Blockade of B7-H1 and PD-1 by Monoclonal Antibodies Potentiates Cancer Therapeutic Immunity

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Abstract

Contemporary approaches for vaccination and immunotherapy are often capable of eliciting strong T-cell responses against tumor antigens. However, such responses are not parallel to clinical tumor regression. The development of evasion mechanisms within tumor microenvironment may be responsible for poor therapeutic responses. We report here that constitutive or inducible expression of B7-H1, a B7 family molecule widely expressed by cancers, confers resistance to therapeutic anti-CD137 antibody in mice with established tumors. The resistance is accompanied with failure of antigen-specific CD8+ CTLs to destroy tumor cells without impairment of CTL function. Blockade of B7-H1 or PD-1 by specific monoclonal antibodies could reverse this resistance and profoundly enhance therapeutic efficacy. Our findings support that B7-H1/PD-1 forms a molecular shield to prevent destruction by CTLs and implicate new approaches for immunotherapy of human cancers. (Cancer Res 2005; 65(3): 1089-96)

Introduction

Immunotherapeutic and vaccine approaches, such as genetically modified cancer cells, tumor antigen vaccine, and costimulation, could often elicit T-cell responses in experimental animals and in cancer patients (1, 2). These responses, however, are often not correlated with regression of cancers in clinical trials. This has been attributed to functional deficiencies or tolerance on T cells, including suppression, anergy, ignorance, and programmed cell death in the microenvironment of cancers (1, 2). In several studies, however, tumor-infiltrating T cells in draining lymph nodes are found functionally intact at least *in vitro* (3–7). These findings indicate that mechanisms other than T-cell tolerance may be present within tumors and may reflect our limited understanding of tumor-escaping mechanisms and suggest the importance of the cancer microenvironment in the resistance of immune destruction.

B7-H1 (PD-L1) is a cell-surface molecule of the B7 family with a profound effect on the regulation of T- and B-cell responses (8–10). Whereas B7-H1 mRNA is distributed broadly in normal tissues and organs (8–11), protein expression of B7-H1 is found on macrophage-like cells in the liver, lung, and tonsil (12). The expression of B7-H1 is also found in placenta trophoblasts (13–15), some human epithelial tissues, and myocardium (14, 15) as well as a fraction of dendritic cells (8, 10, 12, 15). B7-H1 could be stimulatory and inhibitory for T-cell functions, perhaps due to ligation of different receptors as well as reverse signaling of B7-H1 to T cells (16, 17). Recent *in vivo* studies indicate that interaction of B7-H1 to programmed death-1 (PD-1), a member of the immunoglobulin superfamily found on activated T, B, and myeloid cells, may induce a negative regulatory signal and inhibit T-cell responses. PD-1-deficient mice develop systemic and organ-specific autoimmune diseases (18, 19). In addition, infusion of neutralizing monoclonal antibody (mAb) against PD-1 increased incidence of experimental autoimmune encephalitis and experimental diabetes (20, 21). On the other hand, B7-H1, as well as its homologue ligand, B7-DC (22, 23), is capable of costimulating growth of T cells from PD-1$^{-/-}$ mice (9, 24, 25). Moreover, mutants of B7-H1 and B7-DC, which have lost their ability to bind PD-1, costimulate normal responses to T cells from both PD-1$^{+/+}$ and PD-1$^{-/-}$ mice *in vitro* (9). Blockade of B7-H1 by neutralizing mAb accelerates progression of inflammatory bowel disease in a mouse model (26). Therefore, B7-H1 could potentially regulate T-cell responses in both positive and negative directions.

Although the role of B7-H1 in the regulation of T-cell responses in inflammation and autoimmunity seems to be complex and is still under debate, B7-H1 is consistently inhibitory on tumor immunity. Constitutive expression of B7-H1 has been found on a majority of freshly isolated human cancers including melanoma and carcinomas of lung, ovary, colon, bladder, breast, cervix, liver, head, and neck and glioblastoma (12, 15, 27, 28). Although surface expression of B7-H1 was only found in a small fraction of cultured cancer lines of human (12) and mouse origin (12, 16, 29), its expression could be rapidly up-regulated by IFN-γ (12). These findings suggest that the expression of B7-H1 on tumor cells is controlled by posttranscriptional mechanisms in the tumor microenvironment. Tumor B7-H1 is found to promote apoptosis of activated effector T cells (12) and to decrease susceptibility of tumor cells to lysis by activated T cells in 4-hour ^{51}Cr release assays (29). Whereas these findings suggest a role of B7-H1 in evasion of tumor immunity, the effect of tumor-associated B7-H1 on cancer immunotherapy and vaccination is not yet evaluated. In this study, we show that B7-H1 expression renders tumor resistance to immunotherapy and cancer vaccination in mouse tumor models and suggest that B7-H1 on tumor cells and PD-1 on T cells may form a "B7-H1/PD-1 shield" to prevent lysis by T cells.

Materials and Methods

Mice, Cell Lines, and Reagents. Female DBA/2, C57BL/6, C3H/HeN, BALB/c, and BALB/c *nu/nu* mice were purchased from the National Cancer Institute (Frederick, MD). Age-matched mice, 6 to 10 weeks old, were used for all experiments. All mice were maintained in the Animal Facility at Mayo

Clinic under approved protocol by the Institutional Animal Care and Use Committee. P815 mastocytoma (H-2^d), L1210 lymphoma (H-2^d), 4T1 breast cancer (BALB/c, H-2^d), EL4 thymoma (H-2^b), J558 plasmacytoma (H-2^d), Sp2/0 myeloma (H-2^d), EMT6 breast cancer (H-2^d), and B16 melanoma (H-2^d) cells were purchased from the American Type Culture Collection (Rockville, MD). AG104A sarcoma (H-2^k) was a gift from Dr. Hans Schreiber (University of Chicago, Chicago, IL). C3 epithelial tumor (H-2^b) was a gift from Dr. W. Martin Kast (Loyola University, Chicago, IL). Stable P815 lines expressing mouse B7-H1 (B7-H1/P815), B7-1 (B7-1/P815), and CD137L (CD137L/P815) were described previously (11, 30, 31). P1A CTL, a mouse CD8$^+$ CTL clone specifically recognizing a P1A$_{35-43}$ (LPYLGWLVF) peptide in the context of the H-2L^d, was maintained as previously described (32). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 25 mmol/L HEPES, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 55 μmol/L 2-ME. Recombinant mouse IFN-γ was purchased from Roche (Mannheim, Germany) and was used for treatment of tumor cells at 200 units/mL overnight.

mAbs, Fusion Proteins, and Flow Cytometry Analysis. Purified mAbs against mouse CD3, CD8, CD28, CD16/32, H-2L^d, H-2D^d, and H-2K^d were purchased from PharMingen (San Diego, CA). FITC- or phycoerythrin-conjugated goat anti-mouse antibodies and FITC-conjugated goat anti-hamster antibodies were purchased from eBioscience (San Diego, CA). An Armenian hamster mAb (clone 10B5) against mouse B7-H1 (12), a rat mAb (clone 2A) against mouse CD137 (33), and mouse B7-H1Ig fusion protein (11) were described previously. Hamster mAb (clone G4) against mouse PD-1 was produced by immunizing an Armenian hamster with mouse PD-1Ig fusion protein by established method (33). G4 mAb is specific for mouse PD-1 and does not bind human PD-1 as well as mouse CTLA-4, CD28, and ICOS. Control mouse, hamster, and rat immunoglobulin G were purchased from Sigma (St. Louis, MO). H-2L^d pentamer conjugated with P1A-specific peptide was purchased from Pro Immune Inc. (Springfield, VA). Tumor cells were incubated with the primary mAb at 4°C for 30 minutes in the presence of the CD16/32 mAb to block Fc receptor. The cells were washed and further incubated with phycoerythrin-conjugated secondary mAbs. Fluorescence was detected by FACScan flow cytometry and analyzed with Cell Quest software (Becton Dickinson, Mountain View, CA).

Animal Studies and Tumor Models. Tumor lines were inoculated at their minimal tumorigenicity dose (30) into the shaved left back of mice in a 100-μL volume of PBS. Tumor size, presented as the average of two perpendicular diameters (millimeters), was measured at regular intervals. To detect the expression of mouse B7-H1, solid tumors (14-21 days after injection) were excised, ground to prepare the cell suspension, and subsequently stained with 10B5 mAb plus FITC-conjugated goat anti-hamster antibodies for fluorescence-activated cell-sorting (FACS) analysis. For treatment of established tumors, mice were given i.p. injections of 100 μg control rat immunoglobulin G or 2A mAb on days 7 and 10. In some experiments, combined treatment with 100 μg control hamster immunoglobulin G or 10B5 mAb on days 7, 10, 13, and 16 were also done. To evaluate the role of P1A CTL in the inhibition of tumor growth *in vivo*, 5×10^4 mock-transfected P815 (mock/P815) or B7-H1/P815 cells were mixed with 5×10^5 activated P1A CTLs and immediately inoculated s.c. into the shaved right flank of DBA/2 mice.

CTL Growth, Survival, Division, and Cytolytic Activity. P1A CTL clones were stimulated by irradiated wild-type (WT) P815 cells in the presence of irradiated syngeneic spleen cells as antigen-presenting cells. On days 3 to 5, cells were incubated with ^{51}Cr-labeled target cells at indicated effector/target (E/T) ratios for either 4 or 12 hours as described previously (30, 34). EL4 or L1210 lymphomas were used for controls. The spontaneous releases of ^{51}Cr are <10% in 4-hour assays and <20% in 12-hour assays.

To determine the death of cells, direct counting of viable cells were used. For this assay, 6×10^5 mock/P815 or B7-H1/P815 cells were incubated with 3×10^5 activated P1A CTL clones in 24-well plates. After incubation for 12 hours, cells were harvested and stained with phycoerythrin-conjugated mAb to CD8 and B7-H1 (10B5) for FACS analysis.

In target competition assay, 1.5×10^5 mock/P815 and 1.5×10^5 B7-H1/P815 cells were premixed and incubated with 3×10^5 activated P1A

CTLs in 24-well plates for 12 hours. Cells were stained with phycoerythrin-conjugated anti-CD8 mAb and 10B5 mAb plus FITC-conjugated goat anti-hamster immunoglobulin antibodies, and all cells were counted by flow cytometry. Wells containing P1A CTLs with 3×10^5 mock/P815 cells alone or 3×10^5 B7-H1/P815 cells alone were also included as positive and negative controls, respectively. Percent lysis was calculated as 100−(percentage of viable P815 cells after incubation with CTLs versus P815 cells alone without CTLs). For purification of P1A CTLs after coculture with P815 cells, we used anti-CD90 MicroBeads in the magnetic field of a MACS magnetic separator, as instructed by the manufacturer (Miltenyi Biotec, Auburn, CA).

For the generation of alloreactive CTLs, lymph node cells (2×10^6/mL) from C57BL/6 mice were stimulated with irradiated spleen cells (2×10^6/mL) from DBA/2 mice in 24-well plates for 5 days. Cells were harvested and cocultured with target cells for ^{51}Cr release assays. For assay of proliferative capacity, P1A CTLs at 1.5×10^4 cells/mL and soluble anti-CD28 mAb (2 μg/mL) were added to 96-well plates coated with anti-CD3 (2 μg/mL). Proliferation of CTLs was assessed by the addition of 1 μCi per well [^{3}H]thymidine during the last 18 hours of 3-day culture. [^{3}H]Thymidine incorporation was measured in a MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

For the generation of P815-specific CTLs (30), spleen cells (2×10^6/mL) from preimmunized DBA/2 mice were stimulated with irradiated mock/P815 or B7-H1/P815 cells (2×10^5/mL) in 24-well plates for the indicated days. Cells were harvested and cocultured with WT P815 or L1210 target cells for ^{51}Cr release assays as described previously.

Results

Constitutive and Inducible Expression of B7-H1 by Mouse Tumors. We reported previously that many human cancers constitutively express B7-H1 by immunohistochemistry analysis (12). To establish mouse tumor models for evaluation of tumor-associated B7-H1 in the inhibition of T-cell immunity, we examined the expression of B7-H1 in eight mouse tumor lines that have been extensively applied for evaluation of tumor immune responses. As summarized in Table 1, constitutive expression of B7-H1 could be detected in J558 plasmacytoma and Sp2/0 myeloma, but not in other tumor lines, particularly those lines with nonhematopoietic origin. The expression of B7-H1, however, could be induced by IFN-γ on 4T1, AG104A, and EMT-6 tumor lines, with the exception of P815, EL4, and C3. In addition, expression of B7-H1 on 4T1, a breast cancer line 4T1 that does not constitutively express B7-H1, could be detected by growth in syngeneic mice (Fig. 1*A*), suggesting that B7-H1 could be inducible *in vivo*. To exclude the effect of B7-H1 expression on host cells, the same experiments were also done in B7-H1-deficient mice (BALB/c background) and the results were identical.[3] We conclude that similar to human cancers, B7-H1 is widely expressed by mouse tumors and, in addition to constitutive expression on some tumor lines, its expression could be induced *in vitro* and *in vivo*.

B7-H1 Expression on P815 Cells Does Not Affect Their Progressive Growth. P815 tumor cells do not express B7-H1, even after treatment by IFN-γ, a potent inducer of B7-H1 (Table 1). We first established several stable P815 clones constitutively expressing mouse B7-H1. The expression of B7-H1 in a representative clone, B7-H1/P815, was confirmed by staining with B7-H1 mAb and analyzed by flow cytometry. As indicated in Fig. 1*B*, WT P815 and mock/P815 line did not express B7-H1, whereas B7-H1/P815

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[3] F. Hirano, unpublished results.

did. Furthermore, mouse B7-H1Ig completely blocked the binding of 10B5 mAb to B7-H1/P815 (data not shown), indicating the specificity of the assay. Flow cytometry analysis showed no differences in the expression of H-2D^d, H-2K^d, and H-2L^d among these transfectants (Fig. 1B). To determine the effect of tumor-associated B7-H1 in the growth of P815 tumors, cells from mock/P815 or B7-H1/P815 were inoculated s.c. into naive DBA/2 mice. Both tumor lines were equally capable of inducing progressively growing tumors in both syngeneic and immunodeficient BALB/c *nu/nu* mice (Fig. 1C). Therefore, expression of B7-H1 on P815 tumor cells did not affect both tumorigenicity and immunogenicity of P815 tumors in immunocompetent mice.

Tumor-Associated B7-H1 Confers Resistance to CD137 mAb Therapy for Established Tumors. We next examined the effect of tumor B7-H1 expression in effector function of tumor immunity. Agonistic mAb to mouse CD137/4-1BB has been shown to induce regression of established P815 tumors in syngeneic mice in which tumor-specific CD8+ CTLs play a central role (35). To determine the effect of B7-H1 in this therapy, we first inoculated mice s.c. with cells from mock/P815 or B7-H1/P815. On day 7, the mice developed palpable tumors in the range of 3 to 5 mm in mean tumor diameter. The mice were then treated with 2A, an agonistic mAb specific for mouse CD137 (33). As expected, tumor regression was observed after 2A treatment in 5 of 5 mice inoculated with mock/P815, whereas all mice treated with control rat immunoglobulin G developed tumors progressively (Fig. 2A, *left*). Whereas inoculation of B7-H1/P815 cells also developed tumors progressively after treatment by control mAb, 2A treatment could only temporarily delay tumor growth; eventually, all the mice developed large, established tumors (Fig. 2A, *middle*). There was no survival benefit from 2A treatment for the B7-H1/P815 tumor-bearing mice compared with those with mock/P815 tumors undergoing complete tumor regression after 2A treatment (Fig. 2A, *right*). This result indicates that expression of B7-H1 confers tumor resistance to CD137 costimulatory therapy.

To evaluate whether B7-H1 blockade could be applied to enhance CD137 costimulatory therapy, we examined the effect of
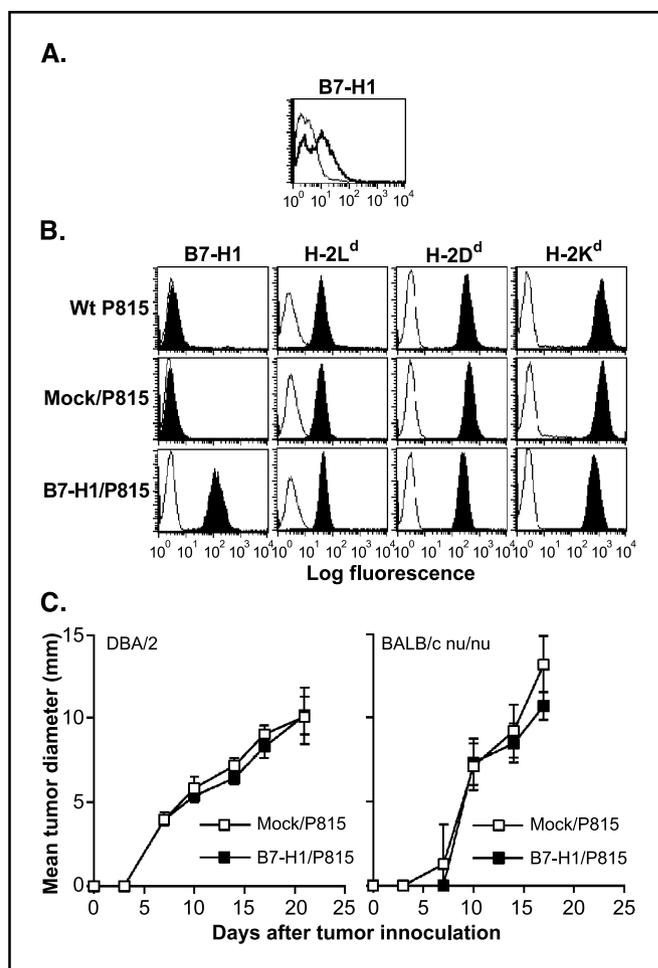

Figure 1. Effect of B7-H1 expression on P815 tumor growth. *A, in vivo* B7-H1 expression on 4T1 cancer cells after reisolation from established tumor. 4T1 cells were inoculated s.c. into BALB/c mice. On day 14, tumors were removed and ground to cell suspension. 4T1 cells were gated based on their size. Cells were stained with control hamster immunoglobulin G (*thin line*) or 10B5 mAb (*thick line*), and detected by FITC-conjugated goat anti-hamster immunoglobulin antibodies. Representative of four independent experiments. *B,* expression of B7-H1 and MHC class I molecules on WT P815, mock/P815, and B7-H1/P815 cells. Cells were stained with control mAb (*open*) or mAbs against mouse B7-H1, H-2L^d, D^d and K^d (*filled*) and at least 50,000 cells were analyzed by flow cytometry. *C,* growth of P815 transfectants in immunocompetent versus immunodeficient mice. DBA/2 mice (*left*) or BALB/c *nu/nu* mice (*right*), five per group, were inoculated s.c. with 5×10^4 mock/P815 or B7-H1/P815 cells. Tumor size was presented as mean tumor diameter in millimeters. *Points,* mean of five experiments; *bars,* SD.

Table 1. Constitutive and inducible expression of B7-H1 on mouse tumor lines

Cell line	Tissue type	Strain	B7-H1 expression	
			Constitutive*	Inducible†
J558	Plasmacytoma	BALB/c	+	+
Sp2/0	Myeloma	BALB/c	+	>+
P815	Mastocytoma	DBA/2	>−	>−
EL4	Thymoma	C57BL/6	>−	>−
C3	Epithelial tumor	C57BL/6	>−	>−
4T1	Breast cancer	BALB/c	>−	>+
AG104A	Sarcoma	C3H/HeN	>−	>+
EMT-6	Breast cancer	BALB/c	>−	>+

*Constitutive expression of B7-H1 was determined by indirect immunofluorescence and flow cytometry analysis using mAb (clone 10B5) against mouse B7-H1.
† The indicated tumor lines were cultured overnight in the presence of recombinant IFN-γ at 200 IU/mL before staining by 10B5.

B7-H1 mAb in the resistance of B7-H1/P815 tumors. 10B5 is a hamster mAb specific against mouse B7-H1 and is capable of blocking the binding of B7-H1 to its receptor, PD-1, in flow cytometry analysis (Fig. 2B). In combination with 2A mAb, growth of B7-H1/P815 tumors were completely inhibited after 10B5 treatment (Fig. 2C, *left*), and the mice lived for >120 days (Fig. 2C, *right*). Similar to the results shown in Fig. 2A, tumors treated with 2A mAb and control hamster immunoglobulin G grew progressively and the mice eventually died. As predicted, treatment with 10B5 alone did not have a significant effect on tumor inhibition (data not shown). In addition, injection with 10B5 did not have an affect on the growth of mock/P815 tumors (data not shown), suggesting that the role of host B7-H1 in this model is minimal.
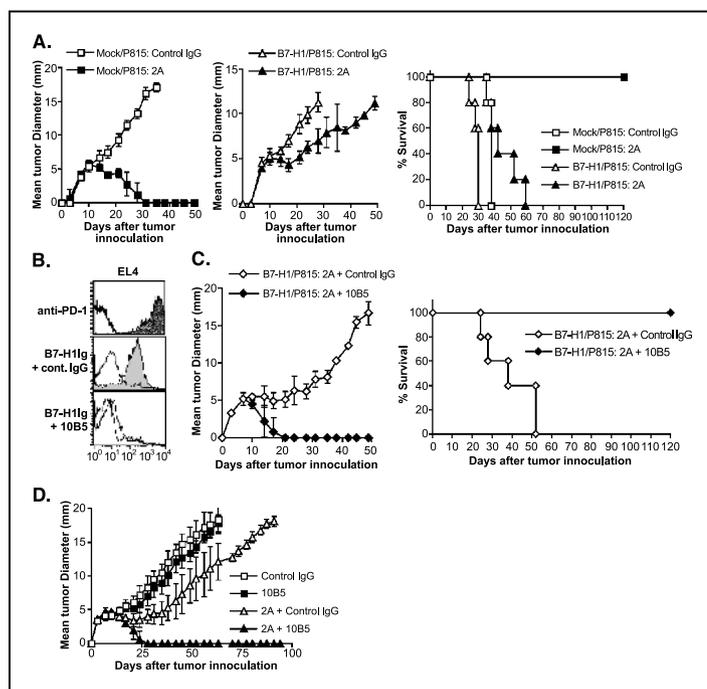
Figure 2. Resistance of B7-H1+ tumors to CD137 antibody therapy. *A,* groups of five DBA/2 mice were given s.c. injections of 5×10^4 mock/P815 (*left*) or B7-H1/P815 (*middle*) cells on day 0. Mice were then treated i.p. with 100 μg control rat immunoglobulin G or anti-CD137 mAb (clone 2A) at days 7 and 10. *Points,* mean tumor diameter of each group; *bars,* SD. Representative of three experiments. In addition to monitoring tumor growth, the mice were also observed for their survival after tumor inoculation and treatment (*right*). *B,* 10B5 blocks B7-H1Ig binding to PD-1 on EL4 cells. EL4 cells were stained with control mouse immunoglobulin G (*open lines*) or B7-H1Ig (*filled lines*) that was preincubated with control hamster immunoglobulin G (*middle*) or 10B5 mAb (*bottom*). The binding of B7-H1Ig was detected by FITC-conjugated goat anti-mouse immunoglobulin antibodies. *C,* 10 DBA/2 mice were given s.c. injections of 5×10^4 B7-H1/P815 tumor cells on day 0. On day 7s and 10, all mice were treated i.p. with 100 μg 2A mAb. On day 7, mice were also divided equally into two groups and subsequently treated with 100 μg control hamster immunoglobulin G or 10B5 mAb on days 7, 10, 13, and 16. *Points,* mean tumor diameter for each group; *bars,* SD (*left*). Representative of three experiments. In addition to monitoring tumor growth, the mice were also observed for their survival after tumor inoculation and treatment (*right*). *D,* BALB/c mice at 5 mice/group were given s.c. injections of 2.5×10^5 4T1 breast cancer cells. Seven and 10 days later, mice were treated i.p. with 100 μg 2A mAb or control immunoglobulin G with or without 100 μg control hamster immunoglobulin G or 10B5 mAb on days 7, 10, 13, and 16. *Points,* mean tumor diameter for each group; *bars,* SD. Representative of two experiments.

Therefore, blocking B7-H1 could be utilized as an approach to augment the therapeutic effect of CD137 costimulatory therapy.

To show the role of *in vivo* inducible B7-H1 on tumor cells in the evasion of tumor immunity, we also tested the effect of B7-H1 blocking mAbs in immunotherapy of 4T1 breast cancer. As we showed above (Fig. 1*A*), 4T1 tumors have up-regulated B7-H1 during progressive growth *in vivo*. S.c inoculation of 4T1 cells induced progressive growth of tumors. Treatment of the mice with 2A mAb starting at day 7 partially inhibited the growth of tumor. However, all mice eventually developed progressive tumors and died, whereas treatment of the mice with 10B5 did not have any effect. Combined treatment by 2A and 10B5 led to complete regression of established 4T1 tumor (Fig. 2*D*). Treatment by 10B5 alone had minimal effect on tumor growth. Taken together, our results suggest that expression of B7-H1 on tumor cells only evade active T-cell immunity in effector cell levels.

Tumor-Associated B7-H1 Confers Resistance to CD8+ CTL Lysis *In vivo* and *In vitro*. To directly test the possibility that B7-H1 confers resistance to activated T-cell immunity in effector phase *in vivo*, we used a T-cell adoptive transfer system in which P815 cells were mixed with *in vitro* preactivated P1A-specific CD8+ CTL clones in a 1:10 ratio and inoculated s.c. into DBA/2 mice. In this test, activated CTLs can directly contact tumor cells *in vivo* to mimic the effector phase of activated T cells at the tumor site. In this experimental setting, growth of mock/P815 tumors was inhibited completely. In sharp contrast, B7-H1/P815 tumors grew progressively and killed the mice (Fig. 3*A*). The progressive growth of B7-H1/P815 tumor, however, could be eliminated by injecting 10B5 mAb (Fig. 3*B*). Our results thus indicate that B7-H1 on tumor cells confers resistance to tumor antigen-specific CTLs and the resistance could be eliminated by B7-H1 blockade *in vivo*.

To establish an *in vitro* cell culture system to study the mechanisms of the resistance, we examined if B7-H1/P815 cells could become resistant to lysis by a P1A-specific CTL clone, P1A CTL (32). In a standard *in vitro* 4-hour ^{51}Cr release assay, mock/

P815 and B7-H1/P815 were lysed equally by P1A CTLs in a wide range of E/T cell ratios (Fig. 3*C*). Because cytolysis in a 4-hour short-term assay is largely mediated through perforin- and granzyme-mediated damage of target cells, our results thus suggest that the expression of B7-H1 on P815 cells does not inhibit the perforin/granzyme pathway by CTLs. This short assay thus does not reflect our *in vivo* findings in T-cell adoptive transfer assay. In contrast, extended incubation of B7-H1/P815 cells with P1A CTLs for 12 hours induced significantly inhibited cytotoxicity to approximately 50% when compared with mock/P815 cells. This cytotoxicity was specific for P815 cells because there was no lysis on L1210 cells, a syngeneic lymphoma that does not express P1A antigens (Fig. 3*C*). Kinetics of tumor killing by P1A CTLs was also determined by sampling tumor cell numbers in 4, 8, and 12 hours after coculture. After exposure to P1A CTLs, the number of mock/P815 cells started to decrease after 4 hours incubation, whereas B7-H1/P815 cells proliferated continuously. Although there was only a small difference between mock/P815 and B7-H1/P815 cells in 4 hours incubation, the difference became significant after 8 to 12 hours incubation. The number of P1A CTLs in the culture, however, remained constant (Fig. 3*D*). Our results thus support that B7-H1 on tumor cells renders the resistance to activated CTLs.

Exposure to B7-H1+ Tumor Does Not Induce T-Cell Tolerance/Anergy. It is possible that inhibition of cytotoxicity by B7-H1/P815 cells is due to rapid induction of T-cell functional deficiency after exposure to B7-H1. If this is the case, exposure to B7-H1/P815 will induce an inhibition of CTL activity, not only against B7-H1/P815 but also against mock/P815 cells. To test this, we mixed mock/P815 and B7-H1/P815 in equal numbers and incubated the mixture with activated P1A CTLs in a T cell/tumor cell ratio of 1:2 to insure sufficient exposure of T cells to tumor cells. Upon 12 hours incubation, cells were counted and the remaining P815 cells were gated based on size, CD8 marker (negative on P815), and B7-H1 expression. As shown in Fig. 4*A*

(*left*), three populations of cells in the culture, P1A CTLs (CD8+, B7-H1⁻), mock/P815 (CD8⁻, B7-H1⁻), and B7-H1/P815 (CD8⁻, B7-H1⁺) could be easily distinguished by FACS analysis after double staining with mAb of CD8 and B7-H1. More important, more than 90% of mock/P815 cells in the mixture were lysed, whereas only ~35% of B7-H1/P815 cells were killed in the mixture (Fig. 4*A*, *right*). This result suggests that P1A-CTL is not functionally defective after exposure to B7-H1/P815 cells. We reisolated P1A CTLs and tested their cytolytic activity, after incubating with B7-H1/P815 cells for 12 hours, against WT P815. There was no inhibition on CTL activity to P815 cells in either 4-hour (Fig. 4*B*, *left*) or 12-hour assays (Fig. 4*B*, *right*). In addition, the reisolated P1A CTLs had a similar proliferative response to anti-CD3 and anti-CD3/CD28 stimulation *in vitro* (Fig. 4*C*). More important, this phenomenon is not limited to P1A CTL because alloreactive CTL from C57BL/6 mice was also significantly inhibited against B7-H1/P815 in comparison with mock/P815 cells (Fig. 4*D*). Taken together, our results indicate that activated CTLs remain functionally normal after exposure to B7-H1 on tumor cells and the inhibition seems to be exclusively due to tumor resistance to CTL lysis.

Resistance of B7-H1+ Tumor to CTL Lysis Could Be Abrogated by Anti-B7-H1 or Anti-PD-1 Antibodies. We next determined whether tumor resistance requires the presence of B7-H1 and one of its receptors, PD-1. Inclusion of neutralizing mAbs to B7-H1 (clone 10B5) significantly reversed inhibition of cytotoxicity that was mediated by B7-H1/P815 cells in 12-hour cytotoxicity assays. We also generated an immunoglobulin G mAb (clone G4) specifically against mouse PD-1 by immunization of a hamster with mouse PD-1Ig fusion protein. G4 is capable of blocking the binding of B7-H1 to PD-1 in flow cytometry analysis (Fig. 5*A*). Similar to 10B5, inclusion of G4 in the culture largely abrogated the inhibition of CTL lysis in the 12-hour assay against B7-H1/P815 cells (Fig. 5*B* and Fig. 4*A*, *right*). In addition, inclusion of 10B5 or G4 also significantly eliminated the inhibition allogeneic CTLs against B7-H1/P815 cells (Fig. 5*C*). Finally, injection of G4 completely abrogated the outgrowth of B7-H1/P815 tumors in the presence of P1A CTLs in adoptive transfer experiments *in vivo* (Fig. 5*D*). Our results show that both B7-H1 and PD-1 are required for tumor resistance to CTLs *in vitro* and *in vivo* and perhaps, through interaction of B7-H1 and PD-1 and blockade of B7-H1 and PD-1 by mAbs, could potentially eliminate tumor resistance as a means to enhance tumor immunity.

Discussion

In this report, we present a clinically relevant finding that expression of B7-H1 in two histologically distinct mouse tumors confers resistance to immunotherapy by anti-CD137 mAb. B7-H1 on tumor cells, perhaps by interacting with PD-1 receptor on effector T cells, forms a temporal "shield" to prevent lysis. Although functions of CTLs are not impaired after B7-H1 and PD-1 interaction, they fail to control tumor growth *in vivo* and are only partially cytolytic *in vitro*. Blockade of B7-H1 or PD-1 by specific mAb rescues the function of therapeutic immunity and leads to regression of established mouse tumors. Our findings thus support B7-H1 and PD-1 blockade as a viable strategy to enhance cancer treatment *in vivo*.

Although the role of B7-H1 in inhibiting T-cell response has been confirmed in a number of studies, underlying mechanisms for this inhibition are different. Currently, three possible mechanisms are observed based on independent experimental systems. Transfection to express B7-H1 into Chinese hamster ovary cells inhibits cell cycle progression of activated DO11.10 T cells in the presence of B7/CD28 costimulation *in vitro* (23). It is worth noting that apoptosis of activated T cells does not increase in this system (23). Moreover, blocking B7-H1 by a mAb stimulated proliferation of anergic human T cells, which were induced by IL-10-treated human dendritic cells, suggesting that B7-H1 is involved in the induction of T-cell anergy (14).
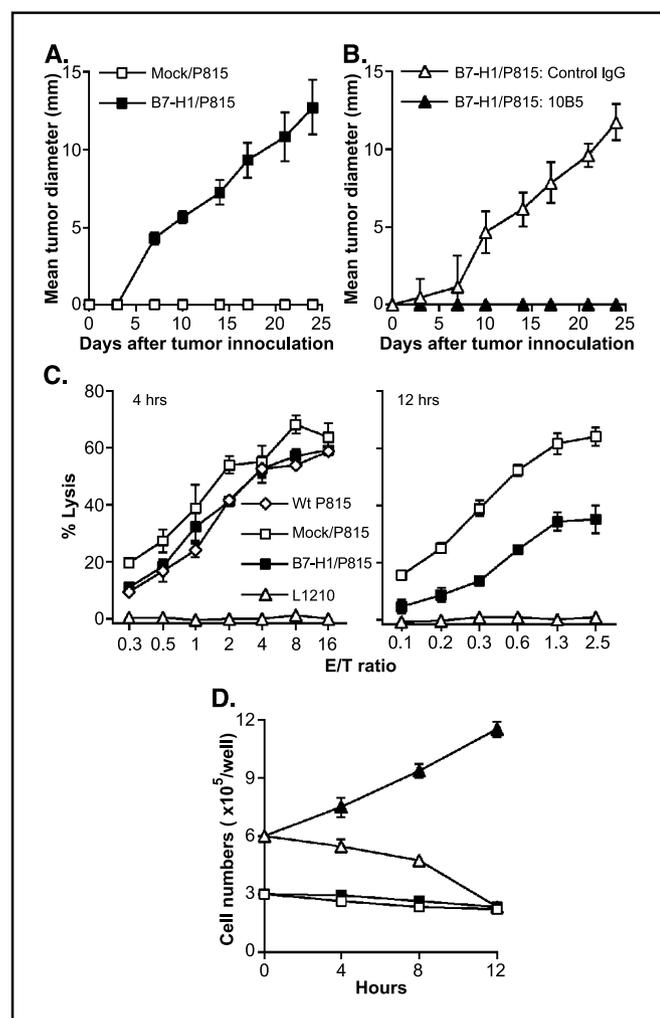

Figure 3. Resistance of B7-H1+ tumors to CTL. *A,* DBA/2 mice, in groups of five, were given s.c. injections of a mix of 5 × 10⁴ mock/P815 or B7-H1/P815 cells with 5 × 10⁵ activated P1A CTL clones. Tumor growth was monitored. *Points,* mean tumor diameter for each group; *bars,* SD. Representative of three experiments. *B,* DBA/2 mice, in groups of five, were given s.c. injections of a mix of 5 × 10⁴ B7-H1/P815 cells and 5 × 10⁵ activated P1A CTL clones with or without 100 μg of control hamster immunoglobulin G or 10B5 mAb on days 0, 3, 6, and 9. *Points,* mean tumor diameter for each group; *bars,* SD. Representative of two experiments. *C,* Activated P1A CTL clones were incubated at indicated E/T ratios with ⁵¹Cr-labeled WT P815, mock/P815, B7-H1/P815, or L1210 cells for 4 (left) or 12 hours (right). CTL activity was determined in a ⁵¹Cr release assay. *Points,* means of triplicates; *bars,* SD. Representative of three experiments. *D,* mock/P815 or B7-H1/P815 (6 × 10⁵) were incubated with purified P1A CTL clones (3 × 10⁵) for indicated hours. Cells were then harvested and stained by anti-CD8 and anti-B7-H1 (10B5) mAbs. P1A CTL (CD8⁺), mock/P815 (CD8⁻, B7-H1⁻), or B7-H1/P815 cells (CD8⁻, B7-H1⁺) cells were counted based on FACS analysis. ▲, B7-H1/P815; △, mock/P815; ■, P1A CTL (after culture with B7-H1/P815); and □, P1A CTL (after culture with mock/P815).

Several studies support that B7-H1 promotes apoptosis of activated T cells. We have shown in a previous report that coculture of a gp100-specific human CTL clone with B7-H1-transfected 624 melanoma cells for 72 hours failed to inhibit their growth, whereas the inhibition could be achieved in B7-H1 negative 624 melanoma cells. During the culture, apoptosis of CTL increased, which could be partially blocked by anti-human B7-H1 mAb (12). Adoptive transfer of activated 2C T cells into the mice bearing B7-H1-transfected P815 cells underwent
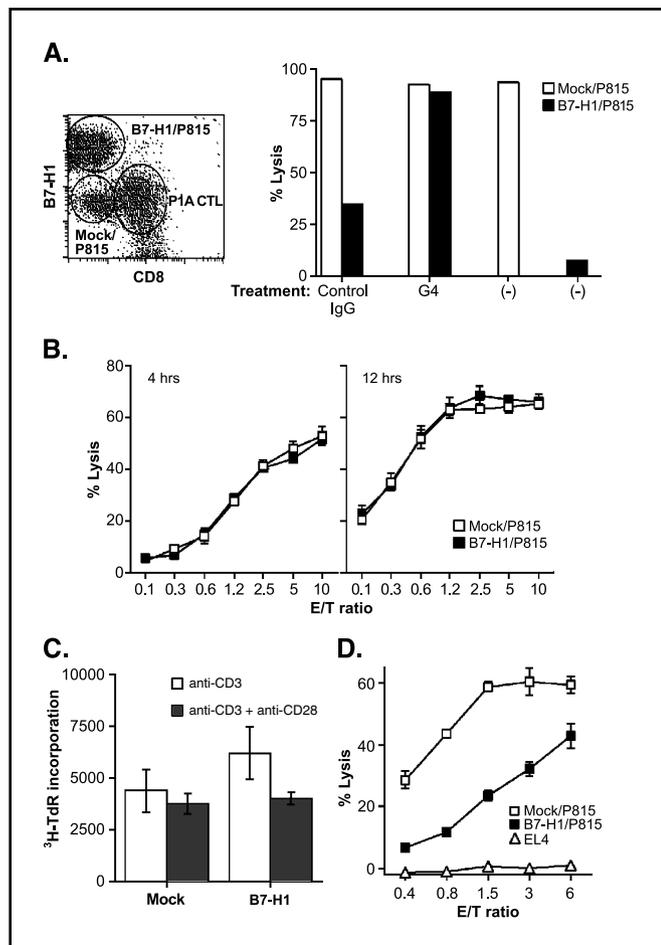

Figure 4. Tumor B7-H1 does not impair T cell functions. *A,* mock/P815, B7-H1/P815, or an equal mix of both lines were coincubated with the same number of activated P1A CTLs in the presence or absence of anti-PD-1 mAb (G4) or control mAb at 10 μg/mL for 12 hours. Cells were harvested and analyzed by flow cytometry (*left*) and live cells for P1A CTL, mock/P815, or B7-H1/P815 cells were counted. Cytolytic activity was calculated as described in Materials and Methods. Representative of three experiments. *B,* P1A CTL retains cytolytic functions after exposure to B7-H1. Activated P1A CTLs were incubated with live mock/P815 or B7-H1/P815 cells for 12 hours. Cells were positively selected by magnetic beads coated with anti-Thy1.2 mAb and incubated with WT P815 cells for 4-hour (*left*) or 12-hour (*right*) ^{51}Cr release assays. *Points,* means of triplicates; *bars,* SD. Representative of three experiments. *C,* P1A CTL responds normally to proliferative signal after exposure to B7-H1. P1A CTLs were incubated with live mock/P815 or B7-H1/P815 cells for 12 hours. CTL was subsequently purified and stimulated with immobilized anti-CD3 mAbs or anti-CD3/CD28 mAbs. Three days later, [3H]thymidine pulsed cells were harvested. Representative of two experiments. *D,* B7-H1/P815 cells are resistant to lysis by allogeneic CTLs. Lymph node cells from B6 mice were stimulated with irradiated spleen cells from DBA/2. Five days later, purified T cells were incubated at indicated E/T ratios with ^{51}Cr-labeled mock/P815 (□), B7-H1/P815 (■), or EL4 cells (△) for 12 hours. CTL activity against each line was determined in ^{51}Cr release assays. *Points,* means of triplicates; *bars,* SD. Representative of three experiments.
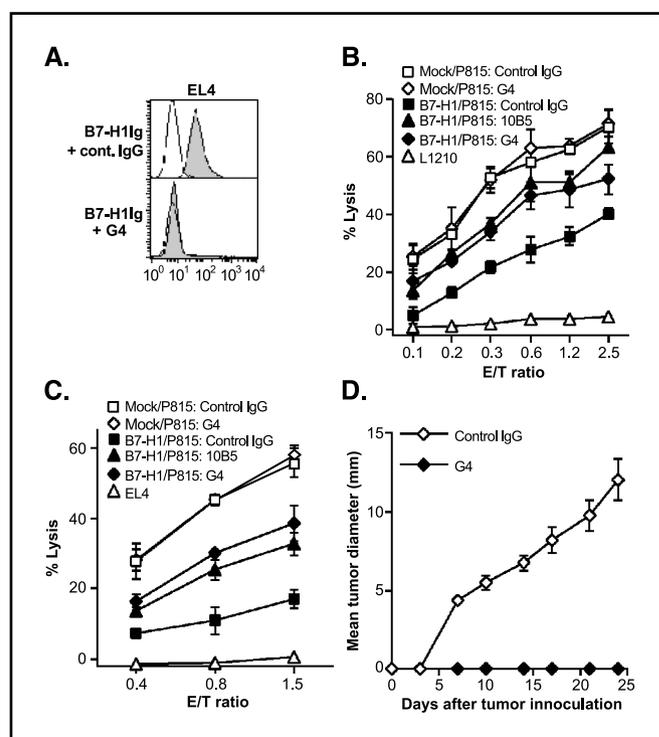

Figure 5. B7-H1/PD-1 interaction is required for the molecular shield. *A,* G4 mAb blocks B7-H1Ig binding to PD-1 on EL4 cells. EL4 cells were preincubated with control hamster immunoglobulin G (*top*) or G4 mAb (*bottom*) and then stained with control immunoglobulin (*open lines*) or B7-H1Ig (*filled lines*). The binding of B7-H1Ig was detected by indirect fluorescence with conjugated secondary mAb for flow cytometry analysis. *B,* activated P1A CTL was incubated at indicated E/T ratios with ^{51}Cr-labeled mock/P815 (□ and ◇), B7-H1/P815 (solid symbols) or L1210 cells (△) for 12 hours. The control mAb (■), 10B5 (▲) or G4 (◆) were included at 10 μg/mL at the beginning of the culture. CTL activity was determined in ^{51}Cr release assays. *Points,* means of triplicates; *bars,* SD. Representative of three experiments. *C,* G4 resumes susceptibility of B7-H1/P815 cells to alloreactive CTL lysis. Lymph node cells from B6 mice were stimulated with irradiated spleen cells from DBA/2. Five days later, purified T cells were incubated at indicated E/T ratios with ^{51}Cr-labeled mock/P815 (□ and ◇), B7-H1/P815 (solid symbols), or EL4 cells (△) for 12 hours. The control mAb (■), 10B5 (▲), or G4 (◆) were included at 10 μg/mL at the beginning of the culture. CTL activity was determined in ^{51}Cr release assays. *Points,* means of triplicates; *bars,* SD. Representative of two experiments. *D,* DBA/2 mice, in groups of five, were given s.c. injections of a mixture of 5 × 10^4 B7-H1/P815 cells mixed with 5 × 10^5 P1A CTLs and subsequently treated with 100 μg control immunoglobulin G or G4 mAb on days 0, 3, 6, and 9. The tumor sizes were monitored on a regular basis. *Points,* mean tumor diameter for each group; *bars,* SD. Representative of two experiments.

profound apoptosis (12). In a recent study, significant decrease of CD8+ T-cell apoptosis was observed in the liver of B7-H1-deficient mice (36). Our experiments show that constitutive and inducible B7-H1 expression by P815 tumors could evade therapeutic immunity *in vivo*, whereas there is no obvious increase in apoptosis of CTL by terminal deoxynucleotidyl transferase–mediated nick end labeling assay.[4] In addition, exposure of effector CTL to B7-H1 *in vitro* does not induce T-cell anergy because these T cells are fully competent to respond to the same antigens *in vitro*. Instead, our *in vitro* findings suggest a new mechanism; that is, B7-H1 on tumor cells formed a temporal shield to prevent the lysis by antigen-specific CTLs. Our findings indicate that B7-H1 may evade T-cell responses utilizing

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[4] Hirano et al., unpublished data.

multiple mechanisms. The reasons for the use of diverse mechanisms by B7-H1 in inhibition of T-cell responses is yet unknown. It is possible that T cells in different stages of activation may express different receptors and it has been suggested that other receptors other than PD-1 may mediate apoptosis and/or costimulation (9, 12, 18, 24, 25).

Iwai et al. observed that B7-H1-expressing P815 cells had decreased susceptibility to lysis by H-2L[d]-specific 2C T cells and by a polyclonal CTL line against P815 in 4-hour ^{51}Cr release assays, suggesting that the expression of B7-H1 on tumor cells could directly affect lysis by CTLs (29). In our system, B7-H1/P815 cells remain sensitive to the lysis by a P1A-specific CTL clone in 4-hour ^{51}Cr release assays. Instead, B7-H1/P815 cells become resistant to CTL lysis only in extended 12-hour culture. Therefore, the inhibition observed in our system could not be simply interpreted as resistance to perforin/granzyme pathway, which is believed to be a dominant effector mechanism in 4-hour ^{51}Cr release assays. Cytolytic activity against B7-H1/P815 cells in 4-hour ^{51}Cr release assays is antigen specific, indicating that TCR-specific recognition takes place. In our system, it is also unlikely that this observation is due to insufficient exposure of effector CTL to tumor cells, because during the stimulation of CTL *in vitro*, T cell/tumor cell ratio was 1:2 (Fig. 4). More important, P1A CTL is also less effective in inhibiting B7-H1+ tumor growth after adoptive transfer into mice, suggesting that this mechanism also operates *in vivo*. A possible interpretation for the observed molecular shield phenomenon is that B7-H1 may be integrated within immunologic synapse when tumor cells contact activated T cells in such a way that T-cell receptor-MHC interaction is disturbed. This hypothesis remains to be tested in the future. Interestingly, a recent study indicates that PD-1 colocalization with TCR is required for its inhibitory function (37), suggesting that B7-H1 on tumor cells may directly bind to PD-1 to prevent TCR-mediated recognition or signaling function. It is worth noting that all proposed mechanisms thus far, including cell cycle arrest, T-cell anergy, T-cell apoptosis, and molecular shield, may not be mutually exclusive, but utilized by cancer cells to evade immune responses during tumor progression. We found that although treatment with anti-CD137 mAb increased P1A-specific CTL in tumor-draining lymph nodes by staining with specific MHC-peptide pentamer, combined treatment with anti-B7-H1 mAbs further increased the frequency of P1A-specific CTLs (see Supplementary Fig. 1). This suggests that other evasion mechanisms in addition to B7-H1 shield formation also play roles in preventing expansion or accumulation of CTLs in tumor-draining lymph nodes.

Recent studies from our laboratory and others indicate that expression of B7-H1 promotes the growth of highly immunogenic P815 tumor cells (12), including a B7-1-transfected P815 (30) and a spontaneously regressing immunogenic P815 mutant (29). Blockade of B7-H1 by 10B5 mAb inhibited apoptosis of activated alloreactive 2C T cells, suppressed growth of P815 cells *in vivo* (12), and blocked B7-H1-mediated promotion of P815 tumor growth (29). These studies, however, did not directly address whether the expression of B7-H1 will make cancer cells resistant to immunotherapy. Our results indicate that B7-H1 expression on tumor cells profoundly affect the tumor's resistance to T-cell destruction after CD137 costimulation therapy, even though this method has been shown to elicit potent T-cell responses and to lead to regression of established P815 tumors as well as other tumor models (33, 35).

T cell–based immunotherapies have been extensively tested in clinical trials for the treatment of patients suffering from advanced cancers but with limited success (1, 2). Similar results have also been obtained by adoptive immunotherapy using *in vitro* activated T cells reactive to tumor antigens (1). B7-H1 is widely expressed by most human cancers. Our studies provide a possible explanation for the limited success of these immunotherapy trials and predict that blockade of B7-H1 may prevent the evasion of T-cell responses and augment the efficacy of immunotherapies and recurrence. Therefore, selective blocking of B7-H1 or PD-1 may represent a new opportunity for the improvement of cancer immunotherapy.

Acknowledgments

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Blockade of B7-H1 and PD-1 by Monoclonal Antibodies Potentiates Cancer Therapeutic Immunity

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