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B7-H3 Enhances Tumor Immunity In Vivo by Costimulating Rapid Clonal Expansion of Antigen-Specific CD8+ Cytolytic T Cells

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B7-H3 is a B7 family molecule with T cell costimulatory function in vitro. The in vivo role of B7-H3 in the stimulation of tumor immunity is unclear. We report here that expression of B7-H3 by transfection of the mouse P815 tumor line enhances its immunogenicity, leading to the regression of tumors and amplification of a tumor-specific CD8+ CTL response in syngeneic mice. Tumor cells engineered to express B7-H3 elicit a rapid clonal expansion of P1A tumor Ag-specific CD8+ CTL in lymphoid organs in vivo and acquire the ability to directly stimulate T cell growth, division, and development of cytolytic activity in vitro. Our results thus establish a role for B7-H3 in the costimulation of T cell immune responses in vivo. The Journal of Immunology, 2004, 173: 5445–5450.

By interacting with cognate counterreceptors on T cells, the B7 family molecules play critical roles in the control and fine-tuning of Ag-specific immune responses. A classic pathway of the B7 family includes B7-1/B7-2 and their receptor CD28/CTLA-4. B7-1 and B7-2 costimulate growth, prevent tolerance, and promote the survival of T cells via the CD28 receptor. Ligation of CTLA-4, however, is postulated to deliver a negative signal. Manipulation of this pathway has profound effects in the control of diseases like cancers, viral infections, autoimmune diseases, and transplantation rejection in animal models and, in some cases, humans (1).

Recent years have witnessed the rapid identification and characterization of new B7 family molecules and their receptors, whereas their role in the positive and negative control of immune responses is just being elucidated (2). B7-H3 was initially identified as a costimulatory molecule with limited amino acid sequence homology to other B7 family molecules for human T cell responses through a distinct receptor from CD28, CTLA-4, ICOS, and PD-1 (3). Although B7-H3 mRNA is widely detected in lymphoid and nonlymphoid organs in human and other species, B7-H3 protein is not constitutively expressed on these tissues (3, 4). Expression of cell surface B7-H3 could be induced on monocytes and dendritic cells by IFN-γ (3). In addition to the originally identified two-domain structure (2IgB7-H3) containing an IgV and an IgC domain for both mouse and human B7-H3, a four-domain structure containing IgV-IgC-IgV-IgC (4IgB7-H3) has also been found in the genomic DNA of humans and monkey, but not in mouse and hamster (4, 5). A recent study using a mAb indicates that the mainly expressed form of B7-H3 on human dendritic cells may be 4IgB7-H3 (6).

Immunological function of B7-H3 is still controversial. Our original study demonstrates that immobilized and cell-associated human B7-H3 costimulates T cell proliferation, selectively induces IFN-γ, and promotes the growth and differentiation of CD8+ CTL against allogeneic Ags, indicating that B7-H3 preferentially costimulates the cellular immune response (3). Injection of mouse B7-H3 plasmid directly into established mouse EL4 lymphoma induced partial regression of tumors and enhanced T cell responses (7). Two recent studies, however, suggest a role of B7-H3 in the inhibition of T cell responses. Ling et al. (5) showed that immobilized 4B7-H3Ig inhibited T cell proliferation in vitro. Lung infiltration of inflammatory cells increased after challenge with a model Ag in B7-H3-deficient mice. The authors concluded that B7-H3 inhibited Th1 T cell responses. In this study, however, inductions of experimental autoimmune encephalitis and CTL to lymphocytic choriomeningitis virus, which are largely dependent on Th1 cytokines, were not affected (8). In this study, we report the role of B7-H3 in the stimulation of T cell-mediated tumor immunity in vivo.

Materials and Methods

Mice and cell lines

DBA/2, CByD2F1/J (DBA/2 × BALB/c), C57BL/6, and BALB/c nu/nu mice were purchased from the National Cancer Institute (Frederick, MD). Transgenic mice expressing TCR specific for tumor Ag P1A in the context of H-2Ld (P1CTL) were described previously (9). P815 mastocytomas were maintained in a complete medium of RPMI 1640 (Invitrogen Life Technologies, Rockville, MD) containing 10% FBS (HyClone, Logan, UT), 25 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate.

Cloning of mouse B7-H3 cDNA and transfection

Full-length B7-H3 cDNA was amplified by RT-PCR from Con A-activated C57BL/6 splenocytes and cloned into pcDNA3 plasmid (BD Clontech, CA58033, and CA69091 and by the Mayo Foundation.

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Palo Alto, CA) as previously described (3, 4). The resulting plasmid mB7-H3/pEMD3 and wild-type (wt) pEMD3 vector were transfected into P815 cells using the Fugene 6 transfection system (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. Cell lines stably expressing mouse B7-H3 were selected by G418 and were screened by flow cytometry analysis with a mouse B7-H3-specific mAb.

**Fusion proteins and mAb**

The fusion protein containing the extracellular domain of mouse B7-H3 and mouse IgG2a Fc was prepared and cloned into pPirGly vector (10). The resulting pmB7-H3Ig plasmid was transfected into Chinese hamster ovary (CHO) cells and cultured in CHO medium (Invitrogen Life Technologies) containing 1% FBS. B7-H3Ig fusion protein was purified from the supernatant by a protein G-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden). A hybridoma (clone 9C11)-secerting Ig Mab against mouse B7-H3 was generated by immunizing an Armenian hamster with purified B7-H3Ig. The specificity of 9C11 was determined by negative staining of CHO cell transfectants expressing mouse B7-1, B7-2, B7-H1, B7-DC, and B7-H4. Hybridomas that produced mAb against CD8 (clone GK1.5) or CD8 (clone 53-6.72) were purchased from American Type Culture Collection. All mAb were purified from culture supernatants of the hybridomas by either protein A- or protein G-Sepharose columns as described previously (11). The mAb to mouse CD3, FITC-conjugated mAb to B220, CD4, or I-A<sup>β</sup>; PE-conjugated mAb to mouse IgG2a; and Cy-Chrome-conjugated mAb to CD8 were purchased from BD Pharmingen (San Diego, CA). Anti-asialo GM1 antisera was purchased from Wako (Osaka, Japan). H-2L<sup>d</sup> Dimer XI was purchased from BD Pharmingen and P1A<sub>Asy</sub>-43 peptide was incorporated into the dimer according to the manufacturer’s instructions.

**T cell proliferation and cytokine assay**

T cell costimulation assays were performed as described previously (12). Briefly, nylon wool-purified T cells from C57BL/6 mice were added at 2.5 x 10<sup>5</sup> cells/well in triplicate into 96-well flat-bottom microplates that were precoated with anti-CD3 mAb overnight at 4°C and subsequently coated with B7-H3Ig or control Ig for 4 h at 37°C. The cells were incubated at 37°C for 72 h and [3H]TdR was added at 1 μCi/well during the last 6 h. The incorporation of [3H]TdR was counted using the MicroBeta Trilux liquid scintillation counter (Wallac, Turku, Finland). To detect cytokines, culture supernatants were collected at 24–72 h and the concentration of cytokines was determined by sandwich ELISA (BD Pharmingen) according to the manufacturer’s instructions.

**P1A-specific CTL generation and cytotoxicity assay**

Naïve or regressor (spontaneous regression after E3 tumor inoculation) mice were challenged with 1 x 10<sup>6</sup> wt P815 cells. Three weeks later, spleens were removed and cultured with irradiated (260 Gy) wt P815 cells at the indicated responder:stimulator ratio for 5 days. The cultured cells were collected on day 5 and CTL activity against wt P815 was measured in a standard 4-h 51Cr release assay. To generate P1A-specific CTL, CD8<sup>+</sup> T cells were negatively selected from a pool of spleen and lymph node cells from P1CTL transgenic mice by incubation with mixed FITC-conjugated mAbs against mouse B220, CD4, and I-A<sup>β</sup>; PE-conjugated mAb to mouse IgG2a; and Cy-Chrome-conjugated mAb to CD8 were purchased from BD Pharmingen (San Diego, CA). Anti-asialo GM1 antisera was purchased from Wako (Osaka, Japan). H-2L<sup>d</sup> Dimer XI was purchased from BD Pharmingen and P1A<sub>Asy</sub>-43 peptide was incorporated into the dimer according to the manufacturer’s instructions.

**Animal studies**

The methods to determine the growth and measurement of tumors in mice were described previously (13). For depletion of CD4, CD8, and asialo GM1 cells in vivo, 500 μg anti-CD4, anti-CD8, or 50 μl of anti-asialoGM1 Ab was injected i.p. on days 0, 7, and 14, respectively, after E3 tumor injection. In a P1CTL reconstitution model, a pool of spleen and lymph node cells from P1CTL transgenic mice were injected i.v. into CByD2F1/J mice at 1 x 10<sup>7</sup>/mouse. The cells were then challenged s.c. with tumor cells on the same day. To determine cell division, the MACS LD-purified CD8<sup>+</sup> T cells from P1CTL mice were labeled with CFSE and adaptively transferred into CByD2F1/J mice that were inoculated with P815/mock or E3 cells 6 days before. Tumor-draining lymph node (TDLN) and spleens were removed on days 2, 3, and 5 and double-stained with P1A/H-2L<sup>d</sup> dimer and CyChroma-conjugated anti-CD8 mAb. Cell divisions were measured by using flow cytometry analysis to calculate the dilution of CFSE intensity as described previously (14, 15).

**Results**

We demonstrated previously that human B7-H3 could costimulate proliferation of human T cells in vitro. As a first step to establish a mouse model for evaluation of the B7-H3 effect in vivo, we examined whether mouse B7-H3 has a similar costimulatory effect in T cell responses. Immobilized B7-H3Ig, a fusion protein between the extracellular domain of mouse B7-H3 and mouse IgG2aFc, enhances proliferation of purified mouse spleen T cells (Fig. 1A) and IFN-γ production (Fig. 1B) in the presence of sub-optimal doses of mAb against mouse CD3. This costimulatory effect of B7-H3Ig is dose dependent. Isotype-matched control IgG2a stimulates neither proliferation nor IFN-γ production of T cells. We conclude that mouse B7-H3 has similar costimulatory functions compared with its human homologue.

We next examine whether expression of B7-H3 on tumor cells could enhance immune responses. To do so, we transfected P815 cells with a plasmid-containing full-length mouse B7-H3 cDNA. Three independently selected transfectants, E3, E6, and D11, that stably express B7-H3 on the cell surface were selected for additional experiments. With a mAb against mouse B7-H3 (clone 9C11), we demonstrated that wt (data not shown) or mock-transfected P815 cells (P815/mock) do not express B7-H3 while the three transfectants express high level of B7-H3 (Fig. 2A). All three transfectants grew similarly to P815/mock line in culture (data not shown). In addition, there are no differences among these lines on MHC class I expression and susceptibility to lysis by activated CTL, specifically against P1A tumor Ag (data not shown).

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<sup>1</sup>Abbreviations used in this paper: wt, wild type; CHO, Chinese hamster Ovary; TDLN, tumor-draining lymph node.

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**FIGURE 1.** Costimulation of T cell response by mouse B7-H3Ig. A, Nylon wool-enriched C57BL/6 mouse T cells were stimulated with the indicated dose of immobilized B7-H3Ig (○) or control Ig (□) in the presence of a suboptimal dose of immobilized mAb to CD3. Data represent one experiment of five and [3H]TdR incorporation is presented as cpm ± SD. B, IFN-γ concentration in 1-, 2-, and 3-day culture supernatants was assessed by sandwich ELISA.
We inoculated P815/mock and the three transfectants s.c. into syngeneic DBA/2 mice to examine their tumorigenicity. All mice inoculated with P815/mock cells developed progressively growing tumors. The mice inoculated with E3 line also developed tumors. However, the growth rate was relatively slow and about one-half of the tumors (44%) regressed completely several weeks after inoculation (Fig. 2B). Similar results were also observed in other lines (data not shown). The survival time of the mice inoculated with the E3 line was extended (Fig. 2C). In contrast, inoculation of E3 cells into BALB/c nu/nu-immunodeficient mice induced progressively growing tumors with similar growth rates to those of P815/mock cells (Fig. 2D). Our results indicate that expression of B7-H3 enhances the immunogenicity of P815 tumor cells.

We next determined the cellular mechanisms of B7-H3-mediated tumor immunity. DBA/2 mice were first inoculated with E3 cells and injected with various Abs to deplete CD4, CD8, or NK cells. Injection of Abs against CD4 or asialo GM1 had a small but not significant effect on the tumorigenicity of E3 cells. However, injection of mAb against CD8 completely eliminated the growth resistance and all mice died of tumors within 30 days (Fig. 3A). Our results thus support a central role for CD8+ T cells in B7-H3-mediated tumor immunity. Furthermore, the mice, which experienced regression of E3 tumor, were also resistant to challenge by lethal doses of wt P815 cells (Fig. 3B) and developed high levels of CTL activity against wt P815 (Fig. 3C), indicating the development of memory T cells. Naive mice after challenged with the same dose of P815 cells developed progressively growing tumors without the induction of specific CTL (Fig. 3D). These results thus offer a more uniform model than regular mice to further analyze B7-H3 costimulation in vivo.

Using the P1CTL adoptive transfer system, we examined the role of B7-H3 on the expansion of tumor-specific CTL. F1 mice were first inoculated with E3 cells. Seven days after inoculation, naive spleen cells from P1CTL transgenic mice were labeled with CFSE and transferred into tumor-bearing F1 mice. Two, 3, and 5 days after T cell transfer, cells from TDLN and spleens were prepared and stained by the P1A dimer, which specifically stains P1A-specific T cells, and gated on CFSE for analysis. Although there was no significant CTL division observed on day 2, a dramatic expansion of P1A-specific CTL were found in both the TDLN and the spleen of E3-bearing mice on day 3. Significant T cell division was also found in P815/mock TDLN, albeit to a lesser degree than those from E3-bearing mice. On day 5, the majority of P1A-specific CTL had undergone at least one cycle of division in E3-bearing mice while some CTL remained unactivated in P815/mock-bearing mice (Fig. 4A). Our results thus support that expression of B7-H3 on tumor cells accelerates division of tumor-specific CTL in lymphoid organs, which may be responsible for enhanced tumor immunity.

Similar to in vivo findings, naive P1CTL cells could be stimulated to proliferate by irradiated E3 cells in a dose-dependent fashion (Fig. 4B) and divide rapidly after stimulation in comparison to mock/P815 cells (Fig. 4C). In addition, cytolytic activity of P1CTL cells against wt P815 cells also increased significantly after stimulation by E3 cells than P815/mock cells (Fig. 4D). Taken together, our results thus support that B7-H3 facilitates the priming of naive T cells to growth and the development of cytolytic activity.

**Discussion**

In this report, we demonstrate that expression of B7-H3 by transfection enhances tumor immunogenicity of a P815 mastocytoma by preferential stimulation of CD8+ T responses in vivo. The role of B7-H3 appears to induce rapid expansion of P1A-specific CTL in vivo, as evidenced by more active division of P1A TCR-transgenic T cells in spleens and TDLN. This observation is correlated
FIGURE 3. Expression of B7-H3 on P815 tumor cells enhances tumor-specific CD8\(^{+}\) CTL responses. A, DBA/2 mice were inoculated s.c. with 5 \(\times\) 10^6 E3 cells. On the same day, the mice were divided into four groups with five mice per group and injected i.p. with mAbs against CD4, CD8, or asialo GM1 on days 0, 7, and 14. The survival of the mice was monitored. B, Mice with regressed E3 tumors (○) were challenged with a s.c. injection of 5 \(\times\) 10^4 wt P815 cells 60 days after primary tumor inoculation. Naïve DBA/2 mice (●) were inoculated with the same number of wt P815 cells as controls. C, Mice after tumor challenge as shown in B were sacrificed at day 21. The splenocytes were prepared and restimulated with irradiated wt P815 cells for 5 days. The CTL activity against wt P815 was assessed in a standard 51Cr release assay. CbyD2F1 mice were injected i.v. with 1 \(\times\) 10^6 splenocytes from P1CTL transgenic mice and inoculated s.c. with 1 \(\times\) 10^6 P815/mock (●) or E3 cells (○) on the same day. The tumor sizes (D) and survival time (E) were monitored.

with their ability to enhance proliferation, division, and development of cytolytic activity of P1A-specific CTL. Our results thus support a role of B7-H3 in the enhancement of CD8\(^{+}\) CTL-mediated tumor immunity in vivo.

We reported previously that the receptor for B7-H3, which could be distinguished from CD28, CTLA-4, and PD-1, is not expressed on resting T cells (3), supporting the hypothesis that B7-H3 is involved in the expansion rather than initial priming of T cell responses. Although our results here support an important role of B7-H3 costimulation in the expansion of T cells in vivo, the effect of B7-H3 in the stimulation of naive or early primed CTL could not be excluded. The observation that B7-H3\(^{+}\) P815 cells induced rapid proliferation and cell division on naive P1TCR CTL in vitro and in vivo (Fig. 4) supports this possibility. Clonal expansion of P1A-specific CTL occurs first in the TDLN and the expression of B7-H3 accelerates this process. Taken together with the observation of the rapid appearance of dividing cells in the spleen, these findings indicate that B7-H3 costimulation rapidly induces not only local expansion of tumor-specific CTL in the TDLN but also systemic responses in the spleen.

High-level expression of costimulatory molecules on tumor cells could enhance direct and bystander costimulation of T cells (16). It is unknown at this time whether these mechanisms, individually or collectively, operate in our model. Direct stimulation/expansion, however, is normally not a major mechanism for tumor Ag-mediated activation of T cells because solid tumors often do not express high levels of such molecules. To test whether the expression of B7-H3 by tumor cells enhances direct stimulation of T cells, we purified P1A-specific CTL CD8\(^{+}\) T cells to move professional APC by MACS bead-guided negative selection (>99% purity) and stimulated them with irradiated P815/mock or E3 cells. Stimulation with E3 cells induced an increased proliferation of T cells, in contrast to P815/mock, in a wide range of responder:stimulator ratios from 1:5 to 1:40. Consistent with this finding, incubation of purified P1CTL with E3 cells induced elevated division of T cells on day 3 and stimulated a stronger CTL response in comparison to the P815/mock cells (Fig. 4C). Our results thus indicate that expression of B7-H3 on tumor cells could directly stimulate T cells in the absence of professional APC. In a recent report, intratumoral injection of a mouse B7-H3 plasmid induced regression of established EL4 tumor in a portion of tumors-bearing mice by CD8\(^{+}\) T cells and NK cells (7). In our system, we did not find the role of NK/NKT cells for the regression of B7-H3\(^{+}\) tumors. One possibility is that plasmid injection led to expression of B7-H3 on APC to enhance an interaction with NK cells because it is unclear whether or not B7-H3 was expressed exclusively by tumor cells or other APC after plasmid injection.

Inoculation of B7-H3\(^{+}\) tumors led to regression on \(\sim\)50% of mice, whereas others eventually developed large tumors and died (Fig. 2). The escaping mechanism underlying this observation is yet to be elucidated. Progressively growing B7-H3\(^{+}\) tumors do not lose the expression of B7-H3 and MHC molecules based on flow cytometry analysis (L. Luo and L. Chen, unpublished data), thus excluding clonal selection of tumor variants. One possible interpretation is elevated regulatory T cell activity in the mice escaping from tumor rejection. We found that depletion of CD25\(^{+}\) T cells by mAb in naive mice before challenge of B7-H3\(^{+}\) tumor led to accelerated rejection (L. Luo and L. Chen, unpublished data), suggesting involvement of regulatory T cells in the regulation of tumor immunity in our system. However, frequency of Ag-specific
T cells may play a key role because adoptive transfer of P1 TCR T cells, which increase frequency of tumor Ag-specific T cells, could lead to regression of 100% tumors in syngeneic mice (Fig. 3). P815 tumors grow very rapidly after inoculation into the mice and it is thus likely that expansion of tumor Ag-specific T cells by B7-H3 expression is suboptimal and does not outpace the growth of tumor cells in the mice.

Experimental tumors could be categorized into immunogenic and poorly immunogenic based on degree of immune responses they elicit in syngeneic hosts and it is, in general, difficult to elicit T cell responses against poorly immunogenic tumors (17). This is partially due to loss of Ag or Ag presentation/processing molecules as well as microenvironment by which a tumor creates during progression. P815 is an immunogenic tumor (17) and, similar to B7-H3, expression of immunomodulatory molecules including the B7 family costimulatory molecules and cytokines could enhance its immunogenicity (18). It remains to be tested whether B7-H3 could also enhance immunogenicity of poorly immunogenic tumors. In our preliminary experiments, we found that transfection to express B7-H3 in a poorly immunogenic C3 tumor (11) failed to enhance its immunogenicity (L. Luo and L. Chen, unpublished data). Since T cells in the mice with growing C3 tumor is in ignorant status (11), our data suggest that the expression of B7-H3 could not break T cell ignorance. In addition, we also found that repeated injection of irradiated B7-H3 + P815 cells did not induce regression of preinoculated P815 tumors (L. Luo and L. Chen, unpublished data). Taken together, our data suggest that B7-H3 costimulation should combine with other immune stimulatory modalities for cancer immunotherapy of established tumors.

A recent study by Suh et al. (8) using B7-H3-deficient mice indicates an enhanced lung inflammatory infiltration by macrophages and lymphocytes and increased IFN-γ secretion after exposure to OVA Ag. They concluded that B7-H3 is inhibitory for Th1-mediated responses. However, there was only a small difference in Th1-mediated responses including experimental autoimmune encephalitis and CTL responses to LCMV viral infection (8), which are believed to be mediated or dependent on Th1 T cell responses. Therefore, it is unclear whether or not B7-H3 preferentially regulates Th1-mediated responses. In our experiments, CD4+ T cells are not required for the induction of CD8+ CTL for tumor immunity since depletion of CD4+ T cells did not diminish the resistance of mice to B7-H3-transfected P815 tumor. One possibility is that B7-H3 may preferentially regulate CD4-independent induction of CD8+ CTL responses as shown in our tumor system. Our findings, however, do not exclude the possibility that absence of CD4+ T cells impairs the generation of memory CD8+ CTL, as indicated in several recent studies (19–21).

Although B7-H3 mRNA has been found in various tissues, surface expression of B7-H3 protein is not evident. B7-H3 is normally undetectable on resting hemopoietic cells and normal organs by immunohistochemistry analysis (L. Luo and L. Chen, unpublished data). However, the expression of B7-H3 protein could be up-regulated by various stimuli on dendritic cells, B cells, and T cells (3). It is thus likely that the expression of B7-H3 is tightly controlled by local assaults such as infection and inflammation. Despite this limited expression pattern, enhanced expression of B7-H3 on tumor cells, as shown in our experiments, has a strong stimulatory function for the expansion of T cell responses. These

**FIGURE 4.** P815 cells expressing B7-H3 induces rapid expansion of tumor-specific CD8+ CTL in vivo. CbyD2F1 mice were first inoculated with 1 × 10^6 P815/mock or E3 cells. On day 6, purified CD8+ P1ACTL cells were labeled with CFSE and injected i.v. into the mice. The TDLN and spleen were removed on days 2, 3, and 5 (A). The cells were stained with P1A/H-2d dimer and CyChrome-conjugated CD8 mAb for gating, and the cell divisions were analyzed by flow cytometry. Purified CD8+ P1ACTL cells were cultured with irradiated P815/mock or E3 cells at the indicated ratio for 48 h. The proliferation of T cells was determined by incorporation of [H]TdR (B) and the CTL activity against P815 was assessed in a standard 51Cr release assay (D). Purified CD8+ P1ACTL cells labeled with CFSE were stimulated with irradiated P815 mock (●) or E3 cells (○) at a 1:20 responder:stimulator ratio. Cell divisions were analyzed by flow cytometry on days 1 and 3 (C).
findings implicate that B7-H3 could be applied alone or incorporated with other immunostimulatory methods, ideally with those promoting T cell priming, to enhance immune responses against cancers.

References


