B7DC/PDL2 Promotes Tumor Immunity by a PD-1–independent Mechanism

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Abstract

B7H1 (PDL1) and B7DC (PDL2) are two new members of the B7 family that can interact with PD-1, a putative negative regulator for immune function. Recent studies have provided evidence for inhibitory functions of both members via PD-1. Meanwhile, compelling evidence exists for costimulatory function of both members. Here we demonstrate that expression of B7DC on the tumor cells promotes CD8 T cell–mediated rejection of tumor cells, at both the induction and effector phase of antitumor immunity. Moreover, B7DC binds to PD-1($-/-$) cells and enhances T cell killing in a PD-1–independent mechanism. Our results demonstrate a novel pathway for B7DC to promote tumor immunity and may reconcile the apparently contradictory findings on the function of B7DC.

Key words: tumor immunity • costimulatory molecules • cytolytic T lymphocytes

Introduction

An important advance in the area of T cell costimulation is the identification of several new functional members of the B7 family, including B7H1/PDL1 (1, 2), B7H/B7RP (3, 4), B7H3 (5), and B7H2/B7DC/PDL2 (6). Although B7H3 remains an orphan, B7RP/B7H is known to bind to inducible costimulator (ICOS;* 3), whereas both B7H1 and B7DC were found to interact with PD-1 (2, 7), a putative negative regulator for immune function (8, 9). These new members have cellular receptors distinct from those of B7-1 and B7-2. In addition, the two known receptors, ICOS and PD-1, are present on activated, but not resting, T cells (10, 11).

Because of its broader tissue distributions, the new members of the B7 family might be primarily involved in the effector function of T cells after they migrate to the tissues. For instance, the blockade of B7H–ICOS interaction, even at a late stage of transplantation, was found to be highly efficient in preventing allograft rejection (12). Correspondingly, expression of B7H on the tumor cells promoted cognate destruction of cancer cells in vivo, in addition to enhanced clonal expansion and recall response (13, 14).

Targeted mutation of ICOS also resulted in reduced Th2-mediated airway allergy and exacerbated experimental autoimmune encephalomyelitis (15–17).

Many have argued that negative regulation is critical for homeostasis of the immune system. The fact that B7H1 and B7DC bind to PD-1, which was proposed as a negative regulator for immune receptors, raises an intriguing possibility that these members may serve as a negative regulator after activation of T cells. In support of this notion, Freeman et al. (2) reported that both B7H1 and B7DC can reduce T cell proliferation when coimmobilized into plastic beads with anti-CD3 mAbs (7). This inhibitory activity appears to depend on PD-1 as B7H1 and B7DC do not inhibit the PD-1($-/-$) T cells (2, 7). Likewise, recent data from Iwai et al. (18) suggest that PD-1–B7H1 interaction may promote tumor escape. However, in earlier studies, Dong et al. (1) found that recombinant B7H1 can costimulate cytokine production of T cells. Meanwhile, Tseng et al. (6) observed a significant enhancing effect of B7DC on T cell clonal expansion.

The disparate conclusions from different investigators remain to be reconciled. Recently, Dong et al. (19) reported that B7H1 is expressed on a large array of cancer cells and induces apoptosis of activated T cells. They also showed that B7H1 induces activation-induced cell death. It is conceivable that B7H1 and B7DC may have non–PD-1 receptors that transduce a positive costimulatory signal. To assess

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Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; CHO, Chinese hamster ovary; ICOS, inducible costimulator.

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this possibility, we expressed B7DC on tumor cells and analyzed the role for B7DC in tumor growth and tumor-specific CTL response. Our results demonstrate that expression of B7DC caused rapid rejection of tumors in syngeneic mice and development of immunity to subsequent tumor challenge, suggesting a positive role of B7DC in vivo. The B7DC-mediated, enhanced immunity can be attributed to both enhanced priming and effector function. More importantly, B7DC binds to PD-1(−/−) cells and enhances T cell killing in a PD-1–independent mechanism. Our results demonstrate a novel pathway for B7DC to promote tumor immunity and may reconcile the apparently contradictory findings on the function of B7DC and B7H1.

Materials and Methods

Construction of Expression Vectors. To clone murine B7DC, cDNA were prepared from dendritic cell culture. The B7DC was amplified by RT-PCR using gctagcatgctgctcctgc as the forward primer and gcggccgcctagatcctctttc as the reverse primer. The PCR products were digested with NheI and NotI and cloned into NheI and NotI sites of pcDNA3.1/Hygro+ vector (Invitrogen). To produce B7DCIg, extracellular domain of B7DC were amplified from B7DC cDNA using atgctgctcctgc as the forward primer and acttacctgtccacgttctggg as the reverse primer. The PCR product was cloned into the pIg vector (Novagen). B7DCIg fusion gene was then inserted into the HindIII/KpnI site of pcDNA3.1/Hygro+ (Invitrogen). The construct was transfected into Chinese hamster ovary (CHO) cells using the Fugene 6 transfection system (Boehringer). The hygromycin B–resistant clones were expanded. The B7DCIg protein was purified using protein G column. Murine PD-1 was amplified from cDNA prepared from mouse spleen cells using aagcttatgtgggtccggc as the forward primer and ggatcctcaaagaggcc as the reverse primer. The PCR products were digested with HindIII and BamHI and cloned into the HindIII and BamHI sites of pcDNA3.1/Hygro+ and used to transfect CHO cells. The PD-1Ig was produced by the same strategy as that used for the production of B7DCIg, with the exception of the primers (forward, atgtgggtccggc; reverse, acttacctgtttgaaaccggcc) used. The primers for B7H1 are: forward, atgaggatatttgctggc; reverse, acttacctgtcctgttctgtgg.

Cell Lines and Experimental Animals. Plasmocytoma J558 cells were transfected with either vector alone (J558-Neo) or B7DC (J558-B7DC) according to a previously described procedure (14). Stable cell lines expressing B7DC were obtained after drug selection and were screened using the PD-1Ig.

Transgenic mice expressing TCR specific for tumor antigen P1A35-43:L^d complex have been described (20). The TCR transgenes were backcrossed with BALB/cByJ for at least eight generations before they were used for this study. Wild-type BALB/c mice were purchased from the National Cancer Institute. BALB/c mice with a targeted mutation of RAG-2 gene were purchased from Taconic. PD-1(−/−)2C mice (21) were provided by T. Honjo (Kyoto University, Kyoto, Japan) and were bred into RAG-1(−/−) background at the University of Chicago. The PD-1(+/+) RAG-1(−/−)2C mice have been previously described (22). The bone marrows from both RAG-1(−/−) PD-1(−/−) and RAG-1(−/−) PD-1(+/+)2C mice were used to produce irradiation chimeras with syngeneic C57BL/6j mice. 4–6 wk after reconstitution, the spleen cells were harvested and stimulated with irradiated syngeneic BALB/c

spleen cells. Essentially all CD8 T cells were from 2C origin, as confirmed by idiotype-specific antibody for the 2C TCR.

Antibodies. PE-conjugated anti–PD-1, CyChrome-conjugated anti-CD8, PE-conjugated anti-Vα8, and biotinylated anti–H2-L^d mAbs were purchased from BD Biosciences. Anti-B7DC mAb TY25 was purchased from eBioscience. Biotinylated clonotypic antibodies against 2C was a gift from J. Chen (Massachusetts Institute of Technology, Cambridge, MA).

Adoptive Transfer of Purified Transgenic T Cells. Pools of spleen and lymph node cells from the RAG-2(+/+) or RAG-2(−/−) P1CTL transgenic mice were incubated with a cocktail of mAbs (anti-CD4 mAb GK1.5, anti-FcR mAb 2.4G2, and anti-CD11c mAb N418). After removal of unbound mAbs, the cells were incubated with anti-Ig–coated magnetic beads. The antibody-coated cells were removed by a magnet. The unbound cells consisted of >95% CD8 T cells with no detectable CD4 T cells. The purified T cells were adoptively transferred into RAG-2(−/−) mice that had large tumors. In some experiments, the CD8 T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). The labeled cells were either stimulated with tumor cells in vitro or adoptively transferred into tumor-bearing hosts as previously described (23).

Tumorigenicity Assay. 5×10^6 J558 cells were injected into the flanks as previously described (24). The tumor size and incidence were determined by physical examination.

CTL Assays. A 6-h ^{51}Cr release assay was used to measure CTL activity. The effector T cells were either P1CTL activated for 96 h with 0.1 μg/ml P1A peptide (20), or PD-1(+/+) and PD-1(−/−)2C cells activated with spleen cells from BALB/c mice.

To determine the relative susceptibility of vector or B7DC-transfected tumor cell lines, vector-transfected cells were labeled with 0.5 ng/ml CFSE, whereas the B7DC transfectants were labeled with 5 ng/ml CFSE. The two cell types were mixed at an ~1:1 ratio and incubated with effector T cells for 2 h. At the end of the incubation, the cultures were fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Flow Cytometry. Cell surface expression of H-2L^d was detected using biotinylated mAb 28-14-8 (BD Biosciences) followed by PE-labeled streptavidin. To determine the binding of H-2L^d–peptide complex to transgenic T cells, we used the H-2L^{d}Ig dimer purchased from BD Biosciences according to the manufacturer's instructions. In brief, 3 μg peptides (P1A sequence, LPYLGWLVF; control peptide from murine cytomegalovirus, YPHFMPTNL) were incubated with 4 μg H-2L^{d}Ig and β$_2$ microglobulin complex at 4°C for 48 h in a total volume of 200 μl. PE-conjugated monoclonal rat anti–mouse IgG1 antibodies were added to the solution 1 h before it was used to stain spleen cells from transgenic mice whose T cells had expressed the TCR specific for the H-2L^d–P1A peptide complex. After washing away the unbound complex, the spleen cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry.

The binding of B7H1 and B7DC to T cells or PD-1 transfected was determined by incubating 100 μg/ml biotinylated IgG1 Fc, B7H1Ig, or B7DCIg with cells at 4°C for 30 min. After washing away unbound fusion proteins, PE-conjugated streptavidin was added to measure the amount of fusion protein bound to cells. In some experiments, CyChrome-conjugated anti-CD8 mAb was used together with the PE conjugate to mark CD8 T cells. After three washes, the cells were fixed and analyzed by flow cytometry.

In some experiments, 40-fold excess of unlabeled IgG1 Fc, B7H1, or B7DC was incubated with PD-1(−/−)2C cells for 30 min before the addition of biotinylated fusion proteins. In yet an-

other setting, 800 μg/ml anti–B7DC mAb or control normal rat IgG was incubated with 100 μg/ml of the biotinylated B7DC for 30 min before the mixtures were used to test their binding to given cells.

Results

Expression of B7DC on Tumor Cells Results in Tumor Rejection and Immunity to Parental Tumors. To determine whether B7DC has a positive or negative role in antitumor immunity, we cloned B7DC by RT-PCR and transfected it into the wild-type and MHC-deficient plasmocytoma J558. As shown in Fig. 1 a, the vector and B7DC transfectants are comparable in expression of H-2L^d but differing in B7DC expression, as revealed by their binding to PD-1Ig.

To test the effect of B7DC on tumorigenicity of the J558 tumor cells, we injected the J558 cells that had been transfected with either vector alone or B7DC into syngeneic BALB/c mice. As shown in Fig. 1 b, most mice that were injected with vector-transfected J558 cells (J558-Neo) developed tumors that grew progressively. In contrast, the mice that received B7DC-transfected J558 cells (J558-B7DC) had initial tumor growth followed by complete remission. To determine if the difference in the growth kinetics was due to the intrinsic tumorigenicity of the J558-Neo and J558-B7DC, we injected the same dose of J558-Neo and J558-B7DC cells into the syngeneic RAG-2(−/−)

mice, which are devoid of T and B cells. As shown in Fig. 1 c, the two cell lines grew at the same rate in the RAG-2(−/−) mice. Thus, the preferential rejection of the J558-B7DC must be mediated by T or B lymphocytes. We then depleted either CD4 or CD8 T cells by treatment with depleting monoclonal antibodies. As shown in Fig. 1 d, the tumor rejection was mediated by CD8 T cells as the immunity can be abrogated by anti-CD8, but not anti-CD4, mAbs (Fig. 1 e).

2 mo after the rejection of B7DC-transfected tumors, we rechallenged the tumor-free mice with the untransfected J558 cells. Although all naive mice develop J558 tumors within 7 d, none of the mice that had rejected the J558-B7DC tumors developed tumors (Fig. 1 e). Continuous observation over a 4-mo period revealed no tumors in the primed mice. Thus, J558-B7DC induced long-lasting immunity to J558 tumor cells in immune-competent hosts.

It has been established that a major tumor antigen in the J588 tumor cells is P1A peptide AA35-43 presented by H-2L^d (25). Therefore, we analyzed the number of P1A-reactive T cells in mice challenged with either J558-Neo or J558-B7DC, using H-2L^d dimer loaded with either the P1A peptide or a control H-2L^d–binding peptide. As shown in Fig. 2, P1A-reactive T cells were barely detectable in mice challenged with the J558-Neo tumor cells. In contrast, the mice that received the J558-B7DC produced high numbers of P1A-reactive CD8 T cells. On average,
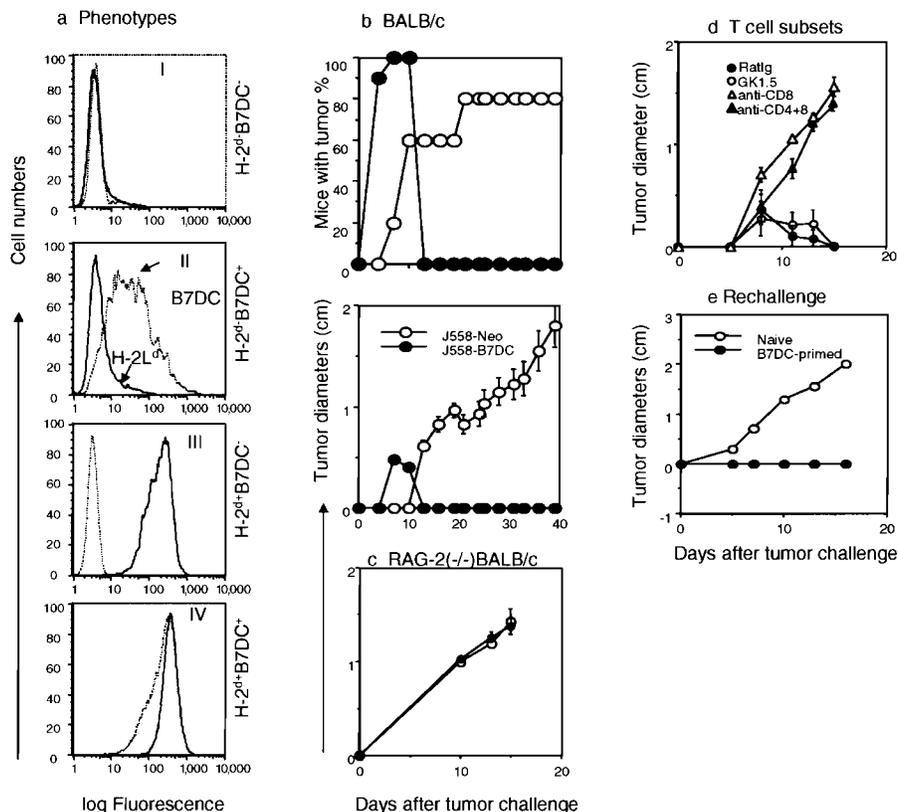

Figure 1. B7DC induced rapid rejection of CD8 T cell–dependent rejection of J558 tumor cells. (a) Expression of H-2L^d and B7DC on J558 tumor variants. Parental J558 cells and its H-2–deficient variants were transfected with either vector alone (I, III) or B7DC (II, IV). The cell lines were stained with either biotinylated anti–H-2L^d mAb (red lines) or PD-1Ig fusion protein (blue lines), followed by PE-conjugated second step reagents. (b) Rapid rejection of J558-B7DC, but not the J558-Neo, tumors by BALB/c mice. 5 × 10^6 tumor cells were injected into the flank and the tumor incidence (top) and growth kinetics (bottom) were monitored by physical examination. Data shown are representative of two independent experiments with two different clones of J558-B7DC. Each experiment consisted of 10 mice per group. (c) Comparable tumorigenicity of J558-Neo and J558-B7DC in RAG-2(−/−) BALB/c mice. As in b, except that RAG-2(−/−) BALB/c mice were used as hosts. Data shown are growth kinetics and are representative of two independent experiments with five mice per group. (d) CD8 T cells are necessary for rejection of J558-B7DC. BALB/c mice were depleted of either CD4, CD8 T cells, or both, with monoclonal antibodies on days −2, 0, and +2 of injection with 5 × 10^6 J558-B7DC tumor cells. (e) Long-lasting immunity to parental J558 tumor cells in mice that have rejected the J558-B7DC.

Naive or J558-B7DC–primed BALB/c mice (*n* = 5) were challenged with 5 × 10^6 J558 tumor cells in the flank and the growth kinetics of tumors in naive mice were monitored. All naive mice developed tumors within 1 wk, whereas no primed mice developed tumors over the 4-mo period observed.

~1% of CD8 T cells in the spleen of the J558-B7DC–primed mice were specific for the P1A peptide, whereas the numbers of antigen-specific T cells were ~10-fold lower in the mice that were challenged with the J558-Neo tumor cells. Thus, B7DC may deliver a positive signal to enhance immunity in vivo.

We had isolated MHC-deficient variants of the J558 tumor cells that lacked multiple antigen-presentation genes, including LMP-2, LMP-7, TAP-1, and TAP-2 (26). Our previous studies revealed that the MHC-deficient tumor cells are capable of cross-, but not direct, priming of P1A-specific T cells (27). To test if expression of B7DC on the tumor cells could enhance cross-priming, we transfected the B7DC into an MHC-deficient variant of J558 (Fig. 1 a). As shown in Fig. 2 a, expression of B7DC on the MHC-deficient cell line was insufficient to induce significant priming of P1A-reactive T cells. Because the nominal antigen P1A gene is expressed on both parental and MHC-deficient J558 cells (Fig. 2 b), the enhanced priming induced by B7DC is most likely due to its effect in enhancing direct stimulation of T cells by the B7DC⁺ tumor cells.

B7DC Promotes Clonal Expansion of Tumor-specific CTL. To determine if B7DC can promote clonal expansion of the P1A-reactive T cells, we injected CFSE-labeled P1CTL into either naive mice or those that had received either J558-Neo or J558-B7DC and analyzed the rate of T cell division in vivo. As shown in Fig. 3, a–d, in the absence of tumor cells, the P1CTL underwent little, if any, homeostatic proliferation during the 3-d period studied. Although no division of T cells could be observed on day 1, significant expansion of T cells was found in mice bearing the J558 tumors by days 2 and 3. In mice bearing the J558-Neo tumors, most cells were at divisions 1–4, with a

few cells at division 5. In mice bearing J558-B7DC, the majority of the cells were at divisions 4–5. Although the difference is less marked on day 3, there were substantially more cells at division 9 in mice bearing the J558-B7DC tumors than in those that bore the J558-Neo tumors. The accelerated division resulted in increased number of antigen-specific T cells in the spleen of the J558-B7DC tumor-bearing mice. Thus, the activated T cells were not immediately eliminated, which contrasted to what was reported for B7H1-expressing tumors (19).

To determine the long-term consequence of the B7DC costimulation, we adoptively transferred purified P1CTL into mice with tumors of ~1.5 cm in diameter. 1 mo after the adoptive transfer, the amounts of P1CTL in the PBL were monitored by flow cytometry. Consistent with the increased clonal expansion during the induction phase, mice that bore B7DC-transfected tumors had substantially higher numbers of tumor-specific transgenic T cells at 1 mo after adoptive transfer (Fig. 3, e–g). On average, >60% of the peripheral blood lymphocytes in the J558-B7DC tumor-bearing mice were the adoptively transferred transgenic T cells, whereas <10% of the PBL in the J558-Neo tumor-bearing mice were transgenic T cells. Taken together, the data in Figs. 2 and 3 demonstrate that expression of B7DC on the tumor cells enhanced, rather than suppressed, tumor-specific T cell response.

B7DC Enhances Rejection of Large, Established Tumors by Promoting Effector Function of T Cells. In addition to its role in promoting the priming of tumor-reactive T cells, the B7DC may also promote effector function of CTL. We performed two types of experiments to evaluate this possibility. As shown in Fig. 4 a, in a conventional CTL assay using activated P1CTL as the effector, we found that the
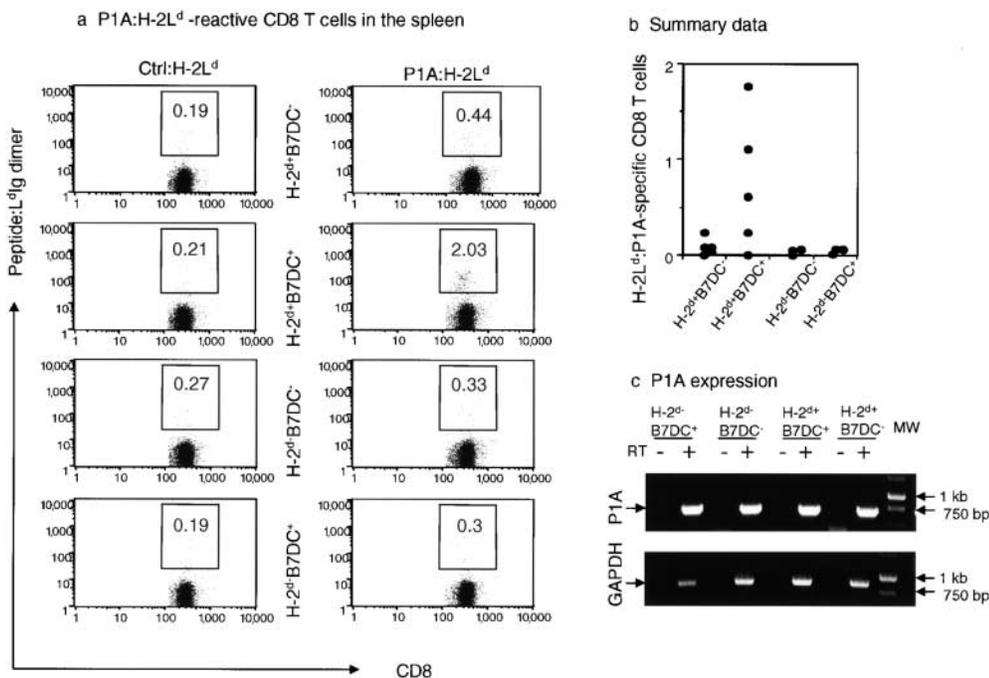

Figure 2. B7DC promotes direct, but not cross-, priming of P1A-specific T cells. (a) P1A–H-2L^d–reactive CD8 T cells in the spleen of mice that were immunized with 5 × 10⁶ of one of the four J558 variants. Spleen cells were harvested 4 wk after tumor cell challenge and stained with FITC-labeled anti-CD8 mAb and H-2L^dIg loaded with either a control peptide or the P1A peptide. Data shown are gated CD8 T cells with the percent of dimer-binding cells indicated in each panel. (b) Summary of percent of P1A-specific cells among the CD8 T cells. The data shown were the percentages of H-2L^dIg–P1A–binding cells after subtracting those of H-2L^d–ctrl peptide binding cells. (c) Expression of tumor antigen P1A among all J558 variants as analyzed by RT-PCR. GAPDH products were used as control for cDNA quality.
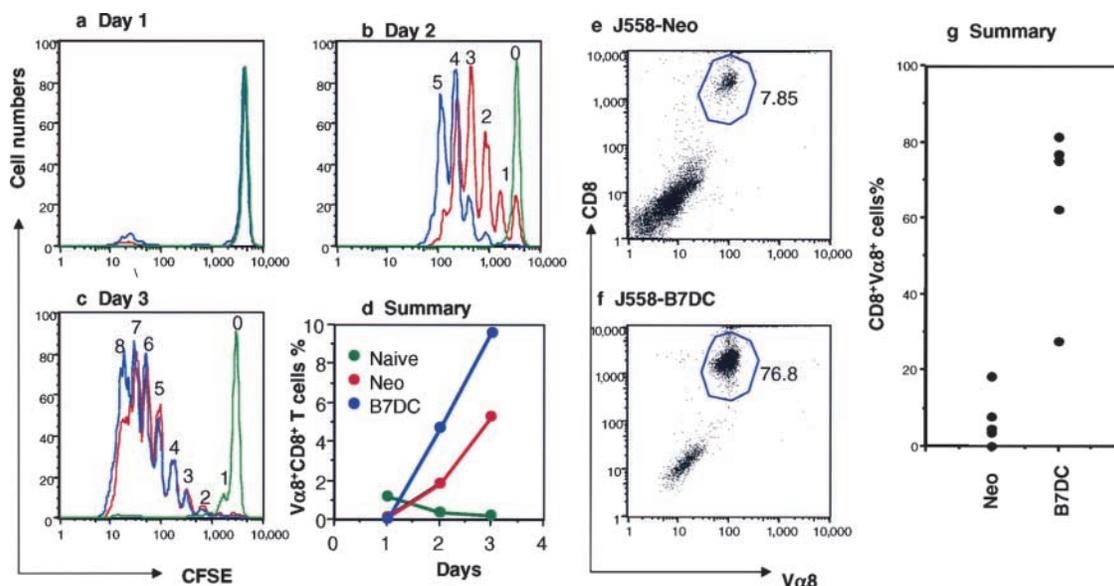
Figure 3. B7DC promotes clonal expansion of P1A-specific transgenic T cells in tumor-bearing mice. (a–d) The effect of B7DC on clonal expansion of the transgenic T cells. CFSE-labeled transgenic CD8 T cells were inoculated in either tumor-free mice (green lines), those that bore J558-Neo (red lines), or J558-B7DC (blue line) tumors. Spleen cells were harvested on days 1 (a), 2 (b), and 3 (c) after inoculation into mice with a comparable tumor size. The percentages of T cells accumulated in the first 3 d are summarized in d. (e–g) The number of transgenic T cells 4 wk after adoptive therapy of large tumor cells. The PBL were analyzed for the presence of transgenic T cells using mAbs to identify the CD8$^+$ Vα8$^+$ T cells. e and f depict representative profiles of PBL from mice that bore either the J558-Neo (e) or J558-B7DC (f) tumors. The percent of gated double CD8$^+$ Vα8$^+$ positive T cells in each mouse ($n = 5$ per group) are summarized in g. See Fig. 5 for the therapeutic effect of transgenic T cells.

J558-Neo was much less efficiently lysed by P1CTL. To confirm this, we labeled the J558-Neo and J558-B7DC with different concentrations of CFSE to make them distinguishable by flow cytometry and mixed them at an ∼1:1 ratio. The P1CTL were added and incubated for 2 h. The cell mixture was then analyzed by flow cytometry to determine the relative amounts of J558-Neo and J558-B7DC cells. As shown in Fig. 4, b and c, P1CTL preferentially re-

moved the J558-B7DC as the ratio of J558-Neo over J558-B7DC increased in a dose-dependent manner. Thus, although tumor cells can be killed by CTL even in the absence of B7DC, the latter substantially increased target susceptibility of the tumor cells.

To test if the expression of B7DC on tumor cells increased their susceptibility to CTL therapy in vivo, we compared the J558-Neo and J558-B7DC tumors for their
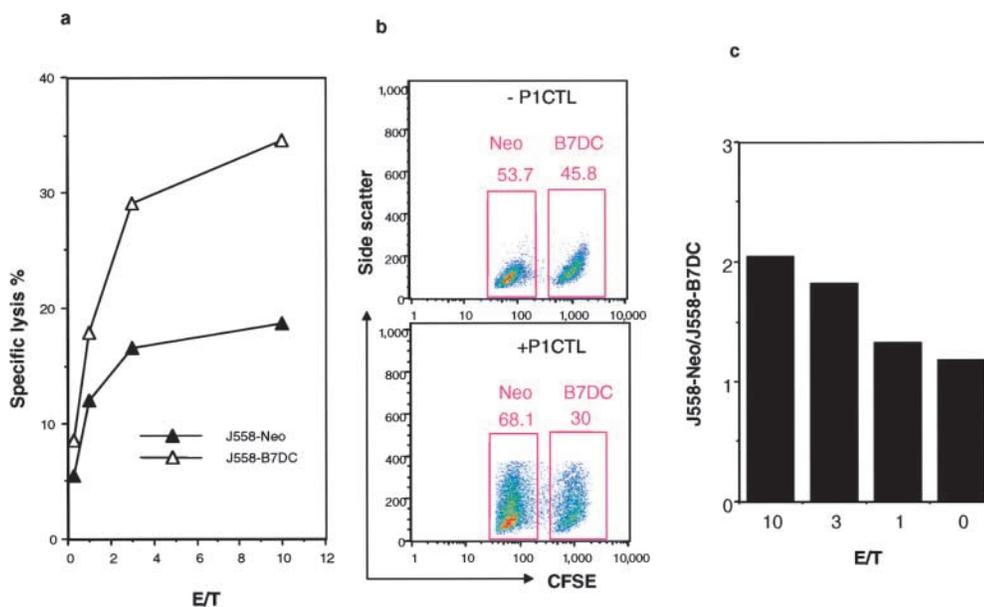

Figure 4. Preferential lysis of J558-B7DC by activated P1CTL as determined by ^{51}Cr release (a) and competitive lysis assay (b and c). The representative FACS® profiles of cell mixture in the absence (top) or presence of P1CTL (bottom). Only gated CFSE$^+$ cells with viable cellular scatters were presented. Data are representative of three independent experiments. (c) Ratios of J558-Neo/J558-B7DC as a function of E/T, calculated based on the percentage of the CFSE^{low} versus that of the CFSE^{high} cells. The number of target cells used for E/T calculation is the sum of two types of tumor cells in the cultures.

response to therapy with P1CTL. As shown in Fig. 5, the P1CTL were injected when the tumors were ~1.5 cm in diameter. In the J558-Neo tumor group, one tumor-bearing mouse died within 1 wk of CTL therapy with no sign of tumor regression, whereas the other four initially had different degrees of regression. However, rapid recurrence was observed in all mice. The recurrent tumors grew progressively, and by 7 wk of CTL therapy, euthanasia became necessary. In contrast, regression was found in all J558-B7DC tumors. With somewhat different kinetics, three mice rejected the large tumors completely and survived for >200 d. Two mice had recurrent tumors and were killed 2 mo after treatment. The fact that the majority of mice with large J558-B7DC tumors achieved long-term survival demonstrates the important role of B7DC in promoting CTL effector function in vivo.

PD-1 Is Not Required for the B7DC-enhanced Induction and Effector Function of CTL: Evidence for a Non–PD-1 Receptor for B7DC. A previous study revealed that B7DC–PD-1 interaction down-regulated T cell activation (7), whereas our data presented here and the original work of Tseng et al. (6) suggest that B7DC may promote T cell function. One hypothesis that may reconcile these observations is that there might be non–PD-1 receptors that mediate the costimulation by B7DC. To test this hypothesis, we set to determine whether B7DC could bind to PD-1(−/−) T cells and if so, whether this interaction can promote the CTL effector function. We produced a fusion protein comprised of the extracellular domain of B7DC and the Fc
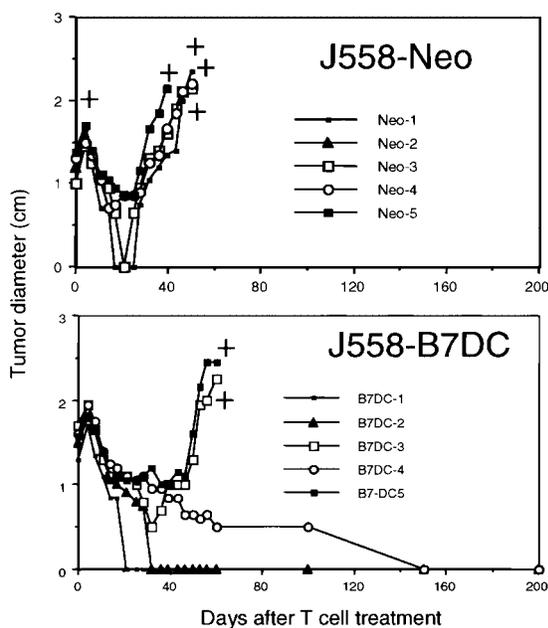
portion of human IgG1. We then compared B7DCIg binding to PD-1(−/−) and PD-1(+/+)2C cells by flow cytometry. As shown in Fig. 6 a, both PD-1(+/+) and PD-1(−/−)2C cells bound strongly to B7DCIg, but not to the control IgG Fc. These results demonstrated that B7DC also has a non–PD-1 receptor on activated T cells.

To determine whether PD-1 and non–PD-1 receptor binds to the same region on B7DC, we tested the effect of a commercially available anti-B7DC antibody (TY25) for its ability to block B7DC binding to PD-1 and the non–PD-1 receptor. Anti-B7DC mAb or control rat IgG were incubated with biotinylated B7DCIg. The mixtures were then tested for B7DC binding to either PD-1–transfected CHO cells or PD-1(−/−)2C cells. As controls, biotinylated Fc fragment or B7DC was used without previous incubation. As shown in Fig. 6 b, biotinylated B7DC, but not the Fc fragment, binds the PD-1–transfected cells. This binding is substantially reduced by preincubation with anti-B7DC mAb, but not with control normal rat Ig. Thus, the anti-B7DC antibodies can block B7DC–PD-1 interaction. Surprisingly, the anti-B7DC antibody significantly enhanced binding of B7DC to PD-1(−/−) cells. Thus, B7DC must have used a different epitope to interact with the non–PD-1 receptor on the PD-1(−/−)2C cells. The enhanced binding is likely due to the multimerization of the B7DCIg by the nonblocking anti-B7DC mAb.

Because B7H1 also appears to have a non–PD-1 receptor (19), we performed a cross-blocking experiment to determine whether B7DC and B7H1 bound to the same non–PD-1 receptor. As shown in Fig. 6 c, PD-1(−/−)2C cells bound to B7H1, but not to control IgG1 Fc. The nature of B7H1 binding is less clear as 40-fold excess of unlabeled B7H1 failed to block its binding. B7DCIg bound significantly better than B7H1Ig. This binding can be substantially blocked by B7DCIg, but not by either B7H1Ig nor control IgG1 Fc. The differential PD-1–independent binding and lack of cross-blocking between B7H1 and B7DC demonstrate that the non–PD-1 receptor for B7DC is distinct from the putative non–PD-1 receptor for B7H1.

To determine whether the putative non–PD-1 receptor can promote the induction of CD8 T cells, we stimulated CFSE-labeled PD-1(−/−)2C T cells with the J558-Neo and J558-B7DC in vitro. At 48, 72, and 96 h after culture, the distribution of CFSE intensity among the transgenic T cells was determined by flow cytometry. The two cell lines expressed the same levels of H-2L^d (Fig. 7 a), yet the B7DC transfectants induced substantially faster division of the T cells (Fig. 7 b). At 48 h, more T cells in the J558-B7DC–stimulated group were undergoing division. At 72 h, most of the T cells in the J558-Neo–stimulated group divided less than three times, but the majority of those in the J558-B7DC–stimulated group divided four or five times. Perhaps due to CFSE over-dilution, the differences were less remarkable at 96 h. These results demonstrate that B7DC can promote T cell proliferation by a PD-1–independent mechanism.

Again, we used two assays to test whether the B7DC enhanced the effector function of 2C transgenic T cells that
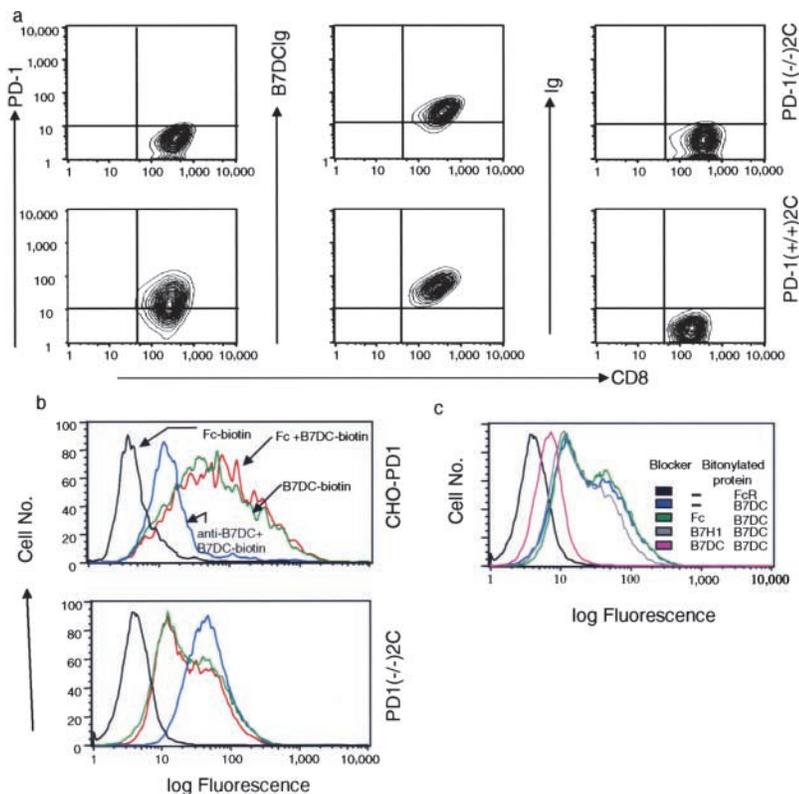

Figure 5. Transgenic T cell therapy of large J558-Neo and J558-B7DC tumors. 5 × 10⁶ P1CTL were purified from the TCR transgenic mice and injected intravenously into mice with large tumor burden. The growth kinetics of tumors was measured using a caliber. The points of mouse death are indicated with a cross. Note that at 4 wk after T cell therapy, the cells in the PBL were analyzed and the data are presented in Fig. 3, e–g.

Figure 6. Characterization of the non–PD-1 receptors for B7H1 and B7DC. (a) Activated CTL express a PD-1–independent receptor for B7DC. Spleen cells from chimera mice with bone marrow from either PD-1(+/+)2C or PD-1(−/−)2C were activated with BALB/c mice for one or two rounds. Activated T cells were harvested and their expression of the 2C receptors on all CD8 T cells was confirmed by flow cytometry. Expression of PD-1 and B7DC receptors were determined using either 10 μg/ml PE-conjugated anti–PD-1 mAb, 100 μg/ml biotinylated B7DCIg, or 100 μg/ml human IgG1 Fc as control. CD8 T cells were marked with CyChrome-conjugated anti-CD8 mAb. Data shown are profiles of gated CD8 T cells and are representative of at least five independent experiments. (b) B7DC binds PD-1 and the putative non–PD-1 receptor via distinct epitopes. PD-1–transfected CHO cells (top) and PD-1(−/−)2C cells (bottom) were stained with biotinylated B7DC or IgG1 Fc as control. The blockers, 800 μg/ml anti-B7DC or control Rat Ig, were incubated with B7DC before the addition of CHO or T cells. The distinct effect of anti-B7DC on PD-1–B7DC binding has been repeated three times. (c) Lack of cross-blocking between B7DC and B7H1. As in b, except that the blockers were 40-fold excess of IgG1 Fc, B7H1Ig, or B7DCIg. The blockers were added to cells 30 min before the addition of biotinylated reagents. The PD-1(−/−)2C cells used in b and c had been stimulated three to five times with irradiated BALB/c cells in the presence of IL-2 and IL-7, and were comprised exclusively of 2C+ CD8 T cells. Data shown are representative of at least three independent experiments.

recognize the H-2L^d on the J558 tumor cells. Using the approach illustrated in Fig. 4, we compared the ratio of J558-B7DC and J558-Neo after a 2-h coincubation with PD-1(+/+) and PD-1(−/−)2C cells. Both PD-1(+/+) and PD-1(−/−)2C preferentially eliminated J558-B7DC cells, as the ratio of J558-Neo/J558-B7DC increased in the presence of CTL (Fig. 8 a). When varying numbers of effector were used, we observed greater preference of B7DC lysis with increasing dosage of CTL (Fig. 8 b). Surprisingly, PD-1(−/−)2C showed a much stronger preference for the J558-B7DC cells. These results demonstrate that PD-1 is not required for the enhanced cytolysis of J558-B7DC.

In the conventional CTL assay, both PD-1(+/+) and PD-1(−/−)2C cells preferentially killed the J558-B7DC over the J558-Neo cells (Fig. 8 c). In multiple experiments, we consistently observed stronger cytolytic activity in PD-1(−/−)2C cells, which is consistent with a previous report (21). Moreover, the difference between J558-Neo and J558-B7DC was not caused by clonal variation in susceptibility to 2C cells, as demonstrated by CTL assays involving two independent clones in each group (Fig. 8 d). Taken together, the data presented in Fig. 7 demonstrate that B7DC cells enhance CTL killing via a non–PD-1 receptor.

Discussion

The recent identification of several new members of the B7 family of costimulatory molecules raises an interesting issue on the function of these molecules. The function of B7H1 and B7DC, which interact with PD-1, remains con-

troversial. Dong et al. (1) and Tseng et al. (6) reported that B7H1 and B7DC promoted T cell proliferation. Recently, Dong et al. (19) reported that B7H1 is widely expressed on multiple lineages of cancer cells and can promote programmed cell death of cancer-specific T cells in both PD-1–dependent and PD-1–independent mechanisms. Tumor expression of B7H1 may allow its evasion of T cell immunity. On the other hand, Freeman et al. (2) have reported that both B7H1 and B7DC can inhibit T cell proliferation by PD-1–dependent mechanisms (7). Here we investigated the function of B7DC in an antitumor CTL response in vivo. Our results revealed that B7DC promotes tumor rejection by T cells via a PD-1–independent mechanism.

Distinct Function of B7DC and B7H1 in Inducing Antitumor Immunity. B7H1 and B7DC share a common receptor, PD-1, and were reported to induce PD-1–dependent inhibition of T cell proliferation (2, 7). However, these two molecules have significant differences in tissue distributions (6, 28). Stimulation of T cells in the presence of B7H1 led to a significant increase in IL-10 production (1). In contrast, coligation with TCR ligand and B7DC promoted Th1-type cytokine production (6). With regard to tumor immunity, B7H1 induces apoptosis of antigen-reactive T cells and inhibits immunity induced by B7-1 (19). Recent data also suggest that B7H1–PD-1 interaction assists tumor evasion of host immunity. The function of B7DC in tumor immunity has not been reported. Here we presented several lines of evidence that support the conclusion that unlike B7H1, B7DC promotes, rather than inhibits, antitumor immunity.
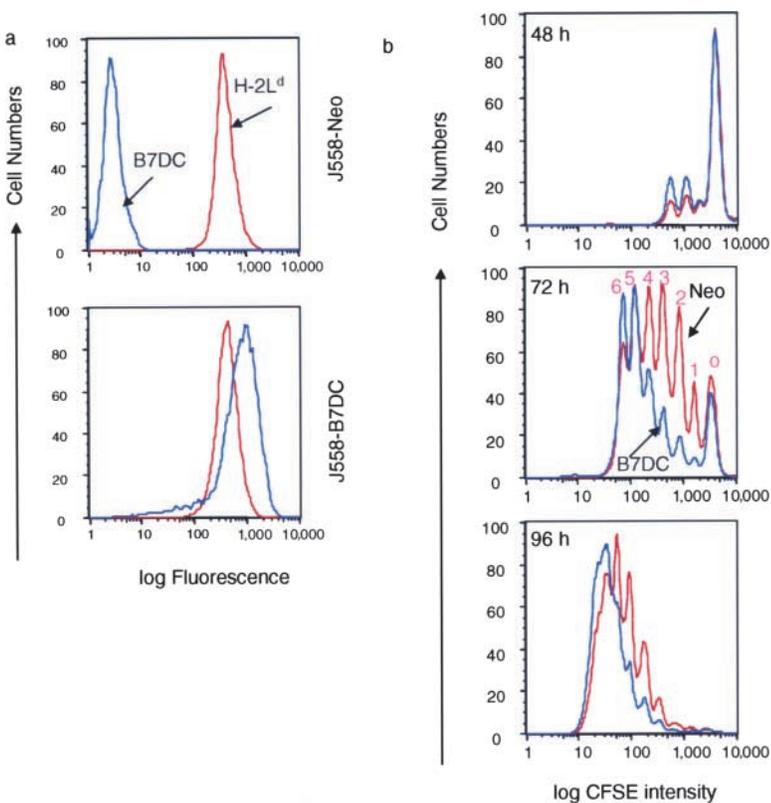
Figure 7. B7DC promotes clonal expansion of PD-1(−/−)2C cells. (a) Characterization of H–2L^d and B7DC expression on the stimulatory cells by flow cytometry. Data shown are histograms depicting expression of H–2L^d (red lines) and B7DC (blue lines) of the J558-Neo (top) and J558-B7DC clones (bottom). (b) Analysis of T cell division. J558-Neo and J558-B7DC cells (2 × 10^5/well of the 6-well tissue culture plate) were irradiated for 10,000 rads and used as stimulators for 4 × 10^6/well CFSE-labeled spleen cells isolated from chimera mice constituted with bone marrow from PD-1(−/−) RAG-1(−/−)2C mice. At 48, 72, and 96 h after stimulation, the viable cells were stained with the 2C TCR-specific mAb and analyzed by flow cytometry for distribution of CFSE intensity. The data shown are the histograms depicting the distribution of CFSE intensity of the gated 2C cells. The B7DC-enhanced proliferation has been repeated twice.

First, B7DC-transfected tumor cells are rapidly rejected by immune-competent hosts, and the mice that rejected B7DC-transfected tumor cells developed immunity against a subsequent challenge with unmodified parental tumors. This is distinct from B7H1-transfected tumor cells, which were not rejected (19).
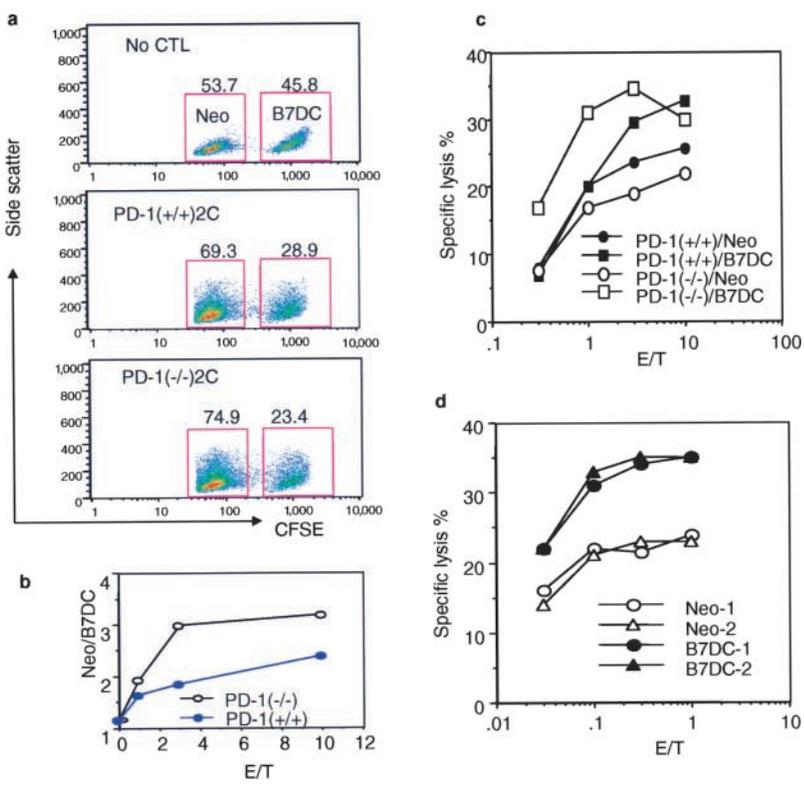

Figure 8. Preferential lysis of J558-B7DC over J558-Neo by a PD-1–independent mechanism. Activated PD-1(+/+) and PD-1(−/−)2C cells were used as effector cells, whereas labeled J558-B7DC and J558-Neo were used as targets. (a) When J558-B7DC and J558-Neo were cocultured with CTL, J558-B7DC cells were preferentially eliminated. E/T = 10. Data shown are representative of three independent experiments, which have been repeated with two independent clones from each group. (b) Ratios of J558-Neo/J558-B7DC at different E/T. (c) Cytolysis of J558-Neo and J558-B7DC as measured by conventional ^{51}Cr release assay. Data shown are representative of three independent experiments. (d) Cytolysis of two independent J558-Neo and J558-B7DC clones by PD-1(−/−)2C. Data are representative of two independent experiments.

Second, unlike B7H1-expressing tumor cells, which reduce the number of antigen-specific T cells in vivo (19), B7DC-transfected tumor cells substantially increased the number of antigen-specific T cells. This has been demonstrated with both transgenic and nontransgenic T cells. In both models, the P1A-specific CTL divided more rapidly and accumulated at higher numbers in response to the J558-B7DC tumors. Using H-2–deficient tumor cells that mediate cross-, but not direct, priming, we were unable to demonstrate any effect of B7DC on cross-priming. Therefore, it is likely that the main function of B7DC in T cell priming is to enhance direct activation of T cells by the tumor cells.

Although both B7H1 and B7DC bind to PD-1(−/−) T cells, we observed two significant differences in their binding to T cells. First, because B7H1 binding to the PD-1(−/−) cells cannot be blocked by 40-fold excess of unlabeled B7H1, the significance of the non–PD-1 receptor for B7H1 in the 2C cells is less certain. In contrast, the B7DC binding to PD-1(−/−) T cells is considerably stronger and can be blocked by unlabeled B7DC. Second, we have failed to detect cross-blocking between the two molecules in their binding to PD-1(−/−) cells, even though both molecules have strong saturable binding to PD-1–transfected CHO cells (unpublished data). These two findings suggest that the non–PD-1 receptor for B7DC may not be shared with B7H1. The distinct non–PD-1 receptors for B7H1 and B7DC may help to explain their distinct biological function.

B7DC Enhances the Effector Function of Cancer-specific T Cells. Several lines of evidence point to an important function of B7DC in promoting cytolysis of tumor cells by CTL. First, B7DC-transfected target cells are preferentially lysed by CTL specific for either P1A–H-2L^d complex or allogeneic H-2L^d. Second, when B7DC-transfected and mock-transfected tumor cells are cocultured with CTL, the B7DC-transfected tumor cells are preferentially eliminated. Third, B7DC transfection conveys high susceptibility of large tumors to therapy by tumor-specific T cells in vivo. Thus, B7DC joins B7H and B7-1 in promoting the effector function of CTL (14, 20, 24, 29) while differing from B7H1, which reduces the efficiency of cancer immunity by inducing apoptosis of CTL (19). Because many cancer patients already have expanded cancer-specific CTL, which can coexist with antigen-bearing cancer cells (30, 31), a critical aspect of cancer immunotherapy is to increase the effector function of cancer-specific T cells. Expression of costimulatory molecules, such as B7–1, B7H, and B7DC, may offer a valuable approach.

B7DC Promotes T Cell Induction and Effector Function by PD-1–independent Mechanisms. Our results documented here differ significantly from both what was described for its PD-1–dependent function and what was described for B7H1 in the tumor model. To address the potential contribution of PD-1 in tumor rejection, we compared the effector function of PD-1(+/+) and PD-1(−/−)2C cells. Our results demonstrated that both PD-1(+/+) and PD-1(−/−) 2C cells preferentially lysed B7DC-transfected tumor cells. Thus, PD-1 is not required for the B7DC-mediated enhancement of T cell effector function. Similarly, the

B7DC-transfected tumor cells are more potent stimulators for the PD-1(−/−) T cells. In fact, we have consistently observed that PD-1(−/−)2C cells are more responsive to B7DC-mediated costimulation for effector function. Although this would argue for the notion that PD-1 may mediate a negative regulation through B7DC, it is also possible that PD-1–deficient T cells and their wild-type counterpart are programmed differently due to a significant function of PD-1 in T cell development (32).

The existence of a PD-1–independent receptor for B7DC is also confirmed by significant binding of PD-1(−/−) 2C cells by B7DC. Interestingly, an anti-B7DC antibody that blocks B7DC–PD-1 interaction enhanced the binding between B7DC and PD-1(−/−) T cells. Because the 2C cells used in this study did not express B7DC, the enhanced binding must be due to anti-B7DC–B7DC interaction, which likely produces higher valancy of B7DC. The opposite effect of the mAb demonstrates that B7DC uses distinct epitope to bind PD-1 and the putative non–PD-1 receptor. This differs from B7-1/B7-2–CD28/CTLA4 interaction, which we and others have demonstrated to use an overlapping site (33, 34).

In conclusion, we have revealed a costimulatory function of B7DC in antitumor immunity. This is achieved by both enhanced T cell priming and effector function. Our studies demonstrate the existence of a non–PD-1 costimulatory receptor(s) for B7DC, which is responsible for the enhanced effector function for B7DC-expressing tumor cells. The involvement of the novel pathway may help to reconcile the conflicting findings on the function of B7DC.

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