the role of B7-H4 in the immune response during tumor growth has been unclear. In this study, we examined the host immune responses in B7-H4-deficient (knockout [KO]) or sufficient (wild-type [WT]) BALB/cJ mice upon transplantation of murine 4T1 carcinoma cells that had little B7-H4 expression. We reveal that host B7-H4 not only dampens the antitumor Th1 responses, but also inhibits the protumor function of myeloid-derived suppressor cells (MDSC). We observed increased expression of both antitumor immune effectors and protumor MDSC-associated transcripts in 4T1 tumors grown in B7-H4 KO mice compared with those grown in WT hosts. Consistently, MDSCs derived from B7-H4 KO mice suppressed T cell proliferation more potently than their WT counterparts. Although the primary growth of 4T1 tumors in B7-H4 KO hosts was similar to that in WT mice, tumors that had grown in B7-H4 KO hosts grew much slower 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 B7-H4 KO hosts allowed outgrowth of 4T1 tumors with reduced immune-evasive capacities against T cells. Thus, B7-H4 can inhibit both antitumor T cells and protumor MDSCs, influencing the immune-evasive character of the outgrowing tumors. These factors should be considered if B7-H4 blockade is to be used for cancer immunotherapy. The Journal of Immunology, 2013, 190:000–000.

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Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; I.E. KO, immunodeleted in B7-H4 knockout; iNOS, inducible NO synthase; KO, knockout; L-NMMA, L-NG-nomonomethyl-arginine; MDSC, myeloid-derived suppressor cell; NSG, NOD- scid IL-2R<sup>g<sub>null</sub></sup>; qPCR, quantitative PCR; TIL, tumor-infiltrating lymphocyte; WT, wild-type.

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the 4T1 tumor transplantation model. We confirm that B7-H4 has a negative regulatory role for Th1-mediated antitumor immunity, yet we also reveal that B7-H4 can inhibit MDSCs. Collectively, we provide evidence that the dual inhibitory roles of B7-H4 on anti- and protumor immune cells have a potential to alter not only the growth of tumors, but the immune-evasive capacities of outgrowing tumor cells. Thus, we uncovered the opposing immune-regulatory functions of B7-H4, which should be considered in the application of B7-H4 blockade in cancer treatment.

Materials and Methods

Mice
Six- to 10-wk-old WT BALB/cJ (The Jackson Laboratory) and B7-H4 KO mice maintained in BALB/cJ backgrounds (N10) were used for all in vitro and in vivo experiments. Generation of B7-H4 KO mice has been described previously (8). Eight-week-old nude mice in BALB/c background (Taconic) and NOD-scid IL-2Rγnull (NSG) mice (The Jackson Laboratory) were used for secondary 4T1 tumor injection experiments. All the animal experiments were performed based on the animal use protocols approved by the Animal Care Committee of Institut de Recherches Cliniques de Montréal.

Cell culture

The murine 4T1 mammary carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). YAC-1 thymoma cells were a gift of A. Veillette (Institut de Recherches Cliniques de Montréal, Montreal, Canada). All the cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS (PeproTech), 100 U/ml penicillin, and 100 µg/ml streptomycin for 7 d. To obtain NK cells, splenic NK cells enriched by EasySep NK Negative Selection kit (StemCell Technologies) were expanded for 5 d in RPMI 1640 media supplemented with murine IL-2 (1000 U/ml; ProSpec-Tech). Cells were cultured in complete RPMI 1640 media supplemented with 10% heat-inactivated FBS (Hyclone), 2-ME, and HEPES. Cells were maintained in CO2 incubator at 37°C in humidified air with 5% CO2. The 4T1 cells were harvested with 0.05% trypsin-EDTA, and viability of cells was determined by trypan blue dye exclusion.

Preparation of MDSCs and NK cells

We generated MDSCs from mouse bone marrow cells, as previously described (31). Briefly, tibiae and femurs of BALB/cJ or B7-H4 KO mice were extracted and minced with scissors in 2 ml HBSS (Life Technologies). Cells and tumor fragments were digested with filter-sterilized collagenase type I (Life Technologies; 10 mg/ml) at 37°C for 1 h on a platform rocker. Subsequently, cells were washed twice with PBS and were prepared for further analysis. Spleen and draining (inguinal) lymph nodes were excised and made into single-cell suspensions by passing the organs through a 70 µm nylon cell strainer. Splenocytes were treated with hypotonic solution to lyse RBCs.

Flow cytometry and cell sorting

Single-cell suspensions prepared as above were washed and resuspended in FACS buffer (1% BSA and 0.05% sodium azide in PBS). After treating with Fc-block (5 min on ice), cells were stained with primary Abs, followed by secondary Abs (20 min at 4°C each). Cells were washed twice with FACS buffer after each staining. Stained cell suspensions were briefly incubated with 7-aminocoumarinycin D (7AAD; BD Pharmingen) and subsequently analyzed using Beckman Coulter Cyan ADP Analyzer. Raw flow cytometry data were analyzed with FlowJo software (Tree Star). Cell populations that are 7AAD− CD45+ were gated as live host hematopoietic cells, and 7AAD− CD45− populations were defined as 4T1 tumor cells. For isolation of tumor MDSC subsets, single-cell suspensions of whole tumors were resuspended in 1% BSA in PBS. Cells were then stained with anti-CD11b, anti-Ly6G, and 7AAD. Following this, 7AAD− viable cells were sorted on MoFlo (Beckman Coulter) based on CD11b−Ly6G− or CD11b−Ly6G+ expression. Anti-CD45 (30-F11), anti-CD11c (N418), anti-CD3 (145-2C11), anti-CD49d (DX5), anti-CD44 (H1/44), anti-CD45 (30-F11), and secondary Abs were purchased from eBioscience. Anti-Ly6G (1A8) Ab was obtained from BioLegend and anti-Ly6C (AL-21) from BD Pharmingen. Anti-mouse B7-H4 Ab, isotype control, and secondary Ab were from R&D Systems.

Quantitative PCR

Total RNA was isolated from tumor single-cell suspensions using the RNeasy Mini Kit (QiaGen), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. The names of genes and their primer sequences are listed in Supplemental Table 1. All reactions were performed as follows: 5 min at 45°C, 3 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles. The amount of a given transcript was normalized against the amount of hypoxanthine phosphoribosyltransferase in the same sample. The relative abundance was determined for both the least abundant sample (set to a unit) and the amount of the relative abundance of each group, and then dividing values from B7-H4 KO samples by respective WT values.

In vitro NK cell killing assay

To analyze NK cell cytotoxicity in the presence of MDSCs, NK cells were obtained from BALB/cJ mice, as described above, and cocultured with MDSCs derived from WT or B7-H4 KO bone marrow cells for 4 h at a ratio of 10:1 (MDSC to NK). Afterward, 1Cr-labeled YAC-1 target cells were added (3 × 105 cells/well) to each well. After a 4-h incubation period, the supernatants were taken to measure 51Cr release using a microplate scintillation counter (Packard). For iNOS inhibition experiments, NK cells were treated with L-NG-monomethyl-arginine (L-NMMA) or L-NG-nitroarginine (L-NNA) for 6 h before analysis.

In vitro MDSC suppression assay

To measure suppressive activities of MDSCs on T cell proliferation, we followed a protocol established by others (32). Briefly, bone marrow–derived MDSCs in complete RPMI 1640 media were added to 96-well plates (at 2-fold serial dilutions starting from 3 × 105 cells/well) that were coated with anti-CD3 (3 µg/ml) and anti-CD28 (2 µg/ml). Afterward, RBC-lysed splenocytes were added to the Ab-coated, MDSC-containing wells (6 × 105 cells/well). Subsequently, [3H]thymidine was added (1 µCi/well) to the wells for the last 8 h of a 1- or 2-d culture periods. DNA was harvested onto 96-well filter plates after lysing the cells with water using Filtermate harvester (Packard), and [3H]thymidine incorporation was measured with a microplate scintillation counter (Packard). For INOS inhibition experiments, WT splenocytes were labeled with CFSE (Invitrogen) and used as responders in cocultures without or with 0.5 mM L-NG-nitroarginine (L-NMMA); Calbiochem). After a 3- or 4-d culture period, cells were harvested and stained with anti-CD4, anti-CD8, and 7AAD. Viable cells were then gated based on CD4+ or CD8+ expression, and CFSE was analyzed via flow cytometry. The proliferation index was calculated using ModFit software. In some experiments, spleens from tumor-bearing mice
were mechanically disrupted, and splenic CD11b+ cells were purified by CD11b microbeads (Miltenyi Biotec). These cells were typically >85% CD11b+Gr1+ MDSCs by FACS analysis. MDSC–splenocyte cocultures were set up as above, and [3H]thymidine was added for the last 8 h of day 1 culture period. Remaining CD11b+ and CD11b+ splenocytes were used for quantitative PCR (qPCR) analysis.

In vitro 4T1 experiments

For proliferation assays, 4T1 tumors were made into single-cell suspensions, as described, and selected in 6-thioguanine–containing medium. After selection, equal numbers of 4T1 cells derived from WT or B7-H4 KO hosts were plated in 96-well flat-bottom plates in triplicates and left overnight in culture. On days 1 and 2, [3H]thymidine was added for the last 8 h of incubation periods to measure the proliferation. For IFN-γ stimulation experiments, 1 × 10⁴ 4T1 cells were plated in a 96-well plate in duplicates and stimulated with 10 ng/ml murine rIFN-γ (PeproTech) for 24 h. Afterward, 4T1 cells were harvested and stained for MHC II, PD-L1, and B7-H4, and then analyzed via flow cytometry.

Statistical analyses

Prism software was used to determine statistical significance by unpaired Student *t* tests (two-tailed).

Results

Absence of B7-H4 in host does not change the growth of 4T1 tumors

The 4T1 mammary carcinoma cell line, derived from a spontaneous mammary tumor in a MMTV+ BALB/c mouse, is a well-studied model of transplantable tumor growth (33). Because 4T1 cells are resistant to 6-thioguanine, cells that have grown as tumors can be isolated by culturing in vitro in the presence of 6-thioguanine for further analyses. The 4T1 cells were also found to be negative for the expression of surface B7-H4 compared with the human breast cancer cell line SKBR3 and murine mammary epithelial cells ectopically expressing B7-H4, NMuMG-B7H4 (Supplemental Fig. 1A). We also confirmed that B7-H4 is not induced on the surface of 4T1 cells by IFN-γ treatment, whereas MHC class II and PD-L1 (also known as B7-1H) can be easily detected under the same conditions (Supplemental Fig. 1B). To determine the role of B7-H4 in the host immune system, 4T1 cells were injected s.c. into the flanks of either WT or B7-H4 KO mice, and tumor growth kinetics and host immune reactions were analyzed.

Within the 21 d of growth, 4T1 tumor volume in B7-H4 KO mice was comparable to that of tumors from WT hosts (Fig. 1A, left). Consistently, final tumor weights were also similar (Fig. 1A, right). To further examine the impact of B7-H4 on primary tumor growth, single-cell suspensions of ex vivo 4T1 tumors were analyzed by flow cytometry. In accordance with tumor growth, the tumor microenvironment of mice deficient or sufficient for B7-H4 had similar percentages of T cells, macrophages, dendritic cells, and NK cells over the total CD45+ host hematopoietic cells (Fig. 1B). Moreover, the level of MDSCs, which accumulate during tumor growth and reflect the degree of tumor burden (28), was also similar in the tumors of WT and B7-H4 KO mice (Fig. 1B). Peripheral lymphoid organs, including the spleen and draining lymph nodes, also displayed comparable percentages of macrophages, T cells, and MDSCs (Supplemental Fig. 2).

Despite similarities in tumor growth, 4T1 tumors from B7-H4 KO host displayed a modest, yet significant increase in the percentage of total tumor-infiltrating immune cells (CD45+ live cells over total live cells) relative to tumors grown in WT hosts (Fig. 1B, total immune cells). However, there were no significant changes in the percentages of immune cell subpopulations, including T cell subsets and MDSC subsets comprising CD11b+Ly6G+ granulocytic MDSCs and CD11b+Ly6C+ monocytic MDSCs (Fig. 1B).

Host B7-H4 influences the cytokine expression profiles within 4T1 tumors

The 4T1 tumors grow more aggressively in IFN-γ-deficient mice (34), and, in the context of a L. major infection, T cells from B7-H4 KO mice showed augmented Th1 responses evidenced by elevated T-bet expression and IFN-γ production (8). Thus, we analyzed IFN-γ expression in tumors taken from WT or B7-H4 KO mice. Intracellular staining of IFN-γ in tumor-infiltrating T cells did not generate meaningful results due to the paucity of IFN-γ-producing cells (data not shown). However, it has been shown that upregulation of MHC class II and PD-L1 on the surface of 4T1 tumors in vivo reliably reflects biologically active IFN-γ in the tumor microenvironment (34). Thus, we examined the expression of these two proteins in 4T1 tumors grown in WT versus B7-H4 KO hosts. Tumors derived from B7-H4 KO mice had elevated expression of MHC class II both in the percentages and in the expression levels (Fig. 2A). Likewise, IFN-γ mRNA levels in the total tumor lysates showed a consistent difference (Fig. 2B). Therefore, our data suggest that 4T1 tumors grown in the absence of B7-H4 experienced elevated levels of IFN-γ compared with those grown in WT hosts.

Despite higher levels of IFN-γ in tumors grown in the absence of B7-H4, the overall tumor growth rate was unaltered. Thus, we hypothesized that the presence of antitumor and protumor immune factors counterbalanced each other in tumors grown in B7-H4 KO mice. We tested this idea by a comprehensive examination of the cytokine expression patterns in the 4T1 tumors by qPCR.

Consistent with the known negative regulatory role of B7-H4 for Th1 responses in bacterial infection and keeping in line with the elevation of IFN-γ in 4T1 tumors grown in B7-H4 KO hosts, we saw that multiple Th1-associated factors were upregulated in tumors derived from B7-H4 KO mice, as follows: T-bet, IL-2, and IL-12 subunits (Fig. 2B). In contrast, the expression pattern of cytokines associated with cytotoxic immune cells (CTL and NK) was similar between tumors grown in WT versus B7-H4 KO hosts, as follows: granzyme B, granzyme A, perforin, IL-15, and NKG2D (data not depicted).

Examination of immune-suppressive factors yielded more insightful results. The levels of transcripts encoding iNOS were ~4-fold higher in tumors harvested from B7-H4 KO mice compared with those from WT control (Fig. 2C). Similar differences were found in both monocytic and granulocytic MDSC subsets (Fig. 2C). We also noticed a marginal increase of arginase-1 mRNA (Fig. 2C). Because iNOS and arginase-1 are the hallmark effector proteins used by MDSCs (27), these data strongly suggest that B7-H4 normally acts as a negative regulator of MDSC as well as Th1 responses.

B7-H4 affects the immunosuppressive activity of MDSCs

Next, we determined whether MDSCs derived from B7-H4 KO mice display differences in T cell-suppressive activity relative to WT MDSCs in vitro. To this end, bone marrow cells harvested from WT and B7-H4 KO mice were cultured in the presence of GM-CSF and IL-6 to induce MDSC differentiation (31). No difference was seen between genotypes in the generation of MDSC populations based on the expression patterns of Gr-1 and CD11b (Fig. 3A). Bone marrow–derived MDSCs from WT or B7-H4 KO mice were cocultured with WT splenocytes in the presence of plate-bound CD3 and CD28 Abs, and T cell proliferation was measured by a thymidine incorporation assay. Remarkably, MDSCs from B7-H4 KO mice were more suppressive on T cell proliferation than MDSCs from WT mice (Fig. 3B).

Because MDSCs are known to suppress the cytotoxicity of NK cells (35–37), we tested whether B7-H4 deficiency affects
MDSC-mediated suppression of NK killing of tumor cells. We measured NK cell cytotoxicity against 51Cr-labeled YAC-1 target cells in the presence or absence of bone marrow–derived MDSCs. We found that MDSCs effectively suppressed NK cell killing, but no difference was seen between MDSCs from WT or B7-H4 KO mice (Fig. 3C).

Therefore, these in vitro results indicate that, in the absence of B7-H4, MDSCs have a stronger ability to inhibit T cell proliferation, but an equal capacity to inhibit NK cells compared with WT MDSCs. We next compared the immunosuppressive capacities of splenic MDSCs isolated from WT or B7-H4 KO mice bearing 4T1 tumors. Consistent with results from bone marrow–derived MDSCs, CD11b+ splenic cells (>85% MDSCs by FACS analysis) from B7-H4 KO tumor-bearing mice had much more potent suppressive activities against T cell proliferation compared with their WT counterparts (Fig. 3D). Importantly, B7-H4 KO MDSCs had higher levels of iNOS expression, but similar levels of gp91 [a key component of NOX2, the major reactive oxygen species–generating enzyme in MDSCs (38)] (Fig. 3E) and undetectable levels of arginase 1 (data not depicted). Using bone marrow–derived MDSC, we confirmed that the iNOS inhibitor, L-NMMA, abrogated the ability of MDSC to suppress T cell proliferation (Fig. 3F).

Taken together, these data indicate that MDSCs generated in the absence of B7-H4 have heightened immunosuppressive function that relies on the elevated expression of iNOS.

Gemcitabine treatment differentiates tumor growth rates

Our model predicts that B7-H4’s dual inhibitory effects on T cells and MDSCs counterbalance each other, leading to equal tumor growth rates in WT and B7-H4 KO mice. We reasoned that in-
Inhibition of MDSCs would accentuate the impact of elevated antitumor T cell responses and consequently change tumor growth patterns. To test this idea, we treated 4T1 tumor-bearing mice with gemcitabine, a chemotherapeutic drug that preferentially inhibits MDSCs in addition to its tumoricidal effects (39). As expected, 4T1 tumors grew slower in mice that were treated with gemcitabine compared with untreated mice, with concomitant reduction in iNOS expression (Fig. 4A, 4B). Remarkably, we noticed that, upon gemcitabine treatment, 4T1 tumor growth was more drastically reduced in B7-H4 KO hosts compared with that in WT hosts.

**FIGURE 2.** Differential expression of anti- and protumorigenic genes in 4T1 tumors grown in B7-H4 KO versus WT hosts. (A) Surface expression levels of MHC II and PD-L1 on 4T1 tumor cells. Live tumor cells (7AAD− CD45−) were gated to measure the levels of MHC class II and PD-L1. Data depict one of three independent experiments with similar results. Each data point represents one tumor. (B and C) Levels of immune effector transcripts in ex vivo 4T1 tumors. Tumor lysates from WT (n = 6 tumors) and KO (n = 8 tumors) were analyzed via quantitative PCR for the indicated transcripts, and the relative abundance or the fold change relative to WT was calculated, as described in Materials and Methods. Subsets of tumor MDSC were sorted, as described in Materials and Methods, prior to qPCR analysis. All qPCR data were mean ± SEM and show one of two independent experiments with similar results. *p < 0.05, **p < 0.01.
The difference in tumor growth rates was well correlated with signs of elevated IFN-γ expression (Fig. 4C). These data strengthen our view that MDSCs negate the enhanced antitumor T cell activities in B7-H4 KO hosts, and that a combination of gemcitabine and B7-H4 blockade may be a good therapeutic option.

Host B7-H4 influences the immune-evasive capacity of 4T1 tumors

Accumulating evidence indicates that there is ongoing immune–tumor interactions during tumor progression that shape the immune-evasive nature of tumors (immunoediting processes) (40), such that tumors developed in Rag KO mice grow slower than those developed in WT mice when reinfected into immune-competent WT hosts (40, 41). Furthermore, an intact T cell compartment has been shown to be important in preventing occult tumor cells from outgrowing (42). We reasoned that if augmented immunosuppressive components prevail over antitumor immune components in B7-H4 KO hosts, 4T1 tumor cells that have grown in B7-H4 hosts should grow slower when reinjected into immune-competent WT hosts compared with 4T1 tumors that have grown in the presence of B7-H4.

To test this possibility, 4T1 primary tumors were extracted and selected in media containing 6-thioguanine. Pure populations of 4T1 cells that had grown in either B7-H4 KO or WT mice were reinjected into WT mice. Remarkably, tumors that had grown in the absence of B7-H4 (immunoedited in B7-H4 KO [I.E. KO hereafter]) grew substantially slower than tumors that had grown in B7-H4–sufficient host (I.E. WT hereafter) (Fig. 5A, left). Consistent with this, when mice were sacrificed after 13 d, the weights of I.E. KO tumors were 3 times less than I.E. WT tumors (Fig. 5A, right). To rule out the possibility that I.E. KO tumors intrinsically proliferate at a slower rate than I.E. WT, we performed an in vitro proliferation assay. Notably, tumors from both groups had comparable rates of proliferation (Supplemental Fig. 3), indicating that the discrepancies in tumor growth in vivo were most likely due to differential immune-evasive capacities stemming from differences in immunoediting during the primary 4T1 growth in either WT or B7-H4 KO hosts.
Next, we tested whether the diminished tumor growth of I.E. KO cells after reinjection into WT hosts correlated with their greater immunogenicity (as a consequence of reduced immune-evasive capacity) by examining the host response toward I.E. KO versus I.E. WT tumors. Indeed, we observed a substantially higher number of CD4 T cells infiltrating into I.E. KO tumors and a marginal increase of T cells in the spleen (Fig. 5B). Consistent with this, higher levels of transcripts for immune factors involved in antitumor responses were detected in I.E. KO tumors, as follows: IL-2, IL-12, T-bet, granzyme B, and perforin (Fig. 5C).

Taken together, these data suggest that the enhanced immunosuppression observed in the primary 4T1 tumor growth in B7-H4 KO mice allowed for the expansion of 4T1 cells with diminished immune-evasion capacities and higher immunogenicity, which led to slower growth when reinjected into immune-competent hosts.

**B7-H4–mediated immunosuppression primarily targets T cells in vivo**

To determine the immune components that were responsible for the slow growth of I.E. KO 4T1 tumors compared with I.E. WT counterparts, I.E. KO and I.E. WT 4T1 cells were injected s.c. into the flanks of NOD-scid IL-2Rγnull (NSG) mice that lack T cells and NK cells, two main antitumor immune cell subsets. Strikingly, I.E. KO and I.E. WT tumors displayed similar growth kinetics and comparable final weights (Fig. 6A). Consistently, tumor immune cell infiltration (CD45+) was also similar, as was the percentage of MDSCs within the tumor milieu (Fig. 6A). These results demonstrate that it is the immune pressure that differentiates the secondary growth of I.E. KO versus I.E. WT 4T1 tumors. More importantly, based on the phenotype observed in NSG mice, the immune cell components responsible for the immune–tumor interactions are most likely T and/or NK cells as opposed to myeloid cells.

To further distinguish the contributions of T cells and NK cells, we repeated the experiment in BALB/c nude mice, which lack only T cells, but have an intact NK cell compartment (43, 44). Akin to our previous observations with NSG mice, I.E. KO and I.E. WT 4T1 tumors grew at similar rates, and also had comparable final tumor weights (Fig. 6B). Furthermore, little differences were observed in the total immune cell population as well as MDSCs and NK cells infiltrating into the tumor microenvironment (Fig. 6B).

We further confirmed that the growth rates of I.E. WT and I.E. KO 4T1 cells were equalized in host BALB/c mice that were depleted of CD4 or CD8 T cells by Ab treatments. In contrast, depletion of NK cells did not abrogate the difference (Fig. 6C). Thus, these data strongly implicate a role for T cells, but not NK cells, in mediating the differential growth of immunoedited 4T1 tumors in immune-competent WT hosts. This is consistent with our view that, during the primary 4T1 tumor growth, MDSC-mediated suppression of antitumor T cell activities in the tumor microenvironment of B7-H4 KO mice facilitates an outgrowth of 4T1 cells that have diminished immune-evasive capabilities against T cells. Also congruent to this finding is the pronounced inhibitory effect of MDSCs from B7-H4 KO mice toward T cells as opposed to NK cells in vitro.
Discussion

To date, the elevated expression of B7-H4 protein has been well documented in human cancers, yet little is known as to how B7-H4 functions to influence tumor growth. Our study provides new insight into the role for B7-H4 in shaping the tumor microenvironment. In addition to confirming that B7-H4 has a negative regulatory role in T cell responses during antitumor immunity, we showed in this work that B7-H4 also has the capacity to hinder the function of MDSCs. This is supported by the data that 4T1 tumors in B7-H4-deficient mice have increased T cell- and MDSC-associated transcripts. Furthermore, treatment with the chemotherapeutic drug gemcitabine, which is known to suppress MDSCs in addition to its antitumor effects, accentuated elevated IFN-γ responses in B7-H4 KO mice and concomitantly delayed tumor growth in B7-H4 KO hosts to a greater extent than it did in WT hosts.

Although the opposing effects of antitumor T cells and protumor MDSCs led to no changes in the growth of primary 4T1 tumors, they significantly influenced the immunoediting process. This was evident, as a secondary injection of 4T1 cells that have grown in B7-H4 KO mice into WT hosts resulted in slower tumor growth and greater immunogenicity relative to 4T1 tumors grown in WT mice. Differences in tumor growth were abrogated when 4T1 cells from either group were injected into T cell-deficient or T cell-depleted mice, supporting the notion that 4T1 tumors that had grown in the absence of host B7-H4 developed reduced resistance to T cell-mediated immune attack. This indicates that the enhanced MDSC suppression in B7-H4 KO mice is mainly targeted toward T cells in vivo, consistent with results from in vitro MDSC suppression assays.

To date, the putative B7-H4 receptor has been detected at a low level on the surface of activated T cells by staining with B7-H4-Fc (2, 7), but its identity remains elusive. In addition, the expression pattern of B7-H4 varies in myeloid cells. For example, B7-H4 is highly expressed on the surface of tumor-associated macrophages in human ovarian cancer and plays a key role in inhibition of antitumor T cell responses (4). However, neither B7-H4 nor the putative B7-H4 receptor was visualized in murine neutrophils despite a clear negative role for B7-H4 in the expansion of neutrophils during Listeria infection (10). Similarly, we had difficulties in detecting B7-H4 or the putative B7-H4 receptor on the surface of MDSCs. Presumably, these proteins are expressed at low levels in most conditions. Identification of the B7-H4 receptor and elucidating its signaling mechanisms should facilitate better understanding of B7-H4 function.
The relative importance of B7-H4 expression in tumor cells versus host immune cells has also been speculative. In the setting of antitumor immunity, B7-H4 overexpression on tumor cells was thought to play a dominant role. It is possible that abundant B7-H4 proteins on the surface of tumor cells can impair the effector functions of tumor-infiltrating lymphocytes akin to a molecular shield model proposed for PD-L1 (45, 46). In support of this concept, it has been shown that the quantity of B7-H4 on the surface of pancreatic β cells positively correlates with their resistance to T cell attack in murine models of type I diabetes (9). However, our data have shown that, regardless of tumor B7-H4 expression, host B7-H4 still contributes to differences in both pro- and antitumor immune components, which drives the differences observed in immunoediting between WT and B7-H4 KO mice. In keeping with this, B7-H4 deficiency or blockade also led to augmented T cell responses during experimental autoimmune encephalomyelitis (2, 9) and anti-Leishmania responses (8), situations in which B7-H4’s role should be pronounced during T cell priming. Given that host B7-H4 affects the antitumor T cell immunity with little B7-H4 in tumors in our 4T1 model, B7-H4-mediated immunotherapies need to be considered for patients even without B7-H4 overexpression.

There is some evidence that B7-H4 may play immune-independent, tumor-intrinsic roles in tumorigenesis. An ectopic overexpression of B7-H4 in human ovarian cancer cells led to enhanced tumor growth in SCID mice (13). In the same study, knockdown of B7-H4 in human breast cancer cells rendered them more susceptible to anoikis in vitro, although the mechanism was not clear (13). We also found that ectopic overexpression of B7-H4 in immortalized murine mammary epithelial NMuMG cells partially protected them from anoikis (J. Leung and W.-K. Suh, unpublished observations). In contrast, Ab-mediated ligation of surface B7-H4 in EBV-transformed human B cells or B cell lymphoma cell lines induced apoptosis or cell cycle arrest, respectively (47, 48). Ligation of B7-H4 overexpressed in NMuMG cells, however, did not cause apoptosis or cell cycle arrest (J. Leung and W.-K. Suh, unpublished observations). Therefore, these apparently conflicting data suggest that B7-H4 may regulate
cell death or proliferation in certain types of cells. In an attempt to address the role of B7-H4 expressed in tumor cells in our model, we sought to overexpress B7-H4 in 4T1 cells by transfection, but failed to obtain 4T1 clones overexpressing B7-H4 despite an extensive effort. Obviously, further investigation is required to establish the tumor-intrinsic and -extrinsic roles of B7-H4 during in vivo tumor progression. We are currently developing mouse models to address these questions.

Many types of solid tumors display abundant expression of B7-H4 to various extents, and the majority of studies have found a link between B7-H4 overexpression and poor prognosis as judged by invasiveness, metastasis, recurrence, and mortality (15, 18–25). Given that another inhibitory B7 protein, PD-L1, has been known to be overexpressed in multiple cancers and blockade of PD-1/PD-L1 pathway is currently in clinical trials (49), comparisons between B7-H4 and PD-L1 can provide insight as to how B7-H4 overexpression may influence tumor progression and immunotherapeutic outcomes. Notably, a recent study revealed that PD-L1 is preferentially expressed in human melanoma subtypes driven by BRAF mutations and PD-L1 expression is geographically colocalized with CD8 tumor-infiltrating lymphocytes (TILs) and IFN-γ (50). Therefore, at least in melanoma patients, PL-L1 overexpression appears to be induced as an adaptive mechanism in response to immune attack, but not driven by oncogenic processes such as loss of PTEN that is known to upregulate PD-L1 expression in gliomas (51). Paradoxically, this study also showed that among the patients with metastatic melanoma who received immunotherapies, PD-L1 overexpression is positively correlated with overall patient survival, contrasting previous studies that showed no difference or a negative correlation (52, 53). This is consistent with a newly emerging notion that a pre-existing T cell–inflamed tumor microenvironment (which correlates with upregulation of PD-L1 and possibly other immunosuppressive markers) predicts better responsiveness to immunotherapies (54). Unlike PD-L1, however, several pieces of evidence suggest that B7-H4 overexpression can be largely driven by oncogenic processes rather than antitumor immunity. First, in contrast to the expression patterns of PD-L1, a small study on melanoma patients found that a high level of B7-H4 was not associated with the degree of CD8 T cell infiltration (24). In fact, several reports documented that B7-H4 expression in other types of tumors negatively correlated with T cell infiltration (24). In fact, several reports documented that B7-H4 is not highly induced in 4T1 tumor cells under conditions in which PD-L1 and MHC class II were abundantly expressed, presumably in response to IFN-γ–producing T cells. Second, expression of B7-H4 in human breast cancer cell lines has been shown to be dependent on phosphoinositide 3-kinase/mTOR/S6kinase signaling, a pathway frequently altered in cancer (55). Third, human B cells express a high level of B7-H4 upon EBV-mediated transformation in vitro without apparent immune attack (47). Taken together, these findings suggest that overexpression of B7-H4 in cancer cells could be largely an outcome of oncogenic processes and may be associated with the low immunogenic nature of the developing tumor. Therefore, more detailed studies are required to validate B7-H4’s value as a predictive biomarker and to optimize therapeutic strategies targeting B7-H4, as overexpression of negative immune modulators may not always predict poor prognosis and immunotherapeutic outcome. Based on what is now known for PD-L1, it is important to examine B7-H4 expression patterns with regard to the tumor subtypes, geographical distributions of TILs/inflammatory cytokines, and the medical history of the patients. In addition, our current study suggests that examination of the relative locations of B7-H4–overexpressing tumor cells, TILs, and MDSCs in the tumor microenvironment may provide additional insights as to how B7-H4 works in human cancer.

Most studies to date have implicated B7-H4 as a biomarker of tumors with poor prognosis (13, 15, 18–25). As such, B7-H4 blockade has been suggested in the treatment of cancers, especially those with elevated B7-H4. Our data show that, in addition to inhibiting T cell responses, B7-H4 also negatively regulates MDSCs, and thus, inhibition of B7-H4 may result in immune-suppression as we have seen in our 4T1 model. Importantly, these differences are driven by a differential expression of B7-H4 in the host immune cells. We also analyzed anti- and protumor immune responses in WT and B7-H4 KO hosts (C57BL/6 background) using a B16F10 murine melanoma (B7-H4–negative) transplantation model. Consistent with our 4T1 model, we observed increased IFN-γ expression in B7-H4 KO hosts (data not depicted). However, the level of MDSC infiltration and iNOS expression was lower in the B16F10 model compared with the 4T1 model (~28% of CD45+ cells in B16F10 versus ~70% of CD45+ cells in 4T1 model), and there was no significant increase of MDSC/iNOS expression in B7-H4 KO mice. One possibility to explain this is that the dual inhibitory effects of host B7-H4 become pronounced for tumors that induce strong MDSC responses. Therefore, our results suggest that, for tumors that elicit robust MDSC responses, beneficial effects of B7-H4 blockade could be maximized when it is delivered in conjunction with treatments that inhibit MDSCs (e.g., gemcitabine). Also, tumors without B7-H4 overexpression may still respond to such treatments.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure Legends

**Supplemental Figure 1.** 4T1 cells do not express surface B7-H4. (A) 4T1 cells maintained in vitro or extracted from WT or B7-H4 KO mice ex vivo, SKBR3 cells, or NMuMG-B7H4 cells were stained with anti-B7-H4 (thick line) or isotype control Ab (shaded) and analyzed by FACS. Cells were gated on 7AAD- populations. (B) 4T1 cells express MHC II, PD-L1 but not B7-H4 upon IFN-γ treatment. 4T1 cells were stimulated with IFN-γ for 24 hours (thick line) or no treatment (shaded). After 24 hours, 4T1 cells were harvested and stained with anti-MHC II, PD-L1 and B7-H4 Ab, and analyzed by FACS. Results shown are representative of two independent experiments.

**Supplemental Figure 2.** WT or B7-H4 KO mice bearing 4T1 tumors have similar levels of macrophages (CD11b+F4/80+), MDSCs (CD11b+Gr-1+) and T cells (CD4+/CD8+) in peripheral lymphoid organs. Viable immune cells were analyzed by FACS as described in Materials and Methods. Data depict one of three independent experiments. Each data point represents one mouse.

**Supplemental Figure 3.** Tumors grown in WT or B7-H4 KO mice proliferate at similar rates in vitro. Equal numbers of I.E. WT or I.E. KO 4T1 cells were plated in a 96-well plate in triplicates, and [3H]-thymidine was added for the last 7 hours on days 1 and 2. Data show mean ± SEM of six I.E. WT and eight I.E. KO 4T1 cell lines and depict one of two independent experiments with similar results.
Supplemental table 1. List of primers used for quantitative PCR (Tm = 60°C)

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Spleen macrophages

Percentage of macrophages (%)

WT  B7-H4 KO

0  10  20  30  40  NS

Spleen T cells

Percentage of T cells (%)

CD8+  CD4+

WT  B7-H4 KO

0  10  20  30  NS

Spleen MDSC

Percentage of MDSCs (%)

WT  B7-H4 KO

0  20  40  60  80  100  120  140  NS

LN T cells

Percentage of T cells (%)

CD8+  CD4+

WT  B7-H4 KO

0  20  40  60  NS