

# Cooperative B7-1/2 (CD80/CD86) and B7-DC Costimulation of CD4<sup>+</sup> T Cells Independent of the PD-1 Receptor

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## Abstract

B7-DC is a recently discovered member of the B7 family that binds to PD-1 and is selectively expressed by dendritic cells (DCs). It has been shown to either costimulate or inhibit T cell responses. To assess the role of B7-DC in DC–T cell interactions, DCs from B7-DC knockout (KO) mice were generated and compared with DCs from wild-type (WT) and B7-1/B7-2 double KO mice. B7-1/B7-2-deficient DCs, while strongly diminished in their ability to stimulate naive CD4<sup>+</sup> T cells, nonetheless retain partial activity. DCs from B7-DC KO mice are diminished in their ability to activate CD4<sup>+</sup> T cells, demonstrating that DC-expressed B7-DC serves a predominantly stimulatory rather than inhibitory function in the initiation of T cell responses. B7-DC costimulates expression of CD40L with faster kinetics than B7-1 and displays potent synergy with B7-1 and B7-2 for T cell proliferation and cytokine production, indicating that these B7 family members work in concert to stimulate T cells. Finally, costimulation with B7-DC alone or in conjunction with B7-1 is PD-1 independent, indicating that B7-DC costimulates T cells via a second receptor.

Key words: B7 • CD40L • costimulatory molecule • PD-1 • T cell activation

## Introduction

DCs play a pivotal role at the intersection of innate and adaptive immune responses (1, 2). The unique character of DCs as APCs for T cells derives from multiple mechanisms including high levels of peptide–MHC complexes to engage the TCR (signal 1), high levels of adhesion molecules, chemokine production, and a large variety of costimulatory signals (so called signal 2). The B7 family of costimulatory molecules is particularly important in regulating T cell responses (3–5). The originally described members of the B7 family, B7-1 and B7-2, both costimulate through CD28. However, T cell activity is down-modulated by a second receptor for B7-1 and B7-2, CTLA-4. While B7-1 and B7-2 were once thought to represent the major

source of signal 2 by DCs, the recent discovery of additional B7 family members highlights the molecular complexity of costimulation.

To elucidate the unique biology of DCs, we have screened subtractive cDNA libraries between DCs and activated macrophages in order to identify genes selectively expressed by DCs. This screen identified a B7 family member, termed B7-DC (also termed PD-L2), which costimulates naive T cells to proliferate and produce cytokines such as IL-2 and IFN- $\gamma$  (6). Murine B7-DC/PD-L2 is highly similar (34% identity) to another B7 family member, B7-H1/PD-L1, and both bind PD-1 (7, 8). Despite their homology and similar ligand binding, B7-DC and B7-H1 have very distinct expression patterns, while B7-H1 is expressed on all hematopoietic cells and many nonhematopoietic tissues and tumors, B7-DC surface expression is generally restricted to DCs and a limited subset of macrophages (6, 9–11).

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More than simply a redundant costimulator, B7-DC is a functionally complex and enigmatic molecule. We recently found that B7-DC cross-linking with an IgM antibody activated DCs, suggesting that B7-DC can serve as a bidirectional amplification signal between DCs and T cells (12). In contrast, Latchman et al. reported this molecule to be inhibitory to stimulation of T cells (particularly preactivated T cells) in keeping with the apparent inhibitory role of its receptor, PD-1 (10). Addition of anti-B7-DC antibodies to DC-T cell cultures enhanced T cell stimulation. This result was interpreted as inhibition of a negative signal delivered to T cells by B7-DC (13).

The apparently discrepant activities of B7-DC have been explained by evoking the ITSM motif in the intracytoplasmic domain of PD-1, which could switch the signal between a costimulatory and an inhibitory pathway (14). Alternatively, a second activating receptor may be operative. Another critical question is the interaction between B7-DC and the B7-1/B7-2 system, as all these molecules are coexpressed on DCs. To directly assess the function of B7-DC alone and together with the B7-1/B7-2 system, we have analyzed T cell activation using a combination of DCs deficient in expression of these B7 family genes as well as Ig fusion molecules and transfectants. These experiments confirm the costimulatory function of B7-DC and further demonstrate a potent synergy with B7-1/2, particularly at low levels of TCR stimulation. The costimulatory activity of B7-DC is mediated by a receptor other than PD-1.

## Materials and Methods

**Mice.** B7-DC KO mice were produced as previously described (12,15). BALB/c, C57BL/6, and B7-1/B7-2 knockout mice were purchased from The Jackson Laboratory. B6-OT-II TCR transgenic mice and BALB/c-6.5 mice and B6-PD-1 knockout mice were gifts from R. Schwartz (National Institutes of Health, Bethesda, MD), H. Von Boehmer (Dana Farber Cancer Institute, Boston, MA), and T. Honjo (Kyoto University, Kyoto, Japan), respectively.

**Antibodies and Reagents.** PE-conjugated anti-mouse B7-DC (TY25) and anti-mouse B7-H1 (MIH6) monoclonal antibodies were purchased from eBioscience. CD3 (2C11) and CD16/32 (2.4G2) mAb, FITC-CD4 (RM4-5), CD11c (HL3) mAbs, PE-CD80 (1G10), CD86 (GL1), CD40L (MR-1), H-2<sup>d</sup> (28-14-8), I-A<sup>b</sup> (25-9-17), and I-E<sup>k/d</sup> (14-4-4s) mAbs were purchased from BD Biosciences.

**Cell Preparations and Cultures.** For T cell assays, CD4<sup>+</sup> T cells were purified by using a CD4<sup>+</sup> T cell negative selection kit (MACS; Miltenyi Biotec). Bone marrow-derived DCs (BMDCs)\* were prepared following the previously described methods (16) with GM-CSF (1,000 U/ml) and IL-4 (1,000 U/ml). A.E7 is a CD4<sup>+</sup> Th1 clone specific for the pigeon cytochrome c peptide 81-104 and was grown and maintained as described previously (17).

**Transfections.** Murine B7-DC and B7-1 cDNA cloned into pCAGGS vector were transfected into RENCA cells (R-DC and R-B7-1, respectively) using GeneJammer (Stratagene). Stable

transfectants were selected with blasticidine and cloned using limiting dilution culture conditions.

**Ag Presentation Assay by RENCA Transfectants.** RENCA cell lines expressing B7-DC, B7-1, or both were used as APC to present HA<sub>110-120</sub> peptide to specific 6.5 TCR transgenic CD4<sup>+</sup> T cells. Prior to the presentation assay, RENCA transfectants were treated 3 d with 100 ng/ml of IFN- $\gamma$  (R&D Systems) to induce surface expression of I-E<sup>d</sup> molecules. Cells were irradiated (13,200 rad), washed, and plated at 10<sup>5</sup> cells/well in 96 flat-bottom plates in presence of 1 to 10  $\mu$ g/ml HA peptide and 10<sup>5</sup> CD4<sup>+</sup> 6.5 TCR transgenic splenocytes. Proliferation was determined by labeling cultures for the last 10-18h of a 3-d assay with 1  $\mu$ Ci [<sup>3</sup>H]thymidine/well.

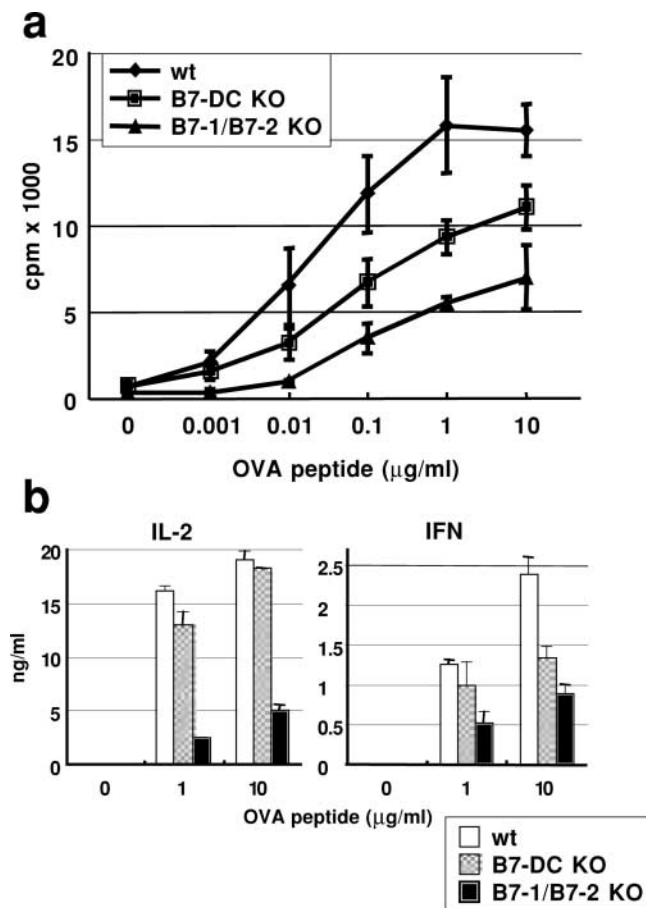
**T Cell Costimulation Assays.** High-bound 96-well plates (Immulon4; Dynex) were coated 90 min at 37°C with 3 ng/well or 30 ng/well of anti-CD3 (2C11; BD Biosciences) in presence or absence of B7-1-Ig or B7-2-Ig (R&D Systems), and/or B7-DC-Ig dimer, which consists of a full mouse IgG1 in which the variable domains of the heavy chain have been substituted with the extracellular domain of mouse B7-DC.

## Results

**Stimulatory Properties of BM-derived DCs from B7-DC KO and B7-1/B7-2 KO Mice.** To definitively determine whether B7-DC expression by DCs enhances or inhibits their ability to stimulate T cells, B7-DC KO mice were generated as a source of DCs. The mice did not display any overt evidence of autoimmunity, immune deficiency, or other pathology up to one year of age. Cell numbers and gross phenotype of the thymus, spleen, and lymph nodes were normal in B7-DC KO animals. For analysis of DC function, BMDCs generated from B7-DC KO mice were assessed for their stimulatory properties. We assessed the stimulatory properties of BMDCs from B7-1/B7-2 KO mice as well in order to determine whether DCs could stimulate CD4<sup>+</sup> T cells in the absence of these primary costimulatory molecules. BMDCs were cultured for 6 d with GM-CSF and an additional 2 d with GM-CSF and IL-4. In a reciprocal fashion, mechanically activated BMDCs from B7-1/B7-2 KO mice failed to express B7-1 and B7-2 but expressed high levels of B7-DC and I-A<sup>b</sup>.

CD4<sup>+</sup> T cells from young OT-II transgenic mice were cocultured with DCs from either wt, B7-1/B7-2 KO, or B7-DC KO mice in the presence of varying peptide concentrations and T cell proliferation, IL-2 and IFN- $\gamma$  production were measured. Fig. 1 shows that unstimulated OT-II T cells responded to OVA<sub>323-339</sub> peptide presented by B7-1/B7-2<sup>-/-</sup> DCs although to a much lesser extent than wt DCs. These results suggested that B7-1/B7-2<sup>-/-</sup> DCs express additional molecules capable of providing costimulation to T cells. Stimulation of OT-II cells by B7-DC<sup>-/-</sup> DCs was diminished relative to wt DCs although it was greater than with B7-1/B7-2<sup>-/-</sup> DCs. In parallel with the proliferation results, IFN- $\gamma$  production by OT-II cells was diminished with B7-DC<sup>-/-</sup> DCs, though not as much as with B7-1/B7-2<sup>-/-</sup> DCs. These results demonstrate that B7-DC provides a positive rather than inhibitory signal for initiating CD4<sup>+</sup> T cell activation.

\*Abbreviation used in this paper: BMDC, bone marrow-derived DC.



**Figure 1.** BMDCs from B7-DC<sup>-/-</sup> mouse are deficient in stimulating T cells. (a) BMDCs ( $10^4$ /well) from wt mice (diamond), B7-DC KO mice (square), and B7-1/B7-2 double KO mouse (triangle) were cultured with CD4<sup>+</sup> OT-II T cells ( $10^4$ /well) in the presence of the indicated concentrations of OVA<sub>323–339</sub> peptide and proliferation was measured after 3 d. (b) Supernatants from these cultures with BMDCs from wt mouse (open bars), B7-DC KO mice (hatched bar), and B7-1/B7-2 double KO mice (filled bars) were collected after 2 d and IL-2 and IFN- $\gamma$  were measured by ELISA.

*Differential Kinetics and Synergy between B7-DC and B7-1-mediated Costimulation of CD4<sup>+</sup> T Cells.* To further assess the costimulatory capacity of B7-DC, alone and in conjunction with B7-1, a series of *in vitro* experiments using immobilized B7-1 and/or B7-DC (as Ig dimers) and RENCA cells expressing B7-1 and/or B7-DC, were performed. Initially, stimulation of CD4<sup>+</sup> T cells was assessed by analyzing the expression of T cell activation markers such as CD40L, CD25 and CD69. Fig. 2 demonstrates that for both high and low concentrations of anti-CD3 (signal 1), B7-DC-Ig costimulates the expression of CD40L with much faster kinetics than B7-1, inducing significant up-regulation after 6 h. In contrast B7-1 costimulation of CD40L was not observed until 18 h. Importantly, B7-DC does not inhibit the up-regulation of CD40L on CD4<sup>+</sup> T cells by B7-1 costimulation. Interestingly, at low concentrations of anti-CD3, B7-DC alone efficiently up-regulated CD40L expression whereas B7-1 did not. The differential

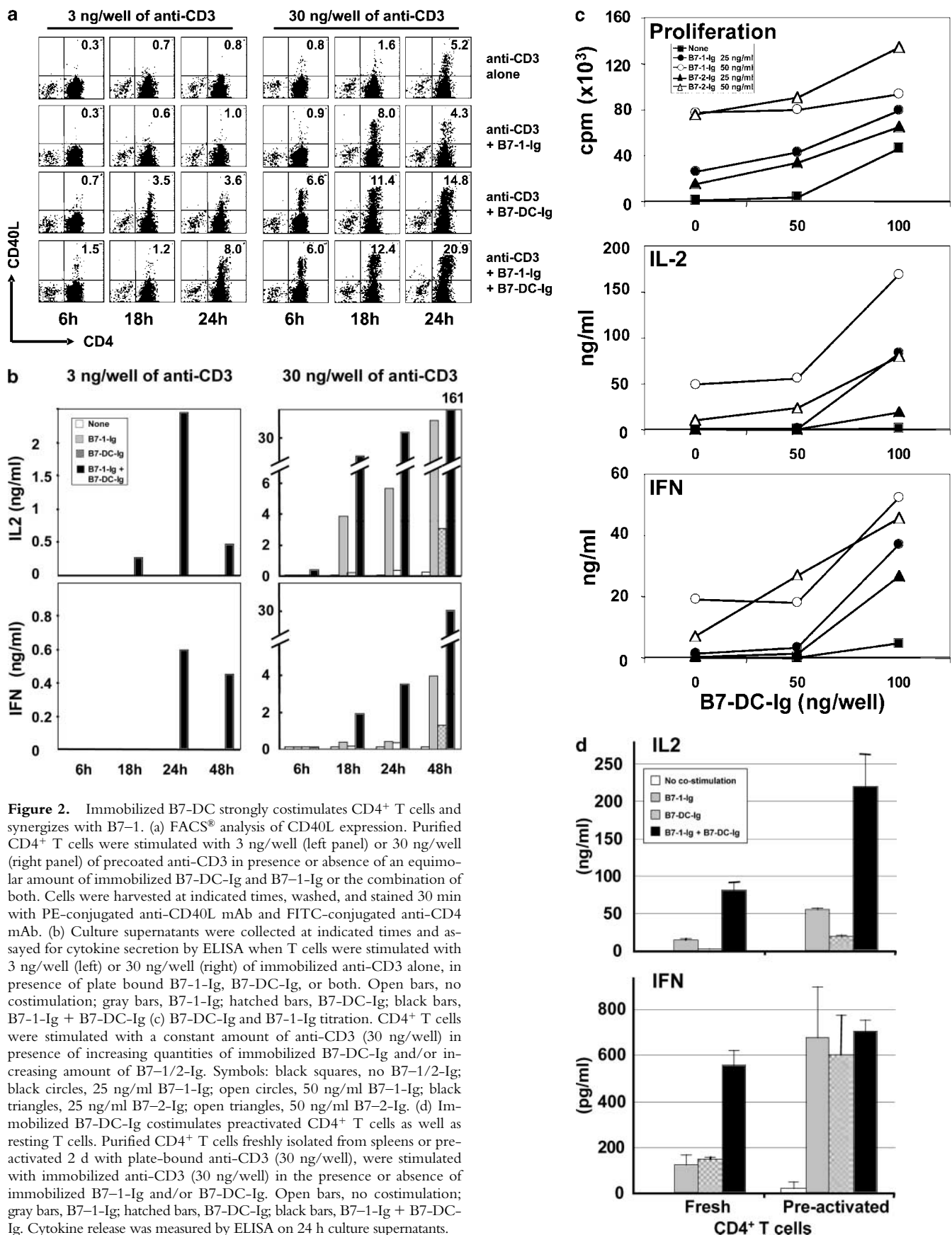
kinetics and sensitivity of CD4 T cells to costimulation with B7-DC versus B7-1 was not observed for other T cell surface molecules such as CD25 and CD69 (data not depicted), which are involved in later stages of T cell activation than CD40L. These results suggest that B7-DC costimulation is particularly important in modulating early events in crosstalk between T cells and DC as CD40L induced on T cells is a potent amplifier of DC function (18, 19) whereas B7-DC cross-linking has been shown to stimulate CD40 expression by DCs (12).

In parallel to the above experiments, culture supernatants were harvested at each time point of stimulation and tested for cytokine secretion. B7-1 costimulated IL-2 secretion much more efficiently than B7-DC (Fig. 2). Whereas B7-1 alone induced high production of IL-2 after 18 h stimulation, significant IL-2 production was only observed after 48 h with B7-DC alone. In contrast, both molecules demonstrated a similar costimulation of IFN- $\gamma$ , which peaked after 48 h incubation. Strikingly, even though B7-DC demonstrated a much weaker influence on IL-2 secretion than B7-1, it synergized tremendously with B7-1 to costimulate IL-2 production. Significant synergy was also observed in the costimulation of IFN- $\gamma$ . Titration experiments (Fig. 2 c) demonstrated that B7-DC synergizes similarly with B7-1 and B7-2.

At low anti-CD3 concentrations, which is thought to mimic limiting signal 1 strength, significant levels of IL-2 and IFN- $\gamma$  were detected only when T cells were costimulated by a combination of B7-1/2 and B7-DC (Fig. 2). These results suggest that the combined activity of B7-DC and members of the B7-1/B7-2 system represent an important mechanism by which DCs uniquely initiate T cell responses even at low antigen dose.

Because of the question of whether B7-DC costimulation versus inhibition was dependent on the state of T cell activation, we compared the function of B7-DC on unstimulated and preactivated CD4<sup>+</sup> T cells using plate bound anti-CD3. Fig. 2 d shows that, rather than inducing inhibition of T cell functions, 2-d preactivation increased the costimulatory effect of B7-1 and B7-DC, with B7-1 costimulation much more efficient than B7-DC. Of note, the synergy between both molecules for costimulation of IL-2 secretion still exists. However, even though the costimulation of IFN- $\gamma$  production by B7-DC or B7-1 is increased, the synergistic effect is no longer observed with preactivated T cells.

*T Cell Costimulation Using B7-1, B7-DC, and B7-1/B7-DC Transfectants.* As an alternative approach to individually reconstitute specific costimulatory signals, we transfected RENCA cells with the B7-1 gene, B7-DC gene, or both. RENCA, a BALB/c-derived tumor does not naturally express B7-1, B7-2, B7-DC, or B7-H1 even after IFN- $\gamma$  treatment. IFN- $\gamma$ -treated RENCA and its various transfectants express I-E<sup>d</sup>, allowing it to present HA<sub>110–120</sub> peptide to 6.5 transgenic CD4<sup>+</sup> T cells. Fig. 3 shows that the optimal stimulation of either unactivated 6.5 transgenic CD4<sup>+</sup> T cells required the presence of costimulatory molecules. In all cases, the presentation of HA<sub>110–120</sub> by RENCA



**Figure 2.** Immobilized B7-DC strongly costimulates CD4<sup>+</sup> T cells and synergizes with B7-1. (a) FACS<sup>®</sup> analysis of CD40L expression. Purified CD4<sup>+</sup> T cells were stimulated with 3 ng/well (left panel) or 30 ng/well (right panel) of precoated anti-CD3 in presence or absence of an equimolar amount of immobilized B7-DC-Ig and B7-1-Ig or the combination of both. Cells were harvested at indicated times, washed, and stained 30 min with PE-conjugated anti-CD40L mAb and FITC-conjugated anti-CD4 mAb. (b) Culture supernatants were collected at indicated times and assayed for cytokine secretion by ELISA when T cells were stimulated with 3 ng/well (left) or 30 ng/well (right) of immobilized anti-CD3 alone, in presence of plate bound B7-1-Ig, B7-DC-Ig, or both. Open bars, no costimulation; gray bars, B7-1-Ig; hatched bars, B7-DC-Ig; black bars, B7-1-Ig + B7-DC-Ig (c) B7-DC-Ig and B7-1-Ig titration. CD4<sup>+</sup> T cells were stimulated with a constant amount of anti-CD3 (30 ng/well) in presence of increasing quantities of immobilized B7-DC-Ig and/or increasing amount of B7-1/2-Ig. Symbols: black squares, no B7-1/2-Ig; black circles, 25 ng/ml B7-1-Ig; open circles, 50 ng/ml B7-1-Ig; black triangles, 25 ng/ml B7-2-Ig; open triangles, 50 ng/ml B7-2-Ig. (d) Immobilized B7-DC-Ig costimulates preactivated CD4<sup>+</sup> T cells as well as resting T cells. Purified CD4<sup>+</sup> T cells freshly isolated from spleens or pre-activated 2 d with plate-bound anti-CD3 (30 ng/well), were stimulated with immobilized anti-CD3 (30 ng/well) in the presence or absence of immobilized B7-1-Ig and/or B7-DC-Ig. Open bars, no costimulation; gray bars, B7-1-Ig; hatched bars, B7-DC-Ig; black bars, B7-1-Ig + B7-DC-Ig. Cytokine release was measured by ELISA on 24 h culture supernatants.



cells transfected with the combination of B7-1 and B7-DC induced the greatest T cell proliferative responses. Similarly, B7-2 + B7-DC double transfectants induced significantly greater proliferative responses among 6.5 CD4<sup>+</sup> T cells than did B7-2 or B7-DC single transfectants (unpublished data). To assess the potential of the transfectants to stimulate truly naive T cells, we used T cells 6.5 transgenic mice with RAG KO background. In contrast with RAG<sup>+/+</sup> 6.5 transgenic splenocytes, 6.5 transgenic RAG<sup>-/-</sup> T cells are extremely dependent on costimulatory signals whereas they are much less costimulation dependent when preactivated. Whereas naive 6.5 transgenic RAG<sup>-/-</sup> CD4<sup>+</sup> T cells were activated neither by plain RENCA nor B7-1 or B7-DC transfectants, they proliferated significantly in response to HA peptide presented by B7-1/B7-DC double transfectants.

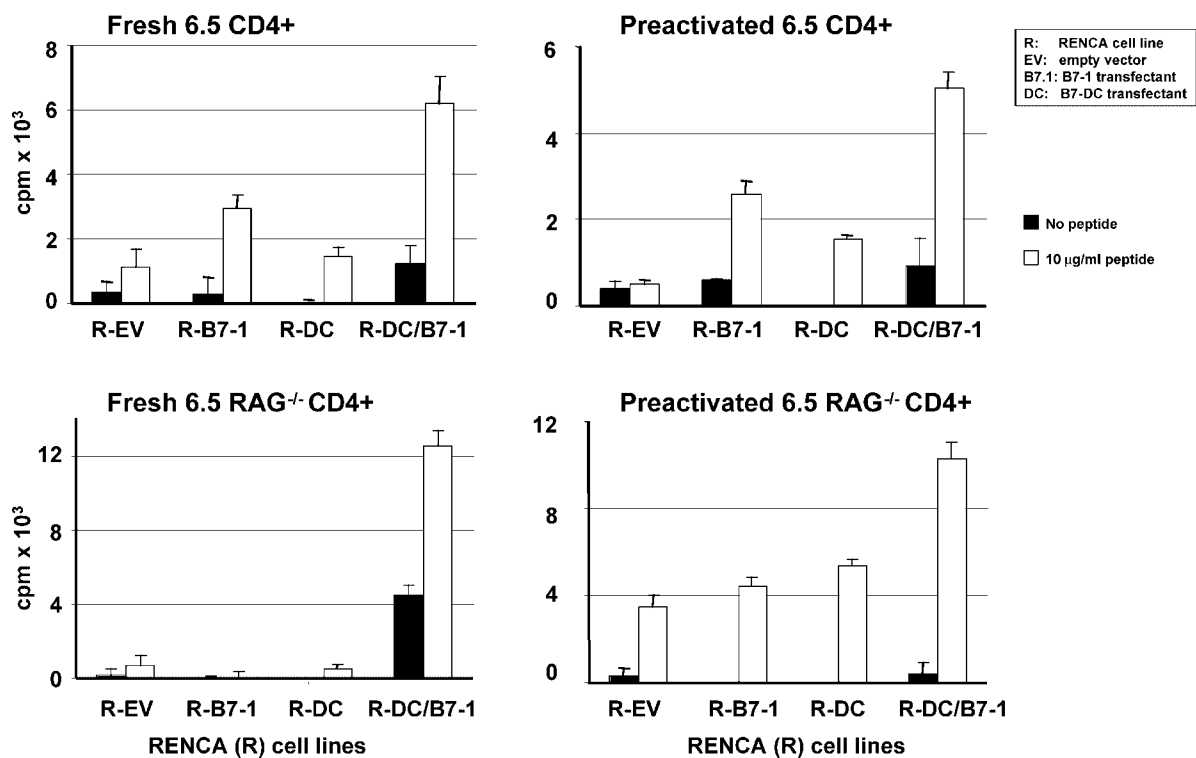
**B7-DC Costimulation of CD4<sup>+</sup> T Cells Is PD-1 Independent.** PD-1 was described as the receptor of B7-DC and B7-H1 and has been shown to negatively modulate immune responses (10, 13, 20). We therefore directly assessed the participation of PD-1 in B7-DC costimulation using CD4<sup>+</sup> T cells from PD-1 KO mice. Fig. 4 shows that immobilized B7-DC costimulates IL-2 and IFN- $\gamma$  secretion by B6 PD1<sup>-/-</sup> as well as wt B6 T cells. Moreover, the synergistic effect of B7-DC + B7-1 costimulation for cytokine production was equivalent for T cells from PD-1

KO as for wt B6 mice. This finding demonstrates that PD-1 is not necessary for the delivery of the positive costimulatory signals by B7-DC. We found that B7-DC similarly costimulates cytokine secretion by preactivated PD-1<sup>-/-</sup> CD4<sup>+</sup> T cells similarly to wt CD4<sup>+</sup> T cells. Finally, even in absence of PD-1 expression on preactivated T cells, the synergy between B7 and B7-DC was observed for IL-2 secretion but not for IFN- $\gamma$  production as observed previously.

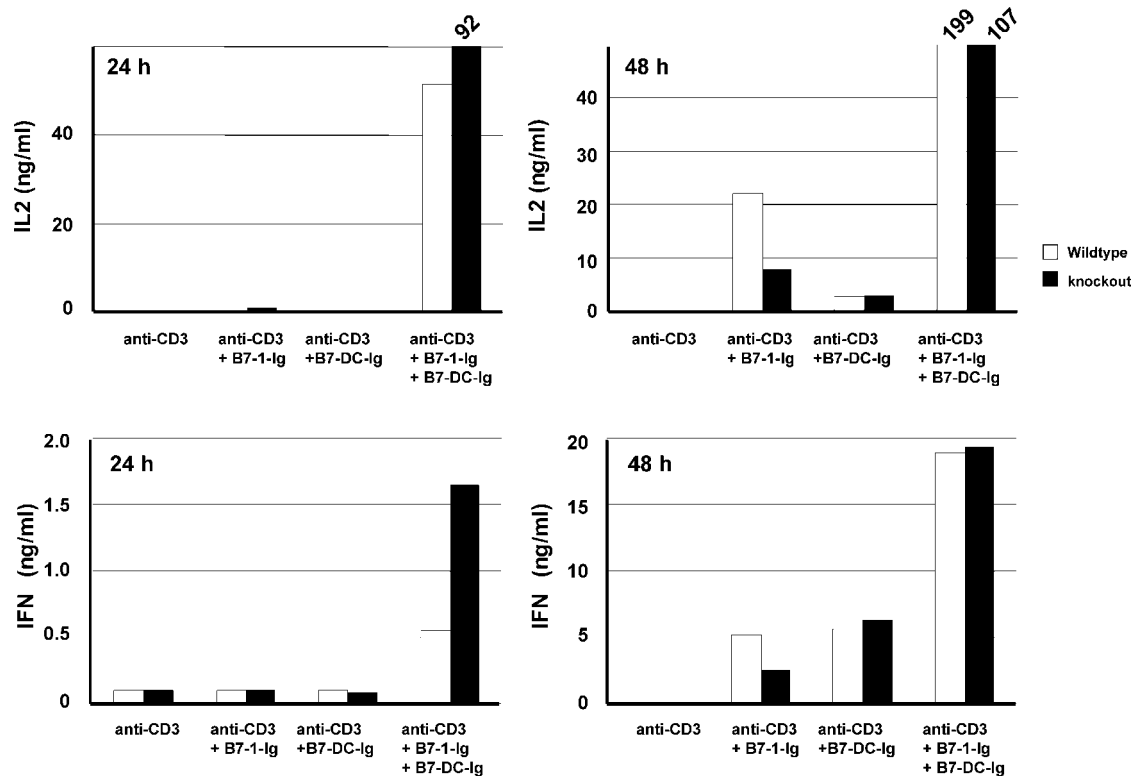
Synergistic effects between B7-1 and B7-DC on activation of the T cell clone AE-7, which expresses constitutively high level of PD-1, were assessed using plate bound anti-CD3 in presence of anti-CD28 or B7-1 and/or B7-DC. Fig. 5 demonstrates that, even in the presence of much higher levels of PD-1 than typically observed on activated splenic or LN T cells directly out of the mouse, B7-DC synergizes very efficiently for costimulation of IL-2 production. These results suggest that interaction of B7-DC with the alternate receptor may override PD-1 mediated effects.

## Discussion

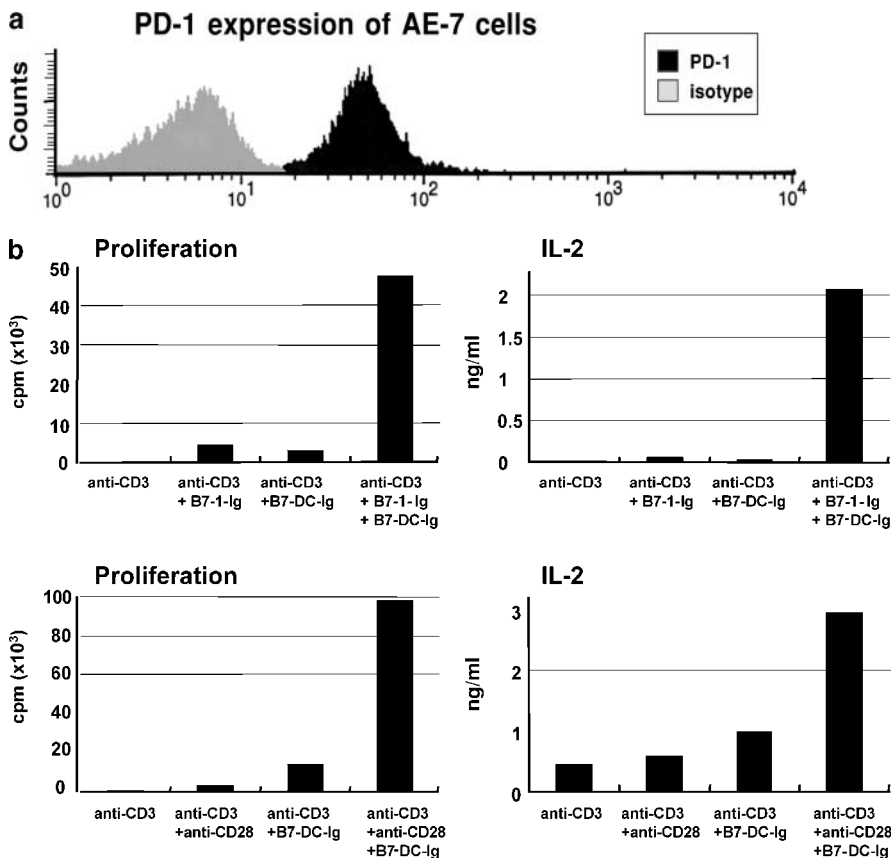
Multiple separate lines of evidence presented here support the notion that B7-DC, which is predominantly expressed on DCs, serves a significant costimulatory function



**Figure 3.** HA<sub>110-120</sub> peptide presentation to specific HA-specific CD4<sup>+</sup> T cells by RENCA cells engineered to express B7-DC and/or B7-1 molecules. CD4<sup>+</sup> T cells were purified from either fresh (left panels) or preactivated (right panels) splenocytes from TCR transgenic 6.5 mice (top panels) or 6.5 RAG KO mice (bottom panels). For the preactivated cells, splenocytes were cultured 4 d in vitro with 1 µg/ml of HA<sub>110-120</sub> peptide. CD4<sup>+</sup> T cells were then negatively selected and rested overnight. The indicated RENCA transfectants were treated 3 d with 10 ng/ml of IFN- $\gamma$  to induce expression of I-E<sup>d</sup> molecules and used to present HA peptide (10 µg/ml) to the CD4<sup>+</sup> T cells. T cell proliferation with either 0 (filled bars) or 10 µg/ml (open bars) HA peptide was measured after 72 h by [<sup>3</sup>H]thymidine incorporation.



**Figure 4.** B7-DC costimulates PD1<sup>-/-</sup> CD4<sup>+</sup> T cells. (a) Purified CD4<sup>+</sup> T cells from wt (open bars) or PD-1 KO mice (filled bars) were stimulated with 30 ng/well of precoated anti-CD3 in the presence or absence of immobilized B7-1-Ig, B7-DC-Ig, or B7-1-Ig + B7-DC-Ig. Culture supernatants were collected at indicated times and assayed for cytokine secretion by ELISA.



**Figure 5.** B7-DC and anti-CD28 mAb synergize for costimulation of IL-2 production even in the presence of high levels of PD-1. (a) AE-7 cells, which express high level of PD-1 were cultured in presence of limiting amounts of immobilized anti-CD3 with either B7-DC-Ig, B7-1-Ig, anti-CD28 (at limiting concentration), or indicated combinations. (b) Culture supernatants were collected at 24 h and assayed for IL-2 by ELISA.

for CD4<sup>+</sup> T cells. Prestimulated T cells demonstrate less dependence on the synergistic action of B7-DC and B7-1, though we never observed inhibition by B7-DC. Furthermore, B7-DC costimulation appears to be both cooperative and synergistic with the B7-1/B7-2 system. This synergy is independent of PD-1, indicating that an alternative B7-DC receptor mediates most, if not all of the B7-DC-dependent costimulation of T cells.

Costimulation by B7-DC alone and in synergy with B7-1 was virtually identical in PD-1<sup>-/-</sup> versus wt T cells, demonstrating the existence of a second B7-DC receptor that transmits an activating signal to CD4<sup>+</sup> T cells. These results are compatible with the findings of Wang et al. who found that mutations in B7-DC that abrogate binding to PD-1 do not inactivate its costimulatory activity (21).

The role of PD-1 in B7-DC physiology remains to be definitively elucidated. Naive T cells engaging a DC would not initially express much PD-1 but live LN imaging studies suggest that T cells remain attached to DCs for 48 h, long enough for PD-1 expression to be induced (3). Our studies with A.E7 cells, which express high levels of PD-1, suggest that B7-DC can costimulate in the presence of ample PD-1.

A number of lines of evidence support the idea that, despite their structural similarity and genomic proximity, B7-DC and B7-H1 have distinct immunologic activity. First, while B7-DC is predominantly expressed on DCs and to a lesser extent on some activated macrophages, B7-H1 is more ubiquitously expressed on both hematopoietic and nonhematopoietic tissues, including many tumors (9, 11, 22, 23). Second, B7-DC and B7-H1 bind to different sites on PD-1 (21), which may mediate distinct signaling. Latchman et al. reported that while B7-H1 inhibited B7-1-mediated costimulation, B7-DC did not (10). Forced expression of B7-H1 on tumors generally inhibits their immunologic rejection whereas forced B7-DC expression appears to activate antitumor immunity (unpublished data, and Yang Liu, personal communication). Taken together, these results suggest a model in which B7-DC plays a major costimulatory role at the T cell priming phase (both as DC activating receptor and costimulator) whereas B7-H1 plays a more complex role. Its expression on DCs likely promotes priming of a subset of naive PD-1<sup>-</sup> T cells while its expression in the periphery (and on tumors) downmodulates effector responses by PD-1<sup>+</sup> T cells. Thus, these two B7 family members likely play diverse and distinct rather than redundant roles in immune regulation.

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