Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion

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B7-H1, a recently described member of the B7 family of costimulatory molecules, is thought to be involved in the regulation of cellular and humoral immune responses through the PD-1 receptor on activated T and B cells. We report here that, except for cells of the macrophage lineage, normal human tissues do not express B7-H1. In contrast, B7-H1 is abundant in human carcinomas of lung, ovary and colon and in melanomas. The pro-inflammatory cytokine interferon-γ upregulates B7-H1 on the surface of tumor cell lines. Cancer cell–associated B7-H1 increases apoptosis of antigen-specific human T-cell clones *in vitro*, and the apoptotic effect of B7-H1 is mediated largely by one or more receptors other than PD-1. In addition, expression of B7-H1 on mouse P815 tumor increases apoptosis of activated tumor-reactive T cells and promotes the growth of highly immunogenic B7-1$^+$ tumors *in vivo*. These findings have implications for the design of T cell–based cancer immunotherapy.

Interactions among subsets of immune cells through costimulatory ligands and their receptors transmit biochemical signals that initiate, amplify, differentiate and terminate immune responses. Abrogation of costimulatory activity, by administration of monoclonal antibodies and soluble receptors that neutralize costimulatory molecules, or by gene-targeted disruption of their expression, results in compromised cellular and humoral immune responses. This can be beneficial when it inhibits autoimmune diseases and rejection of transplanted organs[1,2]. The lack of costimulatory activation can be detrimental, however, if it compromises immune responses against cancer[3]. In addition to their costimulatory function, many receptor–ligand interactions are known to trigger anti-apoptotic pathways that prevent activation-induced T-cell death[4,5]. For example, triggering of CD28, 4-1BB and OX40 receptors costimulates growth of T cells and prevents the death of activated T cells[6–9]. In contrast, engagement of CTLA-4, a second receptor of B7-1 and B7-2, may inhibit the growth of T cells by blocking cell-cycle progression[10,11].

B7-H1 is a cell-surface glycoprotein belonging to the B7 family of costimulatory molecules. Stimulation of primary human T cells *in vitro* with immobilized B7-H1 in the form of an immunoglobulin (Ig) fusion protein (B7-H1Ig), along with monoclonal antibody against CD3 as a surrogate antigen, enhances T-cell growth and IL-10 secretion[12]. In addition, cell-associated B7-H1 costimulates T-cell growth, as demonstrated by stimulating proliferation of resting allogeneic CD4$^+$ T-cells[13]. Administration of B7-H1Ig enhances CD4$^+$ T-cell responses to KLH and increases T helper cell–dependent synthesis of TNP hapten–specific IgG2a[13]. Taken together, our results indicate that B7-H1 may be involved in promoting Th2-biased responses. Ligation of the PD-1 receptor by B7-H1 (PD-L1), however, inhibits proliferation and cytokine production by activated T cells[14]. In addition, PD-1-deficient mice develop systemic autoimmune diseases[15,16]. An alternative explanation for these observations is that receptor(s) other than PD-1 may be engaged by B7-H1 to regulate T-cell responses.

Northern-blot analyses have shown B7-H1 mRNA transcripts in a variety of non-lymphoid parenchymal organs, including the heart, placenta, skeletal muscle and lung[12]. The wide distribution of B7-H1 is suggestive of a broad role in local tissue physiology. Here we detected B7-H1 protein in most human cancers we examined but not in normal tissues. Further study indicated that activated T cells interacting with tumor-associated B7-H1 led to programmed cell death. This suggests a mechanism by which tumors may evade immune destruction.

Human cancers but not normal tissues express B7-H1

We used monoclonal antibody specific for B7-H1 to determine immunohistochemically whether sites of B7-H1 protein expression correlated to sites of mRNA expression. We did not detect immunoreactivity in any normal solid tissue examined, including breast, colon, pancreas, kidney, uterus, skeletal muscle and lung (Fig.1*a* and Supplementary Figs. A and B online). Notably, we detected immunoreactive macrophages in liver, lung and tonsil (Fig. 1*a*). This finding accords with our earlier report that a population of peripheral blood monocytes constitutively expresses B7-H1[12].

FACS analysis of tumor lines derived from a variety of human tissues showed surface B7-H1 expression on 4 of 9 lung carcinomas and 1 of 3 ovarian carcinomas. We did not detect surface expression of B7-H1 on colonic or duodenal cell lines (4), leukemia (5), choriocarcinoma (2) or melanoma lines (6). When treated

with interferon-γ (IFN-γ), however, most of these tumor cell lines were induced to express B7-H1 (Supplementary Table A online). We confirmed expression of B7-H1 mRNA in these tumor cell lines by the reverse transcriptase–coupled polymerase chain reaction (RT-PCR) using primers specific for human B7-H1 (data not shown).

Immunohistochemical analysis showed B7-H1 immunoreactivity in a majority of freshly isolated human lung carcinomas (20/21 patients), ovarian carcinomas (20/23 patients), colon carcinomas (10/19 patients) and melanomas (22/22 patients) (Table 1). We found B7-H1 immunoreactivity in the plasma membrane, cytoplasm or both. In most cases B7-H1 expression was focal, with no expression in adjacent normal tissues (Fig. 1*b*). B7-H1 was expressed on metastatic melanoma cells in the lymph nodes but not on adjacent lymphocytes (Fig. 1*b*).

Tumor-associated B7-H1 increases apoptosis of T cells

To determine the effect of tumor-associated B7-H1 on T cells, we first transfected a melanoma cell line (624mel) with a B7-H1 expression vector. The parental 624mel cells express the gp100 tumor antigen and major histocompatibility (MHC) class I allele, HLA-A2[17], but not B7-H1, even after treatment with IFN-γ (Supplementary Table A online). In addition, 624mel cells do not express the proteins FasL or TRAIL, which are known to participate in the induction of T-cell apoptosis[18–20]. B7-H1-transfected 624mel cells (B7-H1/624mel) express large amounts of immunoreactive B7-H1 protein (stained by B7-H1 monoclonal antibody or PD-1Ig, Fig. 2*a*). We next generated a human $CD8^+$ cytolytic T-cell (CTL) clone, M15, by a previously described method, involving repeated stimulation *in vitro* with an HLA-A2-restricted gp100 epitope peptide[21]. This gp100 peptide-reactive clone expresses PD-1 receptor after stimulation as shown by FACS analysis using human PD-1 monoclonal antibody or B7-H1Ig (Fig. 2*b*), and specifically lyses $gp100^+$ tumor cells (Fig. 2*g* and data not shown). M15 CTLs were cocultured with irradiated B7-H1/624mel cells or mock-transfected 624mel cells (mock/624mel), and T-cell apoptosis was assessed by double staining with annexin V and monoclonal antibodies against CD8. When M15 CTLs were cocultured with mock/624mel cells for 5 days (Fig. 2*c*), 14 ± 5.6% of the M15 CTLs underwent apoptotic death. When cocultured with B7-H1/624mel cells, however, 23 ± 4.7% of the M15 CTL population underwent apoptotic death (Fig. 2*c*). This represented a relative increase of 62% ($P < 0.05$). The ligand specificity of this phenomenon was shown by including monoclonal antibody against B7-H1 or its $F(ab')_2$ fragments in the culture medium to prevent potentially stimulatory cross-linking through the Fc receptor. The antibody against B7-H1 inhibited apoptosis of T cells by >50% ($P < 0.05$) and increased the number of viable T cells by 1.4-fold ($P < 0.05$). The inclusion of high concentrations of PD-1Ig in the cultures also significantly inhibited the induction of apoptosis by B7-H1/624mel (Fig. 2*d*). Consistent with this observation, nearly all mock/624mel cells were eliminated in a 5-day coculture with M15 CTLs, whereas B7-H1/624mel target cells were resistant to destruction by CTLs. Inclusion of antibody against B7-H1 in the culture medium abrogated the resistance of B7-

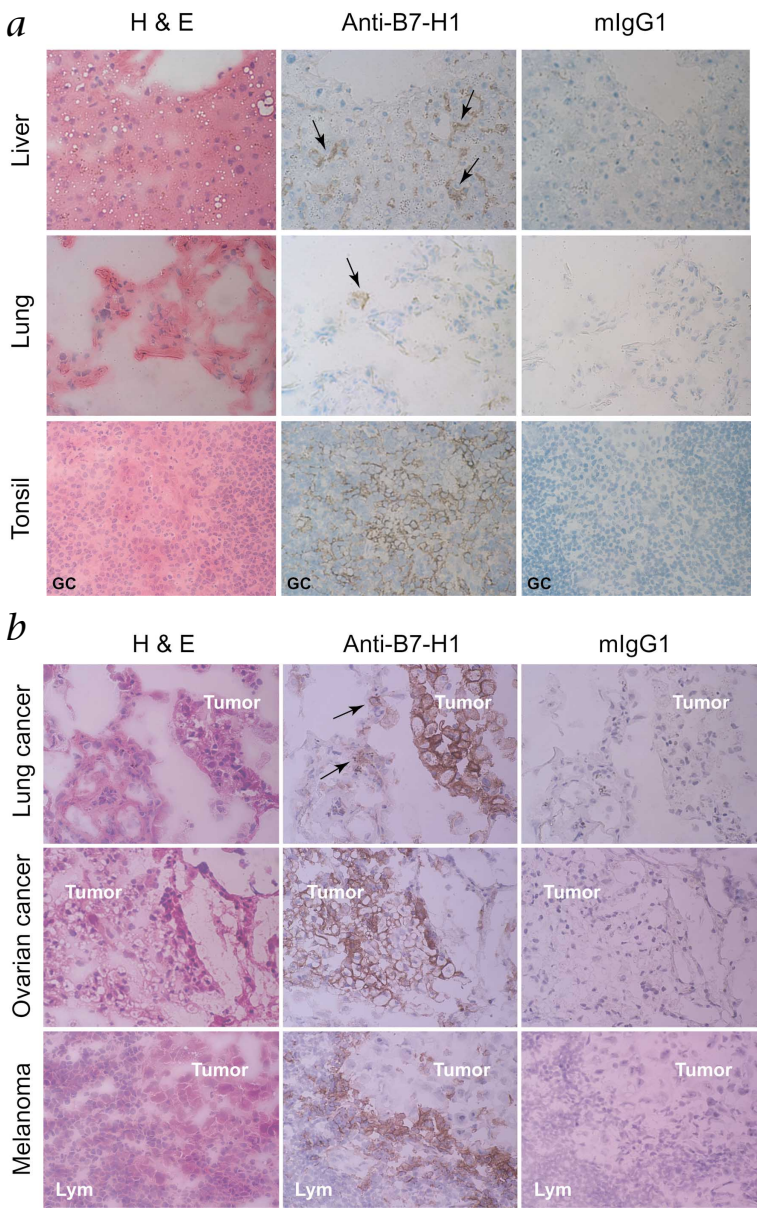


Fig. 1 B7-H1 expression in normal and cancer tissues. Snap-frozen specimens of human tissues, normal or from lung or ovary carcinomas and melanomas, were examined immunohistochemically using monoclonal antibody (5H1) against B7-H1 or control mouse IgG1 (mIgG1). Adjacent sections were also stained with hematoxylin and eosin (H & E) for comparison. ***a***, B7-H1 immunoreactivity was not found in liver parenchyma, the lung or tonsil lymphocytes. B7-H1 expression was limited to macrophage-like cells (arrows) in the Kupffer cells of the liver, in the lung, and in paracortical macrophages near a germinal center (GC) in the tonsil. No B7-H1 expression is found in normal breast, colon, kidney, uterus, muscle or pancreas tissue (see Supplement). Frozen sections of B7-H1/624mel cell pellets were used as positive controls. ***b***, In a lung cancer sample (top), B7-H1 immunoreactivity was limited to the tumor plasma membrane or cytoplasm and was not seen in normal alveolar cells. A few B7-H1-positive pulmonary macrophages are indicated (arrows). In ovarian adenocarcinoma cells (middle), B7-H1 immunoreactivity is in the plasma membrane. In lymph nodes containing metastatic melanoma cells (bottom), B7-H1-positive tumor cells formed a boundary at the interface with B7-H1 negative lymphocytes (Lym).

Table 1 Expression of B7-H1 in human cancer tissues

Diagnosis	Specimen numbers, positive/total (%)	Cases with staining intensity[a] –	+	++	+++
Lung cancer	20/21 (95)	1	9	10	1
Adenocarcinoma	10/10	0	5	5	0
Squamous cell carcinoma	8/8	0	2	5	1
Large cell carcinoma	1/2	1	1	0	0
Neuroendocrine carcinoma	1/1	0	1	0	0
Ovarian cancer	20/23 (87)	3	8	11	1
Adenocarcinoma	19/22	3	7	11	1
Carcinosarcoma	1/1	0	1	0	0
Melanoma	22/22 (100)	0	5	12	5
Skin	13/13	0	4	6	3
Lymph node metastasis	5/5	0	0	4	1
Brain metastasis	1/1	0	0	1	0
Axilla metastasis	2/2	0	1	0	1
Breast metastasis	1/1	0	0	1	0
Colon adenocarcinoma	10/19 (53)	9	6	2	2

[a], Intensity of staining by monoclonal antibody against B7-H1: –, negative; +, focal expression in 10–40% of cancer tissues; ++, focal expression in 40–80% of cancer tissues; +++, diffuse expression in >80% of cancer tissues.

H1/624mel cells to killing by T cells (Fig. 2*e* and *f*). To rule out the possibility that the resistance of B7-H1/624mel cells to M15 CTLs was due to decreased lysis of B7-H1/624mel rather than to inhibition of their M15 proliferation, we compared the sensitivity of mock/624mel and B7-H1/624mel cells to M15-mediated lysis in a 4-hour ^{51}Cr-release cytotoxicity assay. Both were equally sensitive to lysis by M15 CTLs (Fig. 2*g*). In addition, there was no growth advantage of B7-H1/624mel over mock/624mel cells in a proliferation assay *in vitro* (data not shown). Our results thus support that the deletion of M15 CTL clone by exposure to B7-H1 is responsible for the outgrowth of 624mel cells.

We also tested the ability of HBL-100, a human breast cancer–derived cell line that constitutively expresses B7-H1 on its cell surface, to induce T-cell apoptosis. HBL-100 cells express B7-H1, but not FasL or TRAIL, on their surface (Figs 4*a* and 3*a*). HBL-100 cells, however, do express a CTL epitope (IPQQHTQVL) that is derived from carcinoembryonic antigen (CEA), is HLA-B7-restricted and is recognized and lysed by the CD8$^+$ CTL clone M99[21]. Notably, M99 does not express PD-1 throughout the resting and stimulation cycle of the culture. However, B7-H1Ig could still bind the cells after antigen stimulation (Fig. 3*b*). As was seen with B7-H1-transfected 624mel cells, the number of antigen-specific M99 CTLs was reduced and CTLs underwent apoptosis when they were incubated with HBL-100 cells, and T-cell death was inhibited when monoclonal antibody against B7-H1 was included in the medium (Fig. 3*c*). The addition of PD-1Ig up to 10 μg/ml did not inhibit the apoptosis of T cells mediated by HBL-100 cells, a result consistent with lack of PD-1 expression by M99 T cells (Fig. 3*b*). Our result indicate that B7-H1 binds a non-PD-1 receptor on M99 T cells to transmit the apoptotic signal.

To further dissect the mechanisms of B7-H1-mediated apoptosis in human T cells, we used a system in which immobilized B7-H1Ig in the presence of optimal activation dose of monoclonal antibody to CD3 rapidly induced apoptosis of purified human T cells 48 hours after culture (Fig. 4*a*). Apoptosis of T cells could be blocked significantly ($P < 0.05$) by a monoclonal antibody against B7-H1 (data not shown) but not by PD-1Ig (Fig. 4*a*), indicating that PD-1 is not the receptor for apoptotic effect of B7-H1 in this system. Upon stimulation with anti-CD3 antibody and B7-H1Ig, expression of Fas and FasL were upregulated in activated T cells (Fig. 4*b*). In addition, secretion of IL-10 upon B7-H1Ig ligation increased drastically[12]. As both Fas–FasL and IL-10 are involved in the activation-induced T-cell death [18,22], we determined the possible role of these molecules in B7-H1-mediated apoptosis. Inclusion of monoclonal antibody against FasL or IL-10 had minimal effect on apoptosis induced by anti-CD3 monoclonal antibody alone (Fig. 4*c*). Apoptosis of T cells stimulated by anti-CD3 antibody and B7-H1Ig cells could, however, be significantly inhibited (to the level of control $P < 0.01$) by the inclusion of antibodies to FasL, IL-10 or both, but not by control antibody (Fig. 4*c*). Inclusion of antibody against FasL in the coculture system of B7-H1/624mel cells and M15 CTLs (Fig. 4*d*) also inhibited the growth of tumor cells by blocking the interaction of Fas and FasL, leading to the inhibition of M15 CTL apoptosis (Fig. 2*f*). Alone, however, antibody against IL-10 did not affect tumor growth (Fig. 4*d*). Our results indicate that the interaction of Fas and FasL is a component of B7-H1-mediated apoptosis of activated T cells.

Tumor-associated B7-H1 deletes activated T cells *in vivo*

To determine whether T-cell apoptosis *in vitro* correlates with activated T-cell deletion *in vivo*, we used a mouse adoptive transfer model involving T cells containing a transgenic 2C T-cell receptor (TCR), which recognizes a p2Ca peptide in the context of the L^d MHC class I molecule on the P815 tumor line[23,24]. In this model, we injected mock-transfected P815 cells (mock/P815) or B7-H1-transfected P815 cells (B7-H1/P815) intraperitoneally (i.p.) into immunodeficient RAG-1$^{-/-}$ mice to establish progressively growing tumors. We activated the 2C T cells by incubation *in vitro* with BALB/c spleen cells (H-2^d) and then transferred them by i.p. injection into the P815-bearing mice. By 18 hours after injection, the number of 2C T cells greatly increased—accounting for nearly 10% of all peritoneal cells—in mice harboring mock/P815 cells but not in those harboring B7-H1/P815 cells (Fig. 5*a*). In addition, 2C T cells in mice harboring B7-H1/P815 tumor cells underwent significant apoptosis by 8 hours after transfer (Fig. 5*b*). By 42 hours, the number of apoptotic cells had decreased. The absence of 2C T-cell expansion in B7-H1/P815-bearing mice was evidence of the ligand specificity of the initial expansion of the 2C T-cell population in mice bearing mock/P815 cells. The rapid increase in T-cell apoptosis after 18 hours of exposure to B7-H1-transfected tumor cells was suggestive of *in vivo* deletion of activated T cells by tumor-associated B7-H1. Examination of tumor cells in peritoneal cavities after adoptive transfer of 2C T cells, indicated an increase in the number of B7-H1/P815 tumor cells but a proliferation of mock/P815 cells was largely inhibited. Notably, infusion of a neutralizing monoclonal antibody against mouse B7-H1 inhibited the growth of B7-H1/P815 cells *in vivo* (Fig. 5*c*).

To further evaluate the role of tumor-associated B7-H1 in evasion of tumor immunity, we transfected the plasmid encoding mouse B7-H1 into B7-1$^+$P815 cells to establish cell lines express-

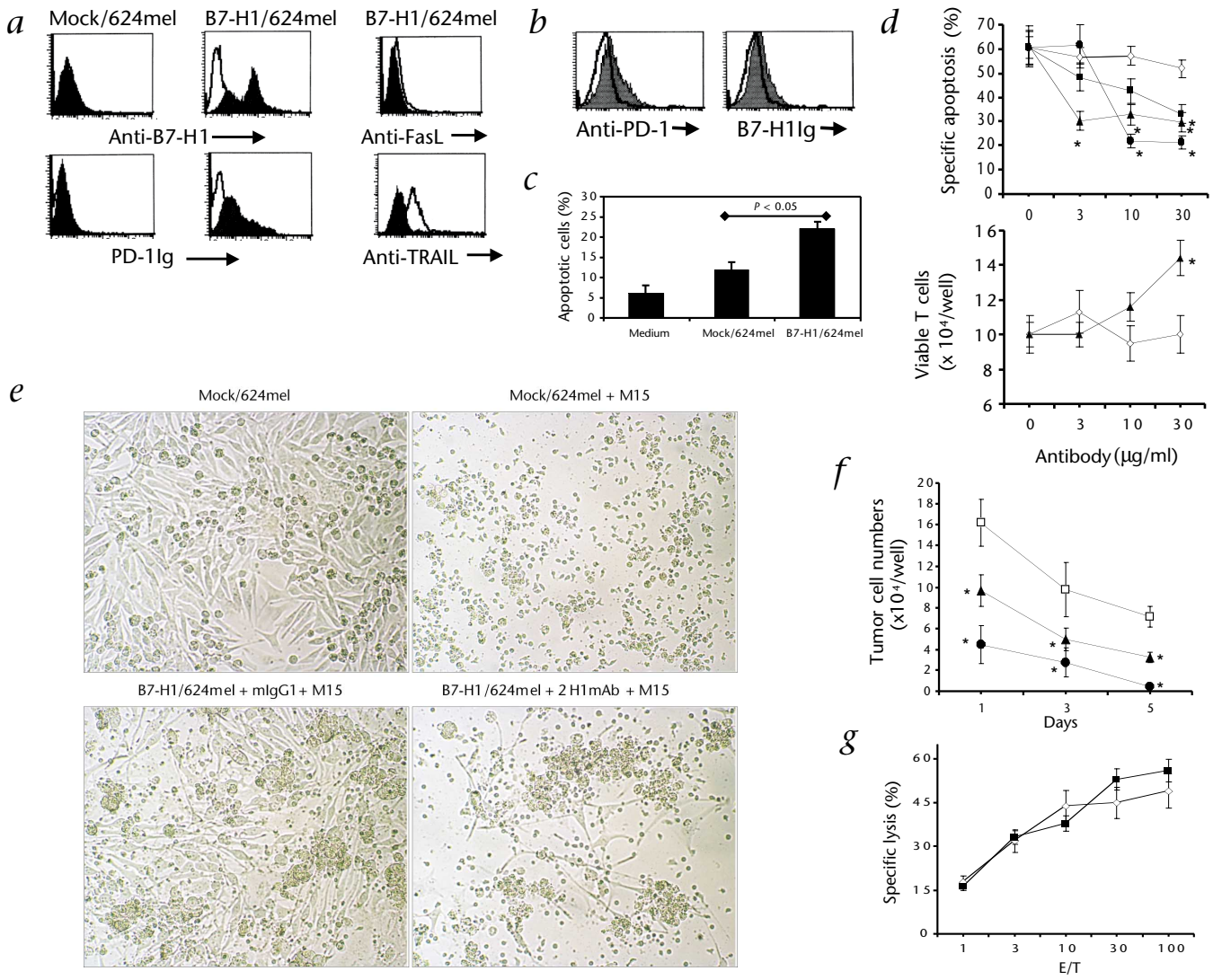


Fig. 2 B7-H1$^+$ 624mel melanoma cells promote apoptosis of tumor-specific M15 CD8$^+$ CTLs and are resistant to growth inhibition by the CTLs. ***a***, FACS analysis of cell-surface B7-H1, FasL and TRAIL on a human melanoma cell line. The melanoma cell line 624mel was transfected with empty pcDNA3 plasmid or plasmid containing full-length human B7-H1 cDNA, and stained with monoclonal antibodies against human B7-H1 (5H1), FasL, TRAIL or PD-1Ig. Positive staining is indicated by the shaded lines and control antibody (mouse IgG1 (mIgG1) or mIgG2a) staining is shown as open bold lines. ***b***, Expression of PD-1 on M15 CTL clone. After 3 d of culture with IL-2 and IL-15, M15 T cells were stained with monoclonal antibody against PD-1 or B7-H1Ig. Positive staining is indicated by the shaded lines and control antibody (mIgG1or mIgG2a) staining is shown as open bold lines. ***c***, Increased apoptosis of M15 CTLs upon incubation with B7-H1/624mel melanoma line. M15 cells at 2×10^5 cells/well were cocultured with medium alone, irradiated mock/624mel or B7-H1/624mel at 1×10^4 cells/well. After 5 d in culture, the cells were harvested and examined for apoptosis by double staining with annexin V$^+$ and antibody against CD8 and subjected to FACS analysis. Apoptosis was calculated as the percentage of annexin V$^+$ cells in viable CD8$^+$ fraction. Data represent means ± s.d. of cells from 3 wells for each treatment condition. ***d***, Effect of monoclonal antibody against B7-H1 and PD-1Ig on apoptosis in a PD-1$^+$M15 CTL line. B7-H1/624mel cells were incubated with control Ig (◇), 5H1 (■), 5H1 F(ab')$_2$ (▲) or PD-1Ig (●) for 1 h at 4 °C before coculture with M15 CTLs. After 5 d, T cells were harvested and stained with annexin V$^+$ and monoclonal antibody against CD8 for apoptosis assay (top) and with Trypan blue for viability assay (bottom). Spontaneous apoptosis in medium alone was 12–15%. Tumor-induced apoptosis of T cells was calculated as [(apoptotic T cells in tumor culture/apoptotic T cells in medium control culture) –1] × 100%, and represented as means ± s.d. of cells from 3 wells for each treatment condition. The results are representative of 3 experiments. *, significantly different from the control Ig group, $P < 0.05$. ***e***, B7-H1/624mel cells are resistant to M15 CTL–mediated growth inhibition *in vitro*. M15 CTLs at 3×10^5 cells/well were cocultured with mock/624mel or B7-H1/624mel cells at an effector to target ratio (E/T) of 1:1 for 5 d in 24-well plates. Monoclonal antibody against B7-H1 (2H1) or control mouse IgG (mIgG1) at 10 μg/ml was included in the culture medium from the beginning. Areas representative of the cell density in each well were photographed at day 5. ***f***, Resistance of B7-H1/624mel to growth inhibition by M15 CTLs can be partially neutralized by antibody against B7-H1 (mean ± s.d. of cell numbers recovered from 3 wells). Tumor cells were collected and their viability examined by Trypan blue exclusion assay on days 1, 3 and 5. ●, Mock/624mel + M15; □, B7-H1/624mel + mIgG1 + M15; ▲, B7-H1/624mel + 2H1 + M15. Results represent 3 experiments. *, significantly different from the B7-H1/624mel + mIgG1 + M15 group, $P < 0.05$. ***g***, Resistance of B7-H1/624mel to M15 CTLs is not caused by a decrease in sensitivity to lysis. Mock/624mel (◇) and B7-H1/624mel (■) cells were labeled with ^{51}Cr and incubated at the indicated ratio of effector to target cells (E/T) with M15 CTLs for 4 h. Cytolytic activity of M15 CTLs against 624mel cells was determined in a standard ^{51}Cr-release assay.

ing both B7-H1 and B7-1. We had shown previously that tumors induced by inoculation of B7-1⁺P815 cells in syngeneic DBA/2 mice regress completely after transient growth, and that tumor resistance is mediated by costimulated T-cell responses[25]. Expression of B7-H1 in B7-1⁺ P815 led to progressive growth of tumors. Expression of B7-H1 alone on P815, however, did not affect tumor growth (Fig. 5*d*). The effect of B7-H1 on tumor growth is not due to clonal variation during the selection of B7-H1 transfectants, because all tumors from transfectants grew progressively at the same rate in immunodeficient BALB/c nude mice (data not shown). Our results thus further support the notion that B7-H1 expression on tumor cells might downregulate active tumor immunity *in vivo*.

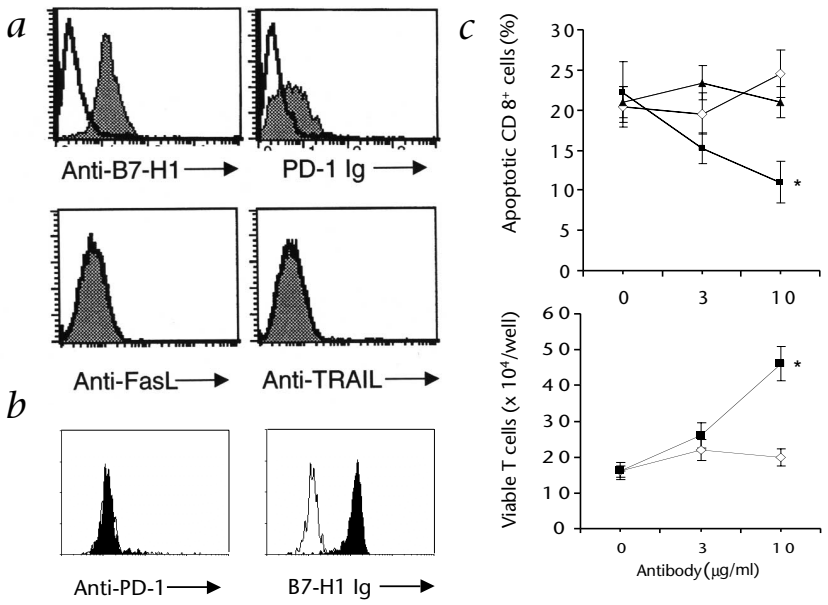


Fig. 3 Human breast carcinoma HBL-100 constitutively expressing B7-H1 promotes apoptosis of the CEA-specific M99 CD8⁺ CTLs. ***a***, FACS analysis of cell-surface B7-H1, FasL and TRAIL on HBL-100 cells. HBL-100 cells were stained by monoclonal antibodies against B7-H1 (5H1), FasL and TRAIL or PD-1Ig. Positive staining is shown as shaded lines and control-antibody staining as open bold lines. ***b***, Absence of PD-1 expression on M99 T cells. After 4 d of culture with HBL100 cells and IL-15, M99 T cells were doubly stained with monoclonal antibodies against CD8 and against PD-1 or with B7-H1Ig. CD8-positive cells were gated for FACS analysis. Positive staining is shown as filled lines and control antibody or immunoglobulin (Ig) staining as open lines. ***c***, Abrogation of M99 CTL apoptosis by monoclonal antibody against B7-H1, but not by PD-1Ig. HBL-100 cells were incubated in the presence of control Ig (◇), 5H1 monoclonal antibody (■) or PD-1Ig (▲) at the indicated concentration for 1 h before coculture with M99 CTLs. After 4 d, T cells were stained with annexin V and antibody against CD8 for apoptosis assay and with Trypan blue for viability assay. Tumor-induced apoptosis of T cells was calculated as [(apoptotic T cells in tumor culture/apoptotic T cells in medium control culture) – 1] × 100%, and represented as means ± s.d. of cells from 3 wells of each treatment. The results are representative of 3 experiments. *, significantly different from the control Ig group, $P < 0.05$.

Discussion

Cancer progression has been attributed to a variety of immune evasion strategies. These include downregulation of cell-surface MHC class I molecules, secretion of immunosuppressive factors (for example, transforming growth factor-β), and lack of T-cell costimulation[26]. Here we describe a new observation that B7-H1 is expressed in many human cancers and promotes apoptotic death of activated tumor antigen-specific human T cells *in vitro* and antigen-specific T cells in a mouse P815 tumor model. Recognition of this new mechanism of tumor evasion will necessitate a new approach to the design of T-cell-based immunotherapy.

FasL is a determinant of immune-privileged status in the eyes and in sites of tumor growth through its induction of programmed cell death in activated T cells[27–28]. Not all malignant human neoplasms express FasL, however, and transfection of tumor cells with FasL does not always promote T-cell apoptosis[29–31]. In this study, we found that cell-surface expression of B7-H1 in normal tissues is limited to macrophages (Fig. 1), a result consistent with our laboratory's earlier report that freshly isolated monocytes express B7-H1[12]. In contrast, we found that B7-H1 immunoreactivity localizes in a majority of melanomas and carcinomas of lung, ovary and colon, in both the plasma membrane and cytoplasm. The mechanisms regulating B7-H1 expression in tumor cells are not known. Inflammatory mediators are implicated by the upregulation of B7-H1 expression on the surface of several tumor lines after exposure to IFN-γ (Supplementary Table 1 online). We also noted that B7-H1 expression was more frequent in freshly isolated cancer tissue specimens than in cultured tumor cell lines. This observation may reflect the cytokine milieu of the cancer patients.

Taken together with our previous report that B7-H1 stimulation promotes production of IL-10, a cytokine implicated in the death of activated T cells[22], our results imply that multiple mechanisms may contribute to the death of activated T cells after exposure to tumor-associated B7-H1. In fact, our results support the finding that both IL-10 and FasL are involved in apoptosis of T cells, as blocking these molecules with neutralizing monoclonal antibody partially inhibited T cell death. Blocking the Fas pathway also resulted in inhibition of tumor cell growth *in vitro*. Although neutralization of IL-10 partially blocked T cell apoptosis (Fig. 4*c*), tumor growth *in vitro* was not affected (Fig. 4*d*). Whereas Fas is the effector molecule that directly triggers apoptosis, the effect of IL-10 is probably indirect and regulated by some unknown factor(s) in the cultures. TRAIL-mediated mechanisms do not seem to contribute to the triggering of apoptosis, because neither 624mel cells nor HBL-100 cells express this protein (Figs. 2 and 3). Recent studies implicate RACS1, a coiled-coil membrane protein, in the apoptosis of activated T cells *in vitro*[32]. It is unknown, however, whether RACS1 is involved in B7-H1 mediated apoptosis.

The proliferation of T cells in the presence of optimal doses of monoclonal antibody against CD3 can be inhibited by B7-H1[13], but this inhibition does not occur if PD-1-deficient T cells are used, indicating that PD-1 may deliver a negative signal for T-cell proliferation[14]. The nature of the inhibition is less clear. It might result from increased programmed cell death after ligation of PD-1 by B7-H1—a particularly attractive hypothesis because PD-1 was originally cloned from a T-cell line undergoing apoptosis[33]. In the M15 clone, apoptosis could be blocked (Fig. 2) by soluble PD-1Ig, suggesting that PD-1 might be a receptor for B7-H1

that delivers an apoptotic signal to M15 T cells. An alternative explanation is that M15 T cells express one or more high-affinity receptors other than PD-1 that compete with the binding of B7-H1 to PD-1 to deliver the apoptotic signal. Similarly, soluble CTLA-4 has been observed to compete with the binding of B7-1 and B7-2 ligands to CD28 (ref. 34). However, PD-1 is expressed on only a fraction of activated mouse and human peripheral T cells[35,36]. Although PD-1Ig significantly inhibited apoptosis of M15, a PD-1$^+$CD8$^+$ CTL line, it did not inhibit apoptosis of M99, a PD-1$^-$ CD8$^+$ CTL line that expresses a B7-H1Ig binding activity. More notably, the induction of apoptosis in polyclonal human T cells by anti-CD3 and B7-H1Ig was not inhibited by PD-1Ig. Our data thus support the possibility that B7-H1 might bind receptor(s) other than PD-1 to transmit the apoptotic signal. B7-H1 might simultaneously bind to PD-1 as well as to the putative alternative receptor, as PD-1Ig does not interfere with the apoptotic effect of B7-H1.

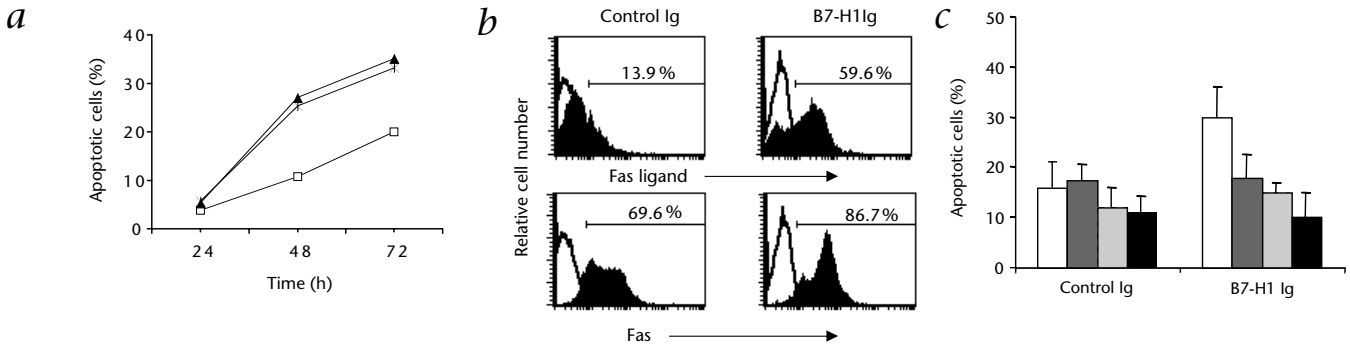
In our experiments, engagement of human primary T cells by B7-H1Ig in the presence of monoclonal antibody against CD3 led to their proliferation (resulting from costimulation) and subsequent inhibition (resulting from programmed death of activated T cells). Neither costimulation or apoptosis of T cells in this system were blocked by PD-1Ig (Fig. 4*a* and Supplementary Fig. C online). These observations suggest that costimulation and induction of apoptosis by B7-H1 might be mediated by the same receptor, which is distinct from PD-1. Taken together with our data showing differential expression of B7-H1 receptors in T-cell clones (Figs 2 and 3), this indicates that B7-H1 might regulate different subsets of T cells through different receptors.

Our results have implications for the design of T-cell-based immunotherapy. For example, adoptive immunotherapy strategies require the infusion of pre-activated T cells. If confronted by an apoptotic molecule such as B7-H1, tumor-specific T cells would be deleted selectively at the tumor site. Our findings also predict that tumors expressing B7-H1 will be more resistant to cancer vaccines, and that blockade of B7-H1 by specific monoclonal antibodies or soluble inhibitors might enhance CTL killing of established cancers.

Methods

Monoclonal antibodies and fusion proteins. Mouse monoclonal antibodies against human B7-H1 were produced by immunizing BALB/c mice with human B7-H1Ig[12]. Two clones (2H1 and 5H1, IgG1) that specifically stained B7-H1/293 cells were used for all studies. The 2 antibodies have similar staining patterns and blocking functions. PD-1Ig was prepared as described[37]. F(ab')$_2$ fragments of 5H1 were prepared by using the Immobilized Pepsin Kit (Pierce, Rockford, Illinois). Monoclonal antibodies against mouse B7-H1 (IgG) were produced by immunizing an Armenian hamster with mouse B7-H1Ig[13], and specifically bound mouse B7-H1 but not mouse B7-H2, B7-H3, B7-1 and human B7-H1 (Zhu *et al.*, unpublished data). Monoclonal antibody against 2C cells was purified from the culture supernatant of 1B2 cells[24]. The monoclonal antibodies specific for CD8 (RPA-T8), Fas (DX2) and Fas ligand (NOK-1) and H-2D^d were purchased from BD PharMingen (San Diego, California), the rabbit antibodies against human TRAIL from Alexis Biochemicals (San Diego, California) and the monoclonal antibody for human PD-1 (J116) from eBioscience (San Diego, California). The cells were analyzed by staining specific monoclonal antibodies and imaging using a FACScan (Becton Dickinson, Mountain View, California) and CellQuest software (Becton Dickinson).

Immunohistochemistry. Human tumor lines were either purchased from the American Type Culture Collection (Manassas, Virginia) or established in the authors' laboratories[38,39]. Human cancer and normal tissue samples were obtained from the Pathology Department and blood samples were from the Department of Transfusion Medicine of the Mayo Clinic with approval of the Institutional Review Board. Frozen tissues were sectioned and stained with monoclonal antibody against B7-H1 (5H1) and with control antibody (mouse IgG1) using a standard protocol.



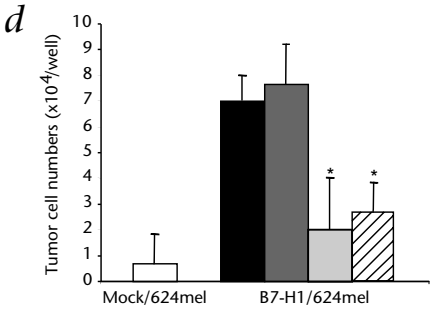


Fig. 4 Role of FasL and IL-10 in B7-H1-mediated apoptosis of activated T cells. Purified human T cells (2×10^5 cells/well) were cultured with plate-bound control Ig or B7-H1Ig at 10 μg/ml in the presence of optimal dose of antibody against CD3 for 72 h, and then analyzed for antigen expression and apoptosis. ***a***, A time course of apoptosis of activated T cells induced by anti-CD3 and B7-H1Ig. Human PD-1Ig or control Ig was included in the culture medium from the beginning. □, control Ig; ▲, B7-H1Ig + control; ✳, B7-H1Ig + PD-1Ig. ***b***, Expression of Fas and FasL. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against Fas, or with purified monoclonal antibody against Fas ligand followed by FITC-conjugated goat antibody against mouse IgG (Fab')$_2$. Numbers on the bar indicate the percentage of positive cells as compared with non-specific staining control. ***c***, Blocking effects of antibodies against IL-10 (■), Fas ligand (■) or both (■) or control antibody (□). Neutralizing monoclonal antibodies against IL-10 or Fas ligand were added from the beginning of culture. Results of a representative of 4 experiments are shown. ***d***, Blockade of Fas–FasL interaction enhanced CTL-mediated inhibition of tumor cell growth. M15 CTLs were cocultured with mock/624mel or B7-H1/624mel cells for 3 d. Antibodies against Fas ligand (■), IL-10 (■) or both (▨), or control antibody (□, ■), at 10 μg/ml were included from the beginning of culture. Tumor-cell viability was assessed by Trypan blue exclusion assay. Data were presented as the mean ± s.d. of cell numbers recovered from 3 wells of each treatment. The results are representative of 3 experiments. *, significantly different from B7-H1/614mel + control antibody group, $P < 0.05$.

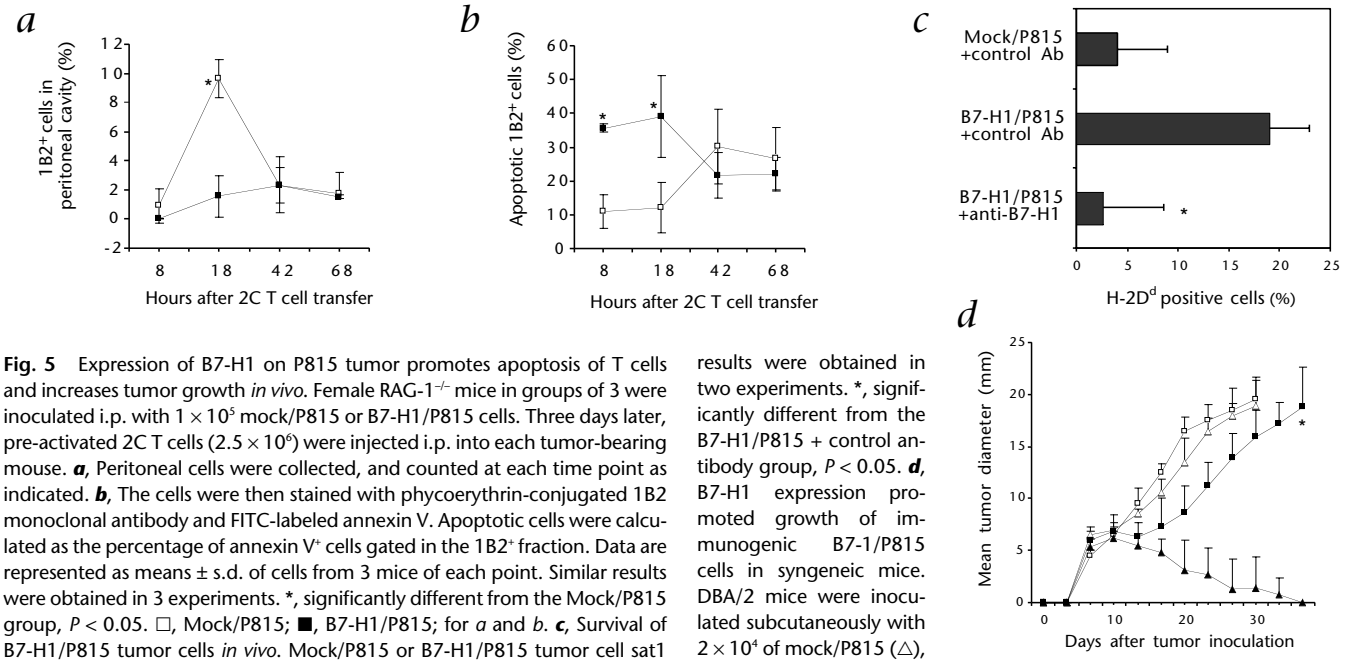


Fig. 5 Expression of B7-H1 on P815 tumor promotes apoptosis of T cells and increases tumor growth *in vivo*. Female RAG-1$^{-/-}$ mice in groups of 3 were inoculated i.p. with 1×10^5 mock/P815 or B7-H1/P815 cells. Three days later, pre-activated 2C T cells (2.5×10^6) were injected i.p. into each tumor-bearing mouse. ***a***, Peritoneal cells were collected, and counted at each time point as indicated. ***b***, The cells were then stained with phycoerythrin-conjugated 1B2 monoclonal antibody and FITC-labeled annexin V. Apoptotic cells were calculated as the percentage of annexin V$^+$ cells gated in the 1B2$^+$ fraction. Data are represented as means ± s.d. of cells from 3 mice of each point. Similar results were obtained in 3 experiments. *, significantly different from the Mock/P815 group, $P < 0.05$. □, Mock/P815; ■, B7-H1/P815; for *a* and *b*. ***c***, Survival of B7-H1/P815 tumor cells *in vivo*. Mock/P815 or B7-H1/P815 tumor cell sat1 $\times 10^5$ were inoculated i.p. 3 d before the transfer of activated 2C T cells. Control antibody (Ab) or monoclonal antibody against B7-H1 was injected i.p. 2 h before the transfer of 2C T cells. One day after the transfer of 2C T cells, the peritoneal cells were harvested and stained with antibody against H-2D^d. The presence of P815 tumor was shown by the percentage of H-2D^d positive cells. Data are represented as mean ± s.d. of cells from 3 mice. Similar results were obtained in two experiments. *, significantly different from the B7-H1/P815 + control antibody group, $P < 0.05$. ***d***, B7-H1 expression promoted growth of immunogenic B7-1/P815 cells in syngeneic mice. DBA/2 mice were inoculated subcutaneously with 2×10^4 of mock/P815 (△), B7-H1/P815 (□), or 5×10^4 of B7-1/P815 (▲), B7-1/B7-H1/P815 (■) tumor cells. Tumor sizes were assessed by measuring 2 perpendicular diameters in millimeters (mm) by a caliper, and the results were expressed as mean ± s.d. of tumor diameter from 5 mice. Similar results were obtained in 4 experiments. *, significantly different from the B7-1/P815 group, $P < 0.01$.

Transfection. Human melanoma 624mel cells were transfected with the pcDNA3 plasmid containing the full-length human B7-H1 gene[12] (B7-H1/624mel) or with the wild-type pcDNA3 plasmid (Mock/624mel), and selected on the basis of G418 resistance. Mock/P815, B7-1/P815[25], B7-H1/P815 and B7-1/B7-H1/P815 tumors were transfected with the corresponding plasmids as described previously[13], and the expression of surface molecules was confirmed by FACS analysis using specific monoclonal antibodies.

Generation of antigen-specific CTLs. Tumor antigen–specific CD8$^+$ human T-cell clones were generated from peripheral blood mononuclear cells from a healthy HLA-A2-positive donor by *in vitro* stimulation using dendritic cells loaded with corresponding peptide epitopes, as described[21]. M15 is a human CTL clone that specifically recognizes an HLA-A2-restricted epitope (IMDQVPFSV) of the gp100 antigen. M99 is an HLA-B7-restricted CTL clone[21] that recognizes an epitope (IPQQHTQVL) of carcinoembryonic antigen. CTL activity was determined by a 4-h ^{51}Cr-release cytotoxicity assay. To activate 2C T cells, 4×10^7 CD8$^+$ T cells were purified from spleen and lymph nodes using MACS beads (Miltenyi Biotech, Auburn, California) and subsequently stimulated with irradiated BALB/c splenocytes and 10 IU/ml human IL-2 for 3 d. Viable 2C T cells were purified with Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada).

Cell apoptosis assays. Human CD8$^+$ CTLs (M15 or M99 clones) at 2×10^5 cells per well were cultured with irradiated tumor cells at 1×10^4–5×10^4 cells per well for 4–5 d. Monoclonal antibodies, fusion proteins and control Ig were included from the start of culture. Cells were harvested at the indicated times and stained with annexin V and monoclonal antibodies against CD8. Apoptosis was calculated as the percentage of annexin V$^+$ cells gated in the CD8$^+$ fractions. To assay for apoptosis of 2C T cells, female RAG-1$^{-/-}$ mice were inoculated i.p. with 1×10^5 Mock/P815 or B7-H1/P815 cells. Three days later, pre-activated 2C T cells (2.5×10^6) were injected i.p. into each mouse. Peritoneal cells were collected, counted at each time point as indicated and stained with 1B2 monoclonal antibody and annexin V. Apoptosis was calculated as the percentage of annexin V$^+$ cells gated in the 1B2$^+$ fraction. Data are represented as means ± s.d. of cells from 3 mice for each point in at least 3 experiments.

To induce apoptosis in polyclonal T cells, purified human peripheral blood T cells (>95% CD3$^+$) at 4×10^5 per well were precoated cultured in plates precoated with 0.5 μg/ml of monoclonal antibody against CD3 (clone HIT3a, PharMingen) and 10 μg/ml of either immobilized B7-H1Ig or control Ig (mouse IgG2a) for 72 h. T cells at 1×10^5 per sample were stained with annexin V (5 μl per test) and propidium iodide (PI; 5 μg/ml; Sigma) for 1 h and the samples were analyzed by FACS. Apoptosis was calculated as the percentage of annexin V$^+$ PI$^-$ cells in the viable cell fraction. For blockade of apoptosis, the human PD-1Ig (30 μg/ml) and the neutralizing monoclonal antibody against human IL-10 (5 μg/ml, JES3-9D7, PharMingen) or against human FasL (8 μg /ml, NOK-1, PharMingen) were added from the outset.

Mouse studies. Female DBA/2 (H-2^d) and RAG-1$^{-/-}$ mice (H-2^b) were purchased from the Jackson Laboratory (Bar Harbor, Maine). 2C (H-2^b) mice were developed by F. Carbone. RAG-1$^{-/-}$ mice (H-2^b) mice were inoculated with 1×10^5 Mock/P815 or B7-H1/P815 cells i.p. After 3 d, 150 μg/mouse of control antibody (hamster IgG) or monoclonal antibody against mouse B7-H1 was injected i.p. 2 h before the transfer of activated 2C T cells. One day later, P815 tumor cells (H-2^d) were detected by staining the peritoneal cells with monoclonal antibody against H-2^d. DBA/2 mice were inoculated subcutaneously with Mock/P815, B7-H1/P815 at 2×10^4 or B7-1/P815, B7-1/B7-H1/P815 cells at 5×10^4 cells per mouse, and tumor sizes were monitored by measuring perpendicular diameters. All studies were approved by the Mayo Foundation's Institutional Animal Care and Use Committee.

Note: Supplementary information is available on the Nature Medicine website.

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Competing interests statement
The authors declare that they have no competing financial interests.

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