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B7-H1 Expressed by Activated CD8 T Cells Is Essential for Their Survival

Vesna Pulko,* Kimberley J. Harris,† Xin Liu,† Rachel M. Gibbons,* Susan M. Harrington,† Christopher J. Krco,† Eugene D. Kwon,*‡ and Haidong Dong*†

An immunoinhibitory role of B7 homologue 1 (B7-H1) expressed by non-T cells has been established; however, the function of B7-H1 expressed by T cells is not clear. Peak expression of B7-H1 on Ag-primecd CD8 T cells was observed during the contraction phase of an immune response. Unexpectedly, B7-H1 blockade at this stage reduced the numbers of effector CD8 T cells, suggesting B7-H1 blocking Ab may disturb an unknown function of B7-H1 expressed by CD8 T cells. To exclusively examine the role of B7-H1 expressed by T cells, we introduced B7-H1 deficiency into TCR transgenic (OT-1) mice. Naive B7-H1-deficient CD8 T cells proliferated normally following Ag stimulation; however, once activated, they underwent more robust contraction in vivo and more apoptosis in vitro. In addition, B7-H1-deficient CD8 T cells were more sensitive to Ca-dependent and Fas ligand-dependent killing by cytotoxic T lymphocytes. Activation-induced Bcl-xL expression was lower in activated B7-H1-deficient CD8 T cells, whereas Bcl-2 and Bim expression were comparable to the wild type. Transfer of effector B7-H1–deficient CD8 T cells failed to suppress tumor growth in vivo. Thus, upregulation of B7-H1 on primed T cells helps effector T cells survive the contraction phase and consequently generate optimal protective immunity. *The Journal of Immunology, 2011, 187: 5606–5614.

B7-H1 1 (B7-H1) is an immunoregulatory molecule in the B7 family. We first described B7-H1 in 1998 (1) and identified its expression in human cancer cells in 2002 (2). Since then, B7-H1 has been reported to be aberrantly expressed by many human cancer cells, and high B7-H1 expression has been correlated with poor prognosis in several human cancers (3). Thus, B7-H1 blockade has been proposed as a means of countering the immunosuppressive effects of tumors to improve tumor immunotherapy (4–7). However, the drive to exploit B7-H1 as a clinical immune target has outpaced any comprehensive understanding of B7-H1 function.

B7-H1 is expressed by several cell types, including T cells. Its constitutive expression is primarily restricted to cells of myeloid lineage, such as macrophages and dendritic cells in both mice and humans (1, 2, 8). In other cell types such as lymphoid lineage cells, endothelial, and epithelial cells, B7-H1 expression can be either induced or upregulated by IFN-γ and TNF-α (7). Naive murine T cells constitutively express low levels of B7-H1, whereas naive human T cells do not, and both murine and human T cells express markedly increased levels of B7-H1 after activation (1, 8, 9). In contrast to accumulating studies on tumor B7-H1, less attention has been paid to elucidating the functional role of B7-H1 expressed by T cells, especially in modulation of protective T cell immunity. Given that T cells are major immune effectors and that B7-H1 has a dynamic expression on activated T cells, it is imperative to address the functional role of T cell-associated B7-H1 in greater detail.

B7-H1 expressed by non-T cells (macrophages, tumor cells) functions as a negative regulator of T cell responses (7). Therefore, B7-H1 blockade strategy has been used to improve immune responses against tumors and various infections (7). However, some deleterious effects of B7-H1 blockade have been reported in animal models of infection (10, 11) and inflammation (12). Pathogen-specific effector T cells have been found to express higher levels of B7-H1, and B7-H1 deficiency has been shown to impair the development and maintenance of effector T cells (13). Common features of studies using infection or inflammation models include the upregulation of B7-H1 on effector T cells during the acute phases of the immune reaction and subsequent reduction and contraction of the effector T cell populations, as well as compromised protective immunity after B7-H1 Ab treatment. Despite these unexpected observations, the detailed function of B7-H1 expressed by T cells in Ag-specific, notable antitumor T cell responses has not been addressed.

The present study was designed to directly examine the role of B7-H1 expressed by Ag-specific T cells in regulation of T cell function and survival. We found that B7-H1–deficient CD8 T cells, while undergoing normal Ag-initiated expansion, exhibited increased contraction in vivo and more apoptosis after Ag stimulation in vitro. B7-H1–deficient CD8 T cells also show increased susceptibility to killing by other CTLs. In addition, we observed that in response to activation, B7-H1–deficient CD8 T cells expressed lower levels of antiapoptotic Bcl-xL, whereas expressions of two other key mediators of intrinsic apoptotic pathway, Bcl-2 and Bim, remained comparable to wild type (WT). Taken together, our studies suggest a prosurvival function of B7-H1 that is essential for effector T cells to survive during contraction phase of the immune response, thereby eliciting protective immunity.

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**Materials and Methods**

**Mice, cell lines, and reagents**

Female C57BL/6 mice and B6.SJL congenic mice (8–12 wk old) were purchased from Taconic Farms (Germantown, NY) and National Institutes of Health. OT-1 TCR (Thy1.2) transgenic mice and Thy1.1 B6.PL-Thy1a/CyJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-1 Thy1.1 mice were provided by T. Tian (Harvard University). B7-H1 knockout (KO) B6 mice (14) were used to breed B7-H1 KO OT-1 TCR transgenic mice. Murine melanoma melanomas (B16-OVA), EG7-OVA, and EL4 thymoma cells were cultured in RPMI 1640 medium (Cellgro, Manassas, VA) with 10% FBS (Life Technologies, Carlsbad, CA), 1 U/ml penicillin, 1 µg/ml streptomycin, and 20 mM HEPES buffer (all from Mediatech, Manassas, VA). Studies were conducted in accordance with the National Institutes of Health guidelines for the proper use of animals in research and with local Institutional Animal Care and Use Committee approval.

**Flow cytometry analysis**

Class I MHC (K^b^OVA peptide 5IINFEKL) tetramer and negative control tetramer were purchased from Beckman Coulter (Brea, CA). Fluorochrome-conjugated Abs against CD8, CD44, CD62L, CCR7, CD43 (1B11), Fas (CD95), Fas ligand, CD122, CD127, CD45.2 (Ly 5.2), and CD90.2 (Thy1.2) were purchased from BD Biosciences (Mountain View, CA), BioLegend (San Diego, CA), or eBioscience (San Diego, CA). Abs to Bcl-xL, Bcl-2, and Bim were purchased from Cell Signaling Technology (Danvers, MA). To detect the intracellular levels of Bcl-xL, Bcl-2, and Bim, T cells were fixed with 2% paraformaldehyde for 10 min at 37°C, followed by permeabilization with ice-cold methanol for 30 min. After blocking with 15% rat serum for 15 min, cells were stained with Abs for 1 h at room temperature. After staining, cells were washed three times with incubation buffer before analysis. At least 100,000 viable cells were live gated on FACSscan or FACSCalibur (BD Biosciences) instrumentation. Flow cytometry analysis was performed using FlowJo software (Tree Star, Ashland, OR).

**T cell immunization, activation, and apoptosis assay**

To induce in vivo CD8 T cell responses, mice were immunized with i.p. injection of 0.5 mg OVA protein (Sigma-Aldrich, St. Louis, MO) and 50 µg poly(I:C) (Sigma-Aldrich), as reported (15). For in vitro T cell activation and apoptosis assay, purified CD8 T cells were labeled with CFSE (Invitrogen-Molecular Probes, Eugene, OR) and incubated with OVA peptide (10 g/ml) for 72 h. Proliferation of T cells was analyzed by CFSE dilution using flow cytometry. Apoptosis of CD8 T cells was analyzed by staining using annexin V (BD Biosciences) and tetramethylrhodamine (TMRE; ethyl ester) (Invitrogen-Molecular Probes). Apoptosis in progress was analyzed by intracellular staining for active caspase-3 using Ab from BD Biosciences (clone C92-605).

**Adoptive transfer of effector CD8 T cells in tumor models**

Purified CD8 T cells were activated for 24–48 h with anti-CD3/CD28 beads or OVA peptide and IL-2 (Chiron, Emeryville, CA) (10 IU/ml) and transferred into sublethally-irradiated B6 mice. For tumor suppression assays, mice were injected with B16-OVA tumors (8 × 10^3, s.c. in the right flank) 1 d after T cell transfer. Tumor growth was monitored by measurement of the longest bisecting diameters of flank tumors. For tumor-infiltrating assays, EG7-OVA tumor cells (1 × 10^6) were injected i.p. on day 7 after T cell transfer. One week after tumor cell injection, cells from peritoneal cavities were harvested for flow cytometry assay.

**CTL function assay**

Degranulation of CTLs was analyzed by CD107a mobilization (16), followed by intracellular staining for IFN-γ. To detect cytotoxicity, target cells were labeled with calcine acetoxymethyl ester (Invitrogen-Molecular Probes) before the CTL assay (17). Calcine release, quantified by an automated fluorescence measurement system with an excitation of 485/20 and an emission filter of 530/25 scanning for s/well, was used to measure target cell lysis. Ag-specific cytotoxic activity was calculated as percentage of specific lysis = 100 × (test release – spontaneous release)/maximum release – spontaneous release).

**T cell–T cell fratricide assay**

Effector CTLs were prepared by activating CD8 T cells from OT-1 mice for 48 h in the presence of anti-CD3/CD28 or Ag peptide and IL-2 (10 IU/ml). Target T cells from WT or B7-H1 KO B6 mice were activated with Con A (5 µg/ml) for 48 h and loaded with or without OVA peptide, followed by labeling with CFSE (1 µM). To block CD8-dependent or Fas ligand-mediated cytolytic activity, graded EGT (Sigma-Aldrich) or 10 µg/ml anti-Fas ligand-neutralizing Ab (clone MFL4, eBioscience, San Diego, CA) was added at the beginning of culture. The survival of CFSE^+ target cells was analyzed by flow cytometry. Cytolytic activity was calculated as percentage of lysis = (1 – % CFSE^+ of target with OVA peptide/% CFSE^+ of target without peptide) × 100%.

**Statistical analysis**

A two-sided, unpaired Student t test was used to assess statistical differences in tumor growth between groups of mice. A p value <0.05 was considered statistically significant.

**Results**

**Late blockade of B7-H1 reduces the numbers of effector CD8 T cells following immunization**

In our attempt to identify the optimal timing of B7-H1 blockade to improve T cell responses, we administrated anti–B7-H1 blocking Ab either during the early (days 0–3) or late (days 7–10) stages following immunization (Fig. 1A). These time periods were set according to the kinetics of T cell response following OVA and poly(I:C) immunization (15). Early B7-H1 blockade greatly increased the expansion of OVA Ag-specific (K^b^OVA tetramer^+^) and functional IFN-γ^+^ CD8 T cells in spleens of immunized mice (Fig. 1B, 1C, 1F; p < 0.05, p < 0.01). Unexpectedly, late B7-H1 blockade decreased the percentages and numbers of Ag-specific (tetramer^+^) and effector (IFN-γ^+^) CD8 T cells in the spleens of mice (Fig. 1D–F; p < 0.05, p < 0.01). Taken together, the results of early blockade of B7-H1 are consistent with our and others’ reports suggesting an inhibitory role of B7-H1 expressed by APCs (dendritic cells) during the early stage of T cell priming (18, 19). However, the opposite effects of late B7-H1 blockade indicate an unknown function of B7-H1 expressed by other cells than dendritic cells during the late stage of T cell responses.

**Effector CD8 T cells upregulate B7-H1 following immunization**

The observed difference in functionality of B7-H1 blockade depending on timing could reflect different role of B7-H1 associated with specific cell type expressing it. It has been reported that T cell activation leads to upregulation of B7-H1 on both human and mouse T cells (8, 9). To determine whether B7-H1 expressed by activated T cells could be a potential target of late B7-H1 blockade, we examined the kinetics of B7-H1 expression on Ag-specific CD8 T cells during the late stage of T cell priming (18, 19). The results of early blockade of B7-H1 are consistent with our and others’ reports suggesting an inhibitory role of B7-H1 expressed by APCs (dendritic cells) during the early stage of T cell priming (18, 19). However, the opposite effects of late B7-H1 blockade indicate an unknown function of B7-H1 expressed by other cells than dendritic cells during the late stage of T cell responses.
B7-H1–deficient CD8 T cells have normal initial proliferation, but cannot accumulate due to increased apoptosis

To directly identify the function of B7-H1 expressed by CD8 T cells, we introduced B7-H1 deficiency into OT-1 TCR transgenic mice and produced B7-H1–deficient OT-1 mice in which CD8 T cells carry OVA-specific TCR, but do not express B7-H1.

We first examined their proliferation to Ag stimulation in vitro and in vivo. Naive B7-H1 KO and WT CD8 OT-1 T cells underwent similar Ag-stimulated proliferation in vitro and homeostatic proliferation in vivo (Supplemental Fig. 1A,1B). Next, we examined whether they have any difference in spontaneous apoptosis.

Freshly isolated WT and B7-H1 KO CD8 T cells underwent comparably low levels of spontaneous apoptosis demonstrated by similar levels of annexin V binding, TMRE staining (measuring mitochondrial trans-membrane potential, which decreases during apoptosis) (21), and active caspase-3 levels (Supplemental Fig. 1C,1D). Comparable rates of apoptosis of WT and B7-H1 KO CD8 T cells were observed up to 3 d of culture in medium.
alone (Supplemental Fig. 1E); however, when stimulated with Ag (OVA), B7-H1 KO CD8 T cells underwent more apoptosis than WT CD8 T cells, as demonstrated with annexin V+ and TMRElow staining (Fig. 3A; p < 0.01) and increased levels of active caspase-3 (Fig. 3B; p < 0.01). Accordingly, the numbers of alive B7-H1 KO CD8 T cells had ~2-fold decrease between days 3 and 5 after activation (Fig. 3C; p < 0.05). To examine whether increased death of B7-H1 KO CD8 T cells was also associated with impaired proliferation, CD8 T cells were labeled with CFSE (an intracellular dye for tracking cell division). On day 3 post-Ag stimulation, B7-H1 KO and WT OT-1 CD8 T cells underwent similar proliferation (up to six divisions), but the percentage of B7-H1 KO CD8 T cells that underwent three or more divisions decreased by ~2-fold compared with WT CD8 T cells (Fig. 3D; p < 0.05). These results suggest the B7-H1–deficient CD8 T cells undergo normal initial proliferation, but cannot accumulate due to increased apoptosis.

**B7-H1–deficient CD8 T cells have more contraction in vivo**

To examine whether increased apoptosis associated with B7-H1 deficiency affects T cell expansion and contraction in vivo, we transferred naive WT or B7-H1 KO OT-1 CD8 T cells (Thy1.2+) into cognate (Thy1.1+) B6 mice that were then immunized 1 d later. Similar frequencies and numbers of transferred Thy1.2+ WT and B7-H1 KO CD8 T cells were detected in the spleens on day 4 after immunization (Fig. 4A, 4B). However, on day 6, a 3-fold more contraction was measured among B7-H1 KO OT-1 CD8 T cells compared with WT OT-1 CD8 T cells, as shown by decreased percentages and absolute numbers of transferred B7-H1 KO CD8 T cells (Fig. 4A, 4B; p < 0.05). Despite their difference in contraction, WT and KO OT-1 CD8 T cells differentiated into equal effector cells, as shown by comparable expression of effector T cell markers (CD62Llow CCR7low and CD43 [1B11]high) and IFN-γ production on day 4 (Fig. 4C). Thus, B7-H1 deficiency does not affect expansion or differentiation of primed CD8 T cells, but may compromise survival of the effector CD8 T cells.

![FIGURE 3](http://www.jimmunol.org/)  
**FIGURE 3.** Increased apoptosis of B7-H1–deficient CD8 T cells following Ag stimulation. Purified CD8 T cells from WT and B7-H1 KO OT-1 mice were incubated (1 × 10⁶ cells/ml) with OVA peptide (1 μg/ml) for up to 5 d in vitro. Apoptosis of Ag-stimulated CD8 T cells was analyzed by staining with TMRE for decreased mitochondrial transmembrane potential and annexin V binding (A), and intracellular staining for active caspase-3 (B). Numbers show percentages (mean ± SD, n = 3) of apoptotic (annexin V+ TMRElow or active caspase-3+) CD8 T cells after 2 d of culture. *p = 0.004, **p < 0.001. C, Numbers of alive T cells were counted based on trypan blue exclusion (n = 3). *p < 0.05. D, CD8 T cells were labeled with CFSE and incubated with OVA peptide, control peptide, or PBS for 3 d. Proliferation was analyzed by evaluation of CFSE dilution using flow cytometry. Numbers show the percentages of proliferating T cells that have undergone three or more times of division. *p < 0.05.

![FIGURE 4](http://www.jimmunol.org/)  
**FIGURE 4.** B7-H1–deficient CD8 T cells have normal expansion, but more robust contraction. Purified CD8 T cells (1 × 10⁶) from WT or B7-H1 KO OT-1 mice (Thy1.2+) were transferred into Thy1.1+ mice. One day after transfer, recipient mice were immunized with OVA protein and poly(I:C). On days 4 and 6 after immunization, the spleen cells were stained with anti-CD8 and anti-Thy1.2 Abs (to identify transferred OT-1 cells). A, Comparison of numbers of transferred Thy1.2+ WT or B7-H1 KO OT-1 CD8 T cells in the spleen (n = 5). B, Numbers in dot-blot figures show the mean percentage ± SD, n = 3. *p < 0.05. C, Phenotype (CD62L, CCR7, CD43) and function (intracellular IFN-γ producing) of WT OT-1 CD8 T cells (shaded) and B7-H1 KO CD8 T cells (open line) on days 4 after immunization. Data are representative of three independent experiments with three mice per group.

Fewer B7-H1 KO effector CD8 T cells survive in vivo

Several reports indicated that B7-H1 expressed by T cells functions in a T cell-suppressive manner (22, 23). Therefore, it was possible that the absence of B7-H1 on CD8 T cells could have enhanced activation-induced cell death due to continued Ag stimulation.
in vivo. To exclude the impact of continued Ag stimulation on effector T cell survival in vivo, we transferred preactivated CD8 T cells into Ag-free syngeneic mice. Thy1.2⁺ WT and B7-H1 KO OT-1 CD8 T cells were preactivated by incubation with anti-CD3/CD28 beads and IL-2 for 24 h. After activation, the expression of T cell activation markers (CD44, CD69, and CD25) and cytokine production were comparable between WT and B7-H1 KO OT-1 CD8 T cells (Supplemental Fig. 2A, 2B). Stimulating beads were removed and CD8 T cells were transferred into naïve Thy1.1⁺ lymphopenic mice that did not have OVA Ag. B7-H1 KO CD8 T cells did not expand as well as their WT counterparts (Fig. 5A). On days 14–21 after the transfer, the frequency of transferred B7-H1 KO Thy1.2⁺ CD8 T cells decreased by 2- to 3-fold compared with the frequency of transferred WT Thy1.2⁺ CD8 T cells (Fig. 5B; p < 0.05).

In addition, we mixed preactivated WT (Thy1.1⁺ CD45.2⁺) and B7-H1 KO (Thy1.1⁺ CD45.2⁻) OT-1 CD8 T cells in equal numbers, cotransferred them into the same CD45.1⁺ B6.SJL mice, and analyzed their numbers on day 15 post-T cell transfer. Again, the percentages and absolute numbers of transferred B7-H1 KO CD8 T cells decreased by 3-fold in the spleen of recipient mice (Fig. 5C, SD; p < 0.01, p < 0.05). These results suggest that the increased contraction or decreased survival of B7-H1⁻ deficient CD8 T cells was not caused by overactivation from continued Ag stimulation.

**B7-H1⁻ deficient CD8 T cells are more susceptible to killing by CTLs**

Because T cell fratricide is one of the proposed mechanisms responsible for T cell depletion (24–27), we examined whether decreased survival of activated B7-H1 KO CD8 T cells could be explained by increased susceptibility to killing by other CTLs. Activated CD8 T cells from WT or B7-H1 KO C57BL/6 mice were used as target cells in an in vivo CTL assay system. Equal numbers of target WT or KO T cells loaded with or without OVA peptide were mixed and injected into C57BL/6 hosts that were immunized with OVA protein/poly(I:C) 7 d earlier. Three and a half hours after the injection, the killing of target cells by host CTLs was evaluated by staining of hosts’ spleens for the presence of transferred B7-H1 KO (CFSEhigh) or WT (CFSElow) target CD8 T cells. Without cognate Ag peptide on their surface, similar frequencies of WT and B7-H1 KO CD8 T cells were detected in the spleens of immunized mice (Fig. 6A). However, when loaded with cognate Ag (OVA) peptide, the frequency of remained B7-H1 KO CD8 T cells decreased by 2-fold compared with WT CD8 T cells (Fig. 6A), indicating that more B7-H1 KO CD8 T cells were killed by host CTLs in an Ag-dependent way (Fig. 6B; p = 0.007). It is important to note that the increased sensitivity of B7-H1⁻ KO target T cells to killing by CTL was not attributed to increased CTL function in the absence of B7-H1 on target cells, as OT-1 CTLs showed similar degranulation and IFN-γ production in

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Fewer preactivated B7-H1⁻ deficient CD8 T cells survive in vivo. Preactivated WT or B7-H1 KO CD8 T cells (1 × 10⁶) were transferred separately (A, B) or together (C, D) into naïve congeneric mice (three mice/group). A. Normalized numbers of transferred Thy1.2⁺ WT or B7-H1 KO CD8 T cells in the recipient spleens. The total number of cells at day 8 after the transfer is normalized to the number transferred (100%). *p < 0.05. Numbers of T cells are shown as mean ± SD of three mice per time point. B. Percentages of recovered Thy1.2⁺ WT and B7-H1 KO CD8 T cells in the Thy1.1⁺ recipient spleens. *p < 0.05. One of three independent experiments is shown. The percentages (C) and numbers (D) of WT (Thy1.1⁺ CD45.2⁺) and B7-H1 KO (Thy1.1⁺ CD45.2⁻) CD8 T cells that were cotransferred into CD45.1⁺ mice. Data show the percentages and numbers (mean ± SD of three mice) of recovered CD8 T cells in the recipient spleens on day 15 post-T cell transfer. **p < 0.01.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** B7-H1⁻ deficient CD8 T cells are sensitive to killing by CTLs in vivo. CD8 T cells from WT or B7-H1 KO B6 mice were activated and labeled with high or low dose of CFSE (2.5 μM for KO cells; 0.25 μM for WT cells). Labeled cells were then incubated with or without OVA peptide. WT and KO CD8 T cells were mixed (1:1, 2 × 10⁶ of each) and injected (i.v.) into B6 mice (three mice per group) that were immunized with OVA protein/poly(I:C) 7 d earlier. A. Histogram of recovered CFSE-labeled cells. Numbers indicate percentages of each population. B. Percentages (mean ± SD, n = 3) of OVA peptide-pulsed CFSE-positive WT or KO CD8 T cells in the spleen of immunized mice. One of two experiments is shown. C. Target WT or B7-H1 KO CD8 T cells were incubated with CTLs for 5 h in the presence of anti-CD107a Ab (degranulation assay), followed by intracellular staining for IFN-γ.
contact with WT or B7-H1 KO target T cells in vitro (Fig. 6C). This result suggests that B7-H1 expressed by targeted T cells could make them more resistant to killing by other CTLs.

We next confirmed our in vivo observations using an in vitro CTL killing assay. Effector OT-1 CD8 T cells and CFSE-labeled WT or B7-H1 KO target T cells were cocultured for 48 h in vitro, and the percentage of CFSE<sup>-</sup> target T cells was measured as an indicator of their loss due to killing by CTLs. As shown in Fig. 7A, the percentage of CFSE<sup>-</sup> B7-H1 KO target CD8 T cells in cultures decreased by ∼3-fold compared with WT target CD8 T cells (Fig. 7B; p = 0.002). WT and B7-H1 KO T cells that were not loaded with OVA peptide were used as control groups, and they had similar percentages of CFSE<sup></sup> population in culture with CTLs (Fig. 7A; upper panel), again suggesting that T cell–T cell killing is Ag dependent and is not caused by any underlying intrinsic death signals alone. To define the exact death signals to which B7-H1 KO T cells are more sensitive, we used EGTA (a Ca<sup></sup>2<sup>+</sup> chelator) or anti-Fas ligand Ab and examined the effects on calcium-dependent perforin/granzyme-mediated lysis or Fas ligand-mediated killing in our in vitro system. As shown in Fig. 7C, addition of EGTA at a suboptimal blocking dose (0.1 mM) dramatically reduced the lysis of B7-H1 KO CD8 T cells (from 84 to 13%, 6-fold decrease) compared with the lysis of WT CD8 T cells (from 41 to 27%, 1.5-fold decrease), suggesting that B7-H1 KO T cells are more sensitive to Ca-dependent lysis by CTLs. In contrast, compared with control Ab, the Fas ligand-neutralizing Ab significantly suppressed Fas ligand-dependent killing of B7-H1 KO T cells (p = 0.016) (Fig. 7D), suggesting that lack of B7-H1 on target T cells makes them more sensitive to Fas/FasL-mediated killing. Taken together, our results suggest that B7-H1 expressed by activated CD8 T cells may facilitate their survival by rendering them resistant to killing by other CTLs.

**Lower levels of Bcl-x<sub>L</sub> in activated B7-H1–deficient CD8 T cells**

Next, we wanted to define the exact molecular mechanisms that are responsible for increased sensitivity of activated B7-H1 KO CD8 T cells to CTL lysis. We first examined the expression of Fas. Both resting and activated WT and B7-H1 KO CD8 T cells expressed comparable levels of Fas on their surface (Supplemental Fig. 2C), suggesting that increased cell death of activated B7-H1 KO T cells is not due to increased Fas expression. As the increased apoptosis of B7-H1 KO T cells was accompanied with decreased mitochondrial transmembrane potential (lower levels of TMRE in Fig. 3A), we hypothesized that B7-H1 deficiency may lead to alterations in mitochondrial apoptotic pathway. To test this, we measured the protein expressions of Bcl-2, Bcl-x<sub>L</sub>, and Bim in both resting and activated T cells. Intracellular staining revealed similar levels of Bcl-2, Bcl-x<sub>L</sub>, and Bim in resting WT and B7-H1 KO CD8 T cells (Fig. 8A). However, in activated T cells, Bcl-x<sub>L</sub> levels were significantly lower in B7-H1 KO CD8 T cells than in WT

![FIGURE 7](http://www.jimmunol.org/)

![FIGURE 8](http://www.jimmunol.org/)
CD8 T cells (Fig. 8A, 8B; p = 0.009). The levels of Bcl-2 and Bim, in contrast, remained comparable between activated WT and B7-H1 KO T cells. To investigate whether B7-H1 ligation would affect Bcl-xL expression, we incubated WT T cells with plate-bound PD-1 Fc fusion protein to engage B7-H1 on T cells in the presence of TCR/CD28 stimulation. Even though TCR/CD28 stimulation upregulated Bcl-xL, Bcl-2, and Bim, ligation of B7-H1 by PD-1 did not have additional effects on their expression (Fig. 8C). This suggests that B7-H1 ligation, at least by PD-1, may not directly affect Bcl-xL protein levels, and that B7-H1 expressed by activated T cells may have an intrinsic, ligand-independent role in regulation of Bcl-xL protein. Taken together, our observations suggest that in the absence of B7-H1, activated CD8 T cells express reduced levels of antiapoptotic Bcl-xL. Although the differences in Bcl-xL levels might not be sufficient to result in initiating the apoptosis of B7-H1 KO CD8 T cells when acting alone, they could render them more susceptible to additional proapoptotic stimuli such as cytokine withdrawal, FasL-, and lytic molecule-mediating CTL lysis.

**B7-H1–deficient effector CD8 T cells fail to mount a protective immunity**

Next, we examined the ability of B7-H1–deficient effector CD8 T cells to mount protective immunity against tumor challenge. WT and B7-H1 KO effector OT-1 CD8 T cells (prepared as in Fig. 5) were transferred (i.v.) into recipient mice that had been irradiated (600 rad) 1 d earlier. One day after T cell transfer, recipient mice were injected (s.c.) with B16 tumor cells. Whereas B16-OVA tumors progressively grew in the control group of mice without effector T cell transfer, they did not grow out in the mice that received WT effector CD8 T cells (Fig. 9A). However, the growth of B16-OVA tumors could not be completely suppressed in mice transferred with B7-H1 KO effector CD8 T cells (Fig. 9A; p < 0.05), suggesting that B7-H1–deficient effector CD8 T cells may have compromised protective function.

To examine the accumulation and function of effector CD8 T cells in tumor site, we injected EG7-OVA tumor cells into the peritoneal cavities 1 wk after T cell transfer and monitored the presence of effector OT-1 CD8 T cells in the peritoneal cavities and spleens. Transferred OT-1 CD8 T cells were identified by K0.8OVA tetramer staining. Following tumor challenge, the frequency and numbers of K0.8OVA-tet+ CD8 T cells decreased by 2- to 5-fold in the peritoneal cavities and spleens of recipients of B7-H1 KO CD8 T cells compared with recipients of WT CD8 T cells (Fig. 9B, 9C; p < 0.05). CTL function of the transferred effector CD8 T cells was also measured in the peritoneal cavities and spleens of recipient mice. In peritoneal cavities, the frequency of CD107a+ IFN-γ+ CD8 T cells that were reactive to tumor cells decreased by 12-fold in recipients of B7-H1 KO CD8 T cells compared with recipients of WT CD8 T cells (Fig. 9D). Observed reduced tumor

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**FIGURE 9.** B7-H1–deficient CD8 T cells show impaired protective immunity against tumor challenge. Preactivated WT and B7-H1 KO OT-1 CD8 T cells (1 × 10^6) were transferred (i.v.) into sublethally irradiated B6 mice. A, One day post-T cell transfer, recipient mice were injected with B16-OVA tumors (5 × 10^5) s.c. in the right flank. Tumor sizes are shown as mean ± SD of five mice per group. *p < 0.05. B and C, EG7-OVA tumor cells were injected (i.p.) on day 7 post-T cell transfer. One week after tumor injection, transferred OT-1 CD8 T cells were identified by staining with anti-CD8 and K0.8OVA-tetramer (tet) in the peritoneal cavities and spleen. Numbers show the percentage of tet+ CD8 T cells (B). Actual numbers of transferred OT-1 CD8 T cells (C). Data show mean ± SD of three mice, *p < 0.05. D, CTL activity of transferred CD8 T cells harvested from peritoneal cavities and spleen of recipient mice. EL-4 cells that were pulsed with OVA peptide (solid lines) or with control peptide (dotted lines) were used as target cells in 4-h calcein release assay. Data are representative of three independent experiments with three mice per group.
reactivity of B7-H1 KO CD8 T cells could be due to Ag-specific anergy, as they could produce IFN-γγ when they were stimulated with PMA/ionomycin that bypasses early TCR/CD28 signaling (Fig. 9D). Consistent with this, cytolytic activity in the spleens of recipients of B7-H1 KO CD8 T cells decreased by 4- to 7-fold compared with recipients of WT CD8 T cells (Fig. 9E). Taken together, our results suggest that B7-H1–deficient effector CD8 T cells could not mount protective immunity due to compromised cytolytic activity resulting from their reduced accumulation.

Discussion
In this study, we show that B7-H1 expressed by activated CD8 T cells is essential for their survival following Ag stimulation. Increased apoptosis results in depletion of B7-H1 KO CD8 T cells during the contraction phase. The decreased levels of Bcl-xL in activated B7-H1 KO CD8 T cells render T cells more sensitive to cytokine withdrawal and killing by other CTLs. The highest level of B7-H1 expression by Ag-primed T cells during the contraction phase helps effector T cells survive by maintaining elevated levels of Bcl-xL that counteract intense apoptotic signals. Therefore, disturbing T cell B7-H1 by blocking Ab at this stage would result in loss of effector cells. Transfer of B7-H1–deficient effector CD8 T cells may not be able to mount a protective immunity against tumor challenge. Thus, this novel prosurvival function of B7-H1 expressed by CD8 T cells has important implications in T cell survival and protective T cell immunity.

Unlike Bcl-2 protein that is constitutively expressed by T cells, Bcl-xL protein levels vary with different levels of T cell activation (28). Its expression is induced by TCR stimulation and upregulated by CD28 signals (28). However, it is not stable and begins to decline at 48 h after activation (28). It has been shown that enhanced Bcl-xL expression prevents T cell death in response to Fas/FasL signaling and cytokine withdrawal (28, 29). In addition, there is a direct regulatory relationship between antiapoptotic Bcl-xL and proapoptotic Fas-mediated pathway, as Fas pathway signaling induces degradation of Bcl-xL via activation of p38MAPK in CD8 T cells (30). In contrast, T cell survival factor IL-7 has been shown to exert its functions through upregulation of Bcl-xL in effector/memory T cells (31). To our knowledge, since the report of CD28 signals enhancing Bcl-xL expression (28), no other B7 costimulatory molecule has been directly linked to Bcl-xL expression. Our finding that the loss of B7-H1 is correlated with lower levels of Bcl-xL is novel and unexpected, because it has been believed that B7-H1 functions as a suppressive regulator for T cells (7). Currently, we do not know how B7-H1 regulates Bcl-xL expression during T cell activation. Because B7-H1 ligation by PD-1 did not affect Bcl-xL expression (Fig. 8C), it is possible that B7-H1 regulates Bcl-xL in an intrinsic ligand-independent manner. However other non–PD-1 ligands cannot be excluded. Further investigation is necessary to address the biochemical mechanisms underlying B7-H1–mediated regulation of Bcl-xL expression.

Several studies have proposed an inhibitory function of B7-H1 expressed by T cells based on proliferation and cytokine production during primary T cell activation (22, 23). In those studies, B7-H1–deficient T cells produced more IFN-γγ, but did not exhibit increased proliferation. However, the apoptosis of B7-H1–deficient T cells in those studies was not examined. We report that B7-H1 expressed by activated T cells may protect them from being killed by other CTLs (Figs. 6, 7). This function of B7-H1 does not seem to be due to its suppressive effects on CTL function (Fig. 6C). Goldberg et al. (32) have previously reported that B7-H1 KO and WT whole spleen cells show identical sensitivity to killing by CTLs in vivo. Although our results agree with their finding to the extent of B7-H1 on target cells not having effect on lytic function of effector CTLs, our in vivo and in vitro killing assays suggest the net effect of B7-H1 absence on target CD8 T cells is to compromise survival of target cells. Goldberg et al. (32) used resting (nonactivated) whole spleen cells, which might have not expressed sufficient levels of B7-H1 and Bcl-xL to provide protection from killing by CTLs. In contrast, purified CD8 T cells used in our systems were first activated for 48 h to upregulate B7-H1 and Bcl-xL (Figs. 6, 7). Taken together, our results suggest upregulation of B7-H1 by activated T cells is essential for upregulation or maintenance of Bcl-xL to prevent activation of the intrinsic apoptotic pathway. Although reduction in Bcl-xL levels alone might not be sufficient to result in apoptosis, it can make CD8 T cells more sensitive to additional apoptotic stimuli such as ligation of Fas or release of lytic granules, both delivered by CTL during the contraction phase of an immune response.

The biological significance of our observations is that disruption of B7-H1’s prosurvival function results in loss of effector cells and compromises generation of protective immunity against malignancy (Fig. 9) or infection. Two groups using similar models of primary infection with Listeria monocytogenes reported that B7-H1 blockade impairs the expansion of pathogen-specific effector CD8 T cells and suppresses the antibacterial protection in the host (10, 11). In addition, B7-H1 deficiency has led to compromised protection to primary Salmonella infection (13). In contrast, suppression of B7-H1’s prosurvival function may mitigate the harm of autoimmunity. In an autoimmune disease model, B7-H1 blockade inhibits the function of effector T cells and alleviates chronic intestinal inflammation (12). Moreover, a transient loss of self-reactive effector T cells has been observed in B7-H1–deficient mice following immunization with myelin oligodendrocyte glycoprotein peptide (33). Whereas those reports support a positive costimulatory role of B7-H1 during the priming of effector T cells (1, 34, 35), our data suggest that B7-H1 blockade or deficiency may disturb its prosurvival function during the late effector and contraction phases of an immune response. It will be important to ascertain the impact of B7-H1 blockade on the expression or degradation of Bcl-xL in effector CD8 T cells to better control loss of protective T cell immunity or to heighten T cell tolerance in autoimmune and transplantation settings.

In summary, our findings suggest that B7-H1 expressed by activated CD8 T cells is essential for their survival. A more thorough understanding of the function of B7-H1 expressed by activated T cells would provide new insights not only into the regulation of T cell survival required for protective immunity, but also into alleviation of autoimmune diseases and preservation of transplants.

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Disclosures
E.D.K. and the Mayo Clinic have received royalties from the licensing of technology related to B7-H1 and have contractual rights to receive future royalties. In addition, E.D.K. and H.D. have filed patents for the potential use of B7-H1 as a prognostic marker for the assessment of cancer. The other authors have no financial conflicts of interest.

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