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J Immunol 2012; 189:2338-2347; Prepublished online 25 July 2012;
doi: 10.4049/jimmunol.1103085
http://www.jimmunol.org/content/189/5/2338
B7-DC-Ig Enhances Vaccine Effect by a Novel Mechanism Dependent on PD-1 Expression Level on T Cell Subsets

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Programmed death receptor 1 (PD-1) is an important signaling molecule often involved in tumor-mediated suppression of activated immune cells. Binding of this receptor to its ligands, B7-H1 (PD-L1) and B7-DC (PD-L2), attenuates T cell activation, reduces IL-2 and IFN-γ secretion, decreases proliferation and cytotoxicity, and induces apoptosis. B7-DC-Ig is a recombinant protein that binds and targets PD-1. It is composed of an extracellular domain of murine B7-DC fused to the Fc portion of murine IgG2a. In this study, we demonstrate that B7-DC-Ig can enhance the therapeutic efficacy of vaccine when combined with cyclophosphamide. We show that this combination significantly enhances Ag-specific immune responses and leads to complete eradication of established tumors in 60% of mice and that this effect is CD8 dependent. We identified a novel mechanism by which B7-DC-Ig exerts its therapeutic effect that is distinctly different from direct blocking of the PD-L1–PD-1 interaction. In this study, we demonstrate that there are significant differences between levels and timing of surface PD-1 expression on different T cell subsets. We found that these differences play critical roles in anti-tumor immune effect exhibited by B7-DC-Ig through inhibiting proliferation of PD-1high CD4 T cells, leading to a significant decrease in the level of these cells, which are enriched for regulatory T cells, within the tumor. In addition, it also leads to a decrease in PD-1high CD8 T cells, tipping the balance toward nonexhausted functional PD-1low CD8 T cells. We believe that the PD-1 expression level on T cells is a crucial factor that needs to be considered when designing PD-1–targeting immune therapies. The Journal of Immunology, 2012, 189: 2338–2347.

It has been shown that when PD-L1 binds PD-1, TCR signaling is dampened, causing decreased cytokine production by the T cell, ultimately resulting in reduced proliferation, anergy, and/or apoptosis (17, 18). In this way, PD-L1 expression by tumor cells serves a protective function, leading to suppression of tumor-infiltrating effector lymphocytes in the tumor microenvironment, thus shielding the tumor from T cell-mediated killing (19–21). To date, using various systems and blocking mAbs, it has been shown that tumor eradication can be enhanced by PD-L1–PD-1 blockade (22–27).

Regulatory T cells (Tregs) are inhibitory CD4+ T cells that are increased in cancer patients and can potentially form a barrier to elicit an effective immune response (28–32). Depletion or inactivation of Tregs has been actively pursued to enhance anti-tumor immunotherapies. Recently, we demonstrated that the combination of CT-011 anti–PD-1 Ab with vaccine and low-dose cyclophosphamide, which reduces the number and function of Tregs (33–38), significantly enhances Ag-specific immune responses, decreases tumor burden, and increases survival of treated mice (26), suggesting that targeting multiple immune checkpoint inhibitors is a promising strategy for cancer immunotherapy.

In this study, we tested the anti-tumor immune effect of B7-DC-Ig—which is a PD-1 binding recombinant protein composed of the extracellular domain of murine B7-DC fused to the unmodified Fc portion of murine IgG2a—in combination with low-dose CPM and model vaccine.

We demonstrated that this treatment synergistically decreases tumor burden and increases animal survival. Notably, we found that B7-DC-Ig is unable to block PD-L1–PD-1 interaction but instead decreases tumor-infiltrated Tregs and reduces the percentage of tumor-infiltrating exhausted PD-1high CD8 T cells. These data suggest that B7-DC-Ig could be used in combination with other immunotherapies to overcome inhibitory checkpoints within the tumor microenvironment, enhance immunogenicity of vaccine, and provide protection against tumors. In addition, while investigating these mechanisms, we showed for the first time to our...
knowledge that different T cell subsets express different levels of surface PD-1, and this difference acts as a breaking point for the effect of B7-DC-Ig.

Materials and Methods
Animals, cells lines, vaccine, and other reagents
Female C57BL6 mice aged 6–8 wk were purchased from NCI Frederick and kept under pathogen-free conditions. All procedures were carried out under guidelines of the National Institutes of Health in accordance with approved institutional animal protocols. TC-1 cells that were derived by cotransfection of human papillomavirus strain 16 (HPV16) early proteins 6 and 7 (E6 and E7) and activated ras oncoprotein to primary C57BL/6 mouse lung epithelial cells were obtained from American Type Culture Collection (Manassas, VA). Female C57BL/6 mice were grown in nonobese diabetic severe combined immunodeficient (NOD-SCID) mice were treated similarly, except only two doses of vaccine and B7-DC-Ig were injected i.p. at a dose of 1 mg/mouse; IgG2a was obtained from AmphiImmuine (Gaithersburg, MD) and injected i.p. at 300 μg/mouse. Anti–CD4 (clone GK1.5) and anti–CD8α (clone 53.6.72) mAbs used for in vitro assays were purchased from BioXCell (West Lebanon, NH). Anti–PD-1 mAb (clone J43) and irrelevant IgG used for in vitro studies were from BD Biosciences (San Jose, CA). All fluorescently labeled Abs and appropriate isotype controls used for flow cytometry were purchased from BD Biosciences or eBioscience (San Diego, CA).

Immunization, tumor implantation, and T cell depletion
In experiments where analysis of tumor growth and survival were the endpoint, mice were implanted with 50,000 TC-1 cells/mouse s.c. in the right flank on day 0. On day 7, when all mice had tumors of ∼3–4 mm in diameter, animals from appropriate groups were injected i.p. with CPM, whereas control mice were injected with a similar volume (100 μl) of PBS. On day 8, mice were injected with vaccine (or PBS) s.c. and/or B7-DC-Ig and harvested 4 d later. Tumors were measured every 3–4 d using digital calipers, and tumor volume was calculated using the formula \( V = \frac{4}{3} \pi r^3 \), where \( V \) is volume, \( r \) is length (longer diameter), and \( w \) is width (shorter diameter). In these experiments, mice were sacrificed when mice became moribund, tumors were ulcerated, or tumor volume reached 1.5 cm\(^3\). In each set of immunologic data assessment experiments, mice were treated similarly, except only two doses of vaccine and B7-DC-Ig were given on days 8 and 15 to be able to analyze spleens and tumors from control mice. Mice were sacrificed 6 d after the second treatment (i.e., on day 21). Spleens and tumors were isolated and analyzed for Ag-specific immune responses and tumor-infiltrated immune cell profiles. In T cell depletion experiments, the same treatment schedule was used, along with anti–CD4 mAb injected i.p. on days 5 and 17 (300 μg/mouse) and anti–CD8 mAb on days 17 and 24 (400 μg/mouse) after tumor implantation. Mice were given a total of three vaccine and B7-DC-Ig treatments and monitored for tumor growth and survival.

Analysis of Ag-specific cellular immune responses
ELISPOT assay was used to detect IFN-γ production in E7-restimulated (10 μg/ml) splenocyte cultures from vaccinated and control mice, as suggested by the manufacturer (BD Biosciences). A CTL Immunospot Analyzer (Cellular Technology, Shaker Heights, OH) was used to analyze spots. The number of spots from irrelevant peptide (hgp 10025–33, KVPQRQDWL; Celtel Bioscience) restimulated splenocytes were subtracted from E7-restimulated cultures.

Direct CTL activity in immunized mice was assessed using a flow cytometry assay as described (40). Briefly, to test effector cell function, freshly isolated splenocytes (effector cells) were mixed with TC-1 target cells labeled with CellTracker Green dye (Invitrogen, Carlsbad, CA) at E:T ratios of 50:1, 25:1, and 10:1. After a 3-h coculture, the E:T T ratios were washed, fixed, and permeabilized before staining with PE-labeled anti– caspase-3–Abs (BD Pharmingen, San Jose, CA). After incubation and washing, the number of activated caspase-3–positive apoptotic cells was detected in the CellTracker Green-positive target cell population, and the percentage of apoptotic cells was calculated using CellQuest software (BD Biosciences).

Detection of tumor-infiltrated T cells
In experiments with immunologic endpoints, tumor tissue was harvested 6 d after the second vaccination (day 21 after tumor implantation, the last day when all mice from control groups were still viable). Tumor samples were processed using GentleMACS Dissociator (Miltenyi Biotec, Auburn, CA) and the solid tumor homogenization protocol, as suggested by the manufacturer. After washing, the number of tumor-infiltrated CD8, CD8PD-1high, CD4Foxp3+, and CD4Foxp3– cells were analyzed using the previously described flow cytometry assay and the following fluorochrome Abs: anti-mouse CD8, anti-mouse CD4, anti-mouse PD-1 (BD Biosciences), and anti-mouse Foxp3 (eBioscience) mAb and appropriate isotype control Ab (BD Biosciences). The level of CD4 Foxp3+ cells (Tregs) was also evaluated in splens of tumor-bearing treated and control mice using the same flow cytometry assay. To confirm that in vivo treatment with B7-DC-Ig does not affect staining of tumor-infiltrated cells with PE-labeled anti–PD-1 Ab, purified and 48-h-stimulated T cells were preincubated with B7-DC-Ig, anti–PD-1 Ab (BD Biosciences), or irrelevant IgG prior to staining with PE-labeled Ab at different ratios. Samples were analyzed using FACS caliber Flow Cytometer (BD Biosciences).

Suppression and proliferation assays
The ability of B7-DC-Ig to inhibit the TC-1 tumor-mediated suppression of CD4+CD25+ T cell proliferation was assessed using a CFSE-based suppression assay. CD4+CD25+ T (Tconv) cells were purified from the spleens of naive mice using the Milteny Biotech MACS T cell purification kit as suggested by the manufacturer (Miltenyi Biotech). Cells were labeled with 1 μM CFSE dye as suggested by the manufacturer (Invitrogen). After washing, CFSE-labeled Tconv cells were stimulated with anti-CD3/anti-CD28 polystyrene Dynal beads (Invitrogen) and coincubated with TC-1 cells at a 1:2 ratio for 4 d, alone or in the presence of 20 μg/ml concentrations of B7-DC-Ig, CT-011 anti–PD-1 Ab (Curetech Ltd, Yavne, Israel), anti–PD-1 mAb (clone J43), irrelevant IgG. Samples were washed and evaluated for T cell expansion via CFSE dye dilution using a FACS caliber Flow Cytometer and CellQuest software (BD Biosciences).

The same CFSE assay was used in experiments investigating the B7-DC-Ig effect on proliferation of Tregs, Tconv cells, and CD8 T cells. All three subsets were purified from the spleens of naive mice using the appropriate Miltenyi Biotech MACS purification kit as suggested by the manufacturer. After CFSE labeling and stimulation, cells were cultured for 3 d in the presence of DMSO or different concentrations of B7-DC-Ig, CT-011, or irrelevant IgG. Proliferation was assessed by CFSE dye dilution using a FACS caliber Flow Cytometer and CellQuest software (BD Biosciences).

Analysis of the number of surface PD-1 molecules on T cells
The actual number of surface PD-1 molecules on nonsustained Tregs, Tconv cells, and CD8 T cells, as well as after 24, 48, and 72 h of stimulation, was analyzed using the Quantum Simply Cellular kit as suggested by the manufacturer (Bangs Laboratories, Fishers, IN). More specifically, all three subsets of T cells were purified from the spleens of naive mice and stained with allopurinol-containing anti–CD4 or anti–CD8, FITC-labeled anti–CD3, and PE-labeled anti–PD-1 mAb (BD Biosciences). The same PE-labeled anti–PD-1 mAb was used to stain the calibration beads (Bangs Laboratories). Mean fluorescent intensity (MFI) in the FL-2 channel (PE) was calculated using a FACS caliber Flow Cytometer, and the number of surface PD-1 molecules was calculated based on the calibration curve from standard beads and the calculation template provided by Bangs Laboratories.

Analysis of phosphorylated S6
Nonstimulated Tregs, Tconv cells, and CD8 T cells, as well as cells stimulated for 24, 48, and 72 h, were incubated at 37°C for 10 min in the presence of 20 μg/ml B7-DC-Ig or irrelevant IgG. Cells were stained with allopurinol-containing CD4 or allopurinol-containing CD8 mAb, fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences), and stained with FITC–Foxp3 and anti–phospho–S6 Ab (Cell Signaling Technology, Danvers, MA), followed by anti-rabbit biotin-conjugated secondary Ab and streptavidin–PE. MFI in the FL-2 channel (PE) was calculated using a FACS caliber Flow Cytometer and CellQuest software (BD Biosciences). In addition, p-S6 levels were analyzed in lysates of Tregs, Tconv cells, and CD8 T cells stimulated for 48 h and treated with
B7-DC-Ig or irrelevant IgG as described earlier using the p-S6 ELISA kit per the manufacturer’s suggestions (Cell Signaling Technology).

Statistical analysis

All statistical parameters (average values, SD, significant differences between groups) were calculated using GraphPad Prism Software. Statistical significance between groups was determined by one-way ANOVA followed by Tukey’s multiple comparison posttest (p < 0.05 was considered statistically significant).

Results

B7-DC-Ig significantly enhances vaccine-mediated Ag-specific immune responses when combined with CPM

We have previously demonstrated that inhibition of Tregs with CPM combined with an anti–PD-1 Ab significantly enhances the antitumor effect of vaccine (26). In addition, Marshall et al. (S.A. Marshall, L. Liu, and S. Langerman, submitted for publication) also demonstrated that inhibition of Tregs with CPM combined with B7-DC-Ig significantly enhanced anti-tumor responses. Accordingly, we tested whether B7-DC-Ig, a PD-1 binding molecule, can enhance immune response to vaccine when combined with Treg inhibition by CPM. To evaluate the immunologic efficacy of B7-DC-Ig in combination with vaccine and CPM, we assessed the levels of Ag-specific IFN-γ-producing cells and direct killing of tumor cells by splenocytes from treated tumor-bearing mice. We used an s.c. TC-1 tumor model based on E7-expressing lung epithelial cells, and CTL epitope from HPV16 E7 Ag was used as a model vaccine. We chose a delayed treatment schedule and implanted a high number of tumor cells to minimize the effect of vaccine alone. Mice were injected with TC-1 cells on day 0, then on day 7 with CPM or PBS, and then vaccine or PBS and B7-DC-Ig or formulation buffer on days 8 and 15. For immune response analysis, mice were sacrificed 6 d after the second treatment, and IFN-γ production was analyzed using a standard ELISPOT assay. Addition of either CPM or B7-DC-Ig alone did not affect the levels of vaccine-induced IFN-γ-producing cells, but the combination of vaccine with both CPM and B7-DC-Ig led to a significant increase of IFN-γ-producing cells compared with all other groups (p < 0.001) (Fig. 1A). Similarly, when we analyzed the direct killing of target TC-1 cells by freshly isolated splenocytes from treated mice, we found that the vaccine–B7-DC-Ig–CPM combination significantly increased the percentage of activated caspase-3–positive cells (apoptotic cells) at E:T ratios of 50:1, 25:1, and 1:10 (p < 0.001 compared with E7, E7–B7-DC-Ig, and p < 0.05 compared with B7-DC-Ig–CPM combination) (Fig. 1B).

B7-DC-Ig and CPM synergize with vaccine to promote tumor rejection in a CD8-dependent manner

To test whether the enhanced immune response shown earlier can lead to an anti-tumor effect, we tested various combination treatments on tumor growth in the same model. As earlier, mice were implanted with 50,000 TC-1 cells s.c. on day 0, and on day 7 mice were treated with a single low dose of CPM or PBS. On days 8, 15, and 22 after tumor implantation, mice were injected with vaccine (or PBS) and/or B7-DC-Ig (or formulation buffer) (Fig. 2A). While vaccine, CPM, vaccine–CPM, vaccine–B7-DC-Ig, and CPM–B7-DC-Ig treatments resulted in different levels of tumor growth inhibition, none led to a significant anti-tumor response or to a complete tumor regression (Fig. 2B). Only the combination of B7-DC-Ig with vaccine and CPM led to complete tumor regression on average in 53.3 ± 11.5% of treated mice (Fig. 2B). Also, this combination led to a significant prolongation in survival (Fig. 2C), compared with all other groups. These experiments reveal that combination of B7-DC-Ig with low dose of CPM and vaccine is a feasible strategy resulting in improved long-term overall survival and complete tumor regression in a significant number of mice and that each of the components of the combinational treatment is required.

To determine the mechanism by which the combination of CPM and B7-DC-Ig with vaccine significantly enhances anti-tumor specific immune responses, we tested the role played by different T cell subsets in tumor protection. Mice were injected with tumor cells and then treated with vaccine–B7-DC-Ig–CPM as described earlier. Animals were depleted of either CD4 T cells or CD8 T cells using anti-CD4 or anti-CD8 mAb. As expected, given that E749–57 is MHC class I restricted, CD8 but not CD4 T cell depletion completely negated the anti-tumor effect of treatment, resulting in survival rates comparable with those of nontreated animals (Fig. 2D).

B7-DC-Ig does not block PD-L1–PD-1 interaction

One known mechanism of action for anti–PD-1 molecules is the ability to block inhibitory PD-L1–PD-1 interaction. We previously demonstrated that anti–PD-1 Ab, CT-011, which we used in combination with vaccine and CPM, is able to block PD-L1–PD-1 interaction leading to significant increase of immune responses and therapeutic efficacy of treatment (26). Accordingly, we tested the ability of B7-DC-Ig to block the interaction between PD-1 on activated CD4+CD25− (Tconv) or CD8+ T cells and PD-L1 on TC-1 tumor cells (26) in an immune suppression assay. Whereas coinubcation of Tconv cells with TC-1 tumor cells almost com-

FIGURE 1. The combination of B7-DC-Ig, CPM, and vaccine induces significant Ag-specific immune responses. C57BL/6 mice (n = 5) were injected s.c. in the right flank with 5 × 10⁶ TC-1 cells. On day 7, CPM or PBS was injected. On days 8 and 15, mice were injected with vaccine and B7-DC-Ig, as well as proper combinatorial controls. On day 21, mice were sacrificed, and spleens were harvested and processed for total lymphocytes. (A) IFN-γ activity in the presence of E749–57 peptide versus irrelevant peptide control was assayed by ELISPOT. Values are presented as number of spots from E749–57 restimulated culture minus irrelevant Ag restimulated culture per million splenocytes ± SD. (B) Freshly isolated lymphocytes were coincubated with TC-1 cells for 3 h at E:T ratios of 50:1, 25:1, and 10:1 ± SD. Percentage of caspase-3–positive TC-1 cells after coincubation was measured by flow cytometry. Data shown are representative of two independent experiments. *p < 0.05, **p < 0.001.
pletely suppressed the proliferation of stimulated T cells, addition of either CT-011 or blocking anti–PD-1 Ab (BD Biosciences) partially rescued tumor-mediated suppression of T cell proliferation (proliferation was significantly increased in the presence of both Abs compared with irrelevant IgG). In contrast, B7-DC-Ig was unable to rescue the proliferation of stimulated Tconv cells.
experiments and representative histograms are shown (Fig. 3). Similarly, tumor-mediated inhibition of CD8 T cells was not affected in presence of B7-DC-Ig (data not shown).

Thus, our data indicate that in contrast to CT-011 Ab, the mechanism of action for B7-DC-Ig is not a direct blocking of the PD-L1–PD-1 interaction.

**B7-DC-Ig decreases PD-1high–expressing CD8+ T cells within the tumors**

To determine further the mechanism by which combination of B7-DC-Ig with vaccine exerts its effect, we tested the effect of treatment on tumor-infiltrated CD8 T cells. Tumor-infiltrated CD8 T cells were tested on day 21 after tumor implantation in mice treated as described earlier. Despite the fact that the combination of B7-DC-Ig in combination with vaccine and CPM elicited a significantly stronger effect on tumor growth and survival compared with the vaccine–CPM combination and that this effect is CD8 T cell dependent, surprisingly we found that addition of B7-DC-Ig to vaccine–CPM did not increase CD8 T cell tumor infiltration on day 21 after tumor implantation (Fig. 4A). To understand further this surprising finding, we assessed whether B7-DC-Ig affects CD8 T cells differentially based on the level of PD-1 expression. It is known that T cells expressing high levels of PD-1 are functionally exhausted and highly susceptible to cell death (41–43). Notably, when we compared the percentage of tumor-infiltrated PD-1high CD8 T cells among the different treatment groups, we found that mice treated with B7-DC-Ig (groups vaccine–B7-DC-Ig and vaccine–B7-DC-Ig–CPM) had more than a 2-fold lower percentage of PD-1high CD8 T cells compared with groups that did not receive the B7-DC-Ig (Fig. 4B). Thus, when we plotted the numbers of tumor-infiltrated CD8+PD-1low T cells, we found that vaccine–B7-DC-Ig–CPM induced significantly higher levels of nonexhausted CD8+PD-1low T cells compared with other treatments (p < 0.05) (Fig. 4C). To confirm that in vivo treatment with B7-DC-Ig does not affect the staining with PD-1 Ab, in a separate experiment we coincubated PD-1–expressing 48-h-stimulated CD4 T cells with B7-DC-Ig or anti–PD-1 Ab prior to staining with PE-labeled anti–PD-1 Ab (BD Biosciences). We showed that whereas preincubation with anti–PD-1 Ab at a 1:1 ratio almost completely blocks the PE staining, even at 25-fold higher level of B7-DC-Ig to PE–anti–PD-1 there was no interference with staining (Fig. 4D).

Thus, we demonstrate that in the combinational treatment, CPM is responsible for the increase of CD8 T cell infiltration into the tumor microenvironment, and although addition of B7-DC-Ig to vaccine–CPM treatment does not alter total number of tumor-infiltrated CD8 T cells, addition of B7-DC-Ig to vaccine–CPM leads to a significantly higher number of nonexhausted functional CD8 T cells.

**B7-DC-Ig decreases tumor-infiltrated Foxp3+CD4+ Tregs and Foxp3+ CD4+ T cells**

To explore further the immune mechanisms by which B7-DC-Ig enhances the effect of vaccine, we tested the effect of the combination on CD4+ T cells. Additionally, we have shown that the combination of CPM treatment with CT-011 anti–PD-1 Ab resulted in maintaining the low level of tumor-infiltrated Tregs (26), B7-DC-Ig–treated mice, either alone or in combination with vaccine, CPM, or both, had significantly lower numbers of tumor-infiltrated Tregs compared with other groups on day 21 after tumor implantation (Fig. 5A). Furthermore, we found that addition of B7-DC-Ig also led to a significant decrease of CPM-induced tumor-infiltrated CD4+Foxp3+ T cells (Fig. 5B).

We previously demonstrated that CPM led to a short-term decrease of splenic Tregs, with the nadir at day 4 after the administration of CPM and returning to posttreatment levels on day 10. Additionally, we have shown that the combination of CPM treatment with CT-011 anti–PD-1 Ab resulted in maintaining the low level of splenic Tregs (26). In this study, we show that treatment of tumor-bearing mice with B7-DC-Ig alone does not affect the level of splenic Tregs. Furthermore, addition of B7-DC-Ig to CPM does not affect the dynamics of CPM-mediated changes in splenic Treg levels, which results in no significant differences between all these groups at day 21 after tumor implantation (Fig. 5C).

![FIGURE 3](http://www.jimmunol.org/DownloadedFrom/B7-DC-Ig%20ENHANCES%20VACCINE%20EFFECT%20BY%20A%20NOVEL%20MECHANISM.png)
Thus, we demonstrate that although CPM is required to decrease the level of peripheral Tregs at early stages and to allow an effective Ag-specific immune response to develop, B7-DC-Ig is the component responsible for the long-term decrease of tumor-infiltrated Tregs without affecting the level of peripheral Tregs.

**B7-DC-Ig inhibits the proliferation of Tregs and Tconv cells but not CD8 T cells in vitro**

To determine the mechanism by which B7-DC-Ig led to a decrease of Tregs in the tumor microenvironment, we tested the direct effect of B7-DC-Ig on different T cell subsets in vitro. We analyzed the proliferation of purified CFSE-labeled CD4+CD25+ Tregs that were stimulated with anti-CD3/anti-CD28/IL-2 alone or in the presence of different concentrations of B7-DC-Ig or irrelevant IgG. We found that B7-DC-Ig significantly inhibits the proliferation of Tregs at 20 and 40 μg/ml concentrations (Fig. 6A). Surprisingly, when we tested the effect of B7-DC-Ig on the proliferation of the other T cell subsets (Tconv and CD8), we found that B7-DC-Ig also inhibits proliferation of Tconv (Fig. 6B) but does not affect the proliferation of CD8 T cells (Fig. 6C).

Thus we demonstrate that B7-DC-Ig differentially affects T cell subsets in vitro.

Upon stimulation, different T cell subsets express different levels of PD-1 molecules that define the “effect threshold” for B7-DC-Ig

Because B7-DC-Ig is a PD-1 binding agent and it differentially suppresses the proliferation of different T cell subsets, we wanted to test whether difference in PD-1 level might be responsible for this effect. Accordingly, we investigated the expression levels of the PD-1 receptor on these T cell subsets after in vitro stimulation by analyzing the actual number of PD-1 receptors on the surface of Tregs, Tconv cells, and CD8 T cells at different time points after stimulation (0, 24, 48, and 72 h).

We showed for the first time to our knowledge that upon stimulation with anti-CD3/anti-CD28/IL-2, both Tregs and Tconv cells express similar numbers of PD-1 receptors at all time points, reaching a maximum of $>10^5$ receptors per cell after 48 h of stimulation (Fig. 7A). We found that at 24 and 48 h poststimulation, CD8 T cells express significantly lower levels of PD-1 compared with both Tregs and Tconv cells. At the 72-h time point, the number of PD-1 receptors on CD8 T cells reaches a level similar to Tregs and Tconv cells (below $8 \times 10^4$ receptors/cell) (Fig. 7A).
Next, we investigated the effect of B7-DC-Ig on Tregs, Tconv cells, and CD8 T cell activation at various time points by analyzing the level of p-S6. We did not observe a significant change in the levels of p-S6 in nonstimulated cells or at 24 or 72 h poststimulation. However, at 48 h poststimulation, when the levels of PD-1 were maximal for Tregs and Tconv cells, we found that B7-DC-Ig significantly decreased the levels of p-S6 in Tregs and Tconv cells but not in CD8 T cells (Fig. 7B, 7C). We also confirmed the effect of B7-DC-Ig on different T cell subsets using a p-S6 sandwich ELISA method. Similar to phospho-flow cytometry data, the level of p-S6 after 48 h of activation was significantly decreased in Tconv cells and Tregs but not CD8 T cell lysates (Fig. 7D). Taken together, the data shown above suggest that there is a specific “effect threshold” that is roughly above 8 × 10^4 of PD-1 molecules for the B7-DC-Ig molecule to exhibit its effect on T cells.

Thus, we demonstrate that different T cell subsets express different levels of surface PD-1 molecules and that this parameter is important and needs to be considered when designing PD-1-targeting immune therapies.

**Discussion**

Upregulation of the PD-1 pathway has been implicated as one of the key mechanisms of tumor immune suppression (19–21). Recently, we demonstrated that the combination of anti–PD-1 Ab CT-011, which blocks the PD-L1–PD-1 interaction, with low-dose CPM and vaccine significantly enhanced Ag-specific immune responses, decreased tumor burden, and increased survival of treated mice (26). Furthermore, the combination of CPM and B7-DC-Ig has also been shown to promote anti-tumor responses (S.A. Marshall, L. Liu, and S. Langerman, submitted for publication).

In this study, we tested the effects of B7-DC-Ig, a molecule that binds and targets PD-1, on the immune efficacy of tumor vaccine and defined the underlying mechanisms responsible for these effects. We showed that B7-DC-Ig fusion protein synergizes with Treg suppression by a single, low dose of CPM, leading to an enhanced therapeutic outcome of cancer vaccine. Combination of CPM and B7-DC-Ig with vaccine inhibited tumor growth and led to complete tumor regression in greater than 50% of mice, with significant prolongation of survival. When we explored the underlying immunologic mechanisms of the treatment, we found that the vaccine–B7-DC-Ig–CPM combination induced the highest number of Ag-specific IFN-γ-producing cells compared with other groups. Similar results were observed when we analyzed direct killing of E7-expressing TC-1 tumor cells. These data indicate that CPM and B7-DC-Ig synergize to enhance vaccine-mediated Ag-specific immune responses. However, we notably found that the mechanism by which B7-DC-Ig exerts its effect is unique and is different from anti–PD-1 blocking molecules.

As others and we have previously reported, one known mechanism by which anti–PD-1 molecules enhance vaccine effect is through their ability to block suppressive PD-L1–PD-1 interaction (26, 27). In the current study, however, we found that unlike blocking anti–PD-1 Ab, B7-DC-Ig is unable to block the PD-L1–PD-1 interaction. To understand the role of B7-DC-Ig in contributing to the effective outcome of the treatment that is leading to enhancement of Ag-specific immune responses, we analyzed tumor-infiltrated T cell repertoire.

We found that the combination of vaccine and CPM with or without B7-DC-Ig led to an increase of CD8+ tumor-infiltrating T cells. Although there were no significant differences in total numbers of these tumor-infiltrated CD8+ T cells between the two groups, analysis of nonexhausted functional CD8+ T cells (PD-1low) revealed that the addition of B7-DC-Ig led to a significant increase in PD-1lowCD8+ T cells within the tumors. Although the effect of B7-DC-Ig on Ag-specific versus nonspecific CD8 T cells remains to be determined, we believe that this effect is solely mediated by B7-DC-Ig, as CD8+ T cells expressing high levels of PD-1 were decreased in all groups treated with B7-DC-Ig.
we concluded that CPM is required to increase the total number of CD8+ T cell infiltration into the tumor, as it decreases the level of Tregs at early phase, allowing a stronger immune response to develop (34), and B7-DC-Ig is responsible for keeping those cells functional through the reduction of exhausted PD-1high CD8+ T cells.

We and others have previously shown that the low-dose CPM selectively ablates Tregs, with the nadir at day 4 and recovery to pretreatment levels by day 10 (26, 34). Accordingly, a decrease in tumor-infiltrated Tregs on day 21 after tumor implantation is not expected to be CPM mediated. We have previously shown, however, that whereas PD-1 blocking Ab does not affect the level of peripheral Tregs, its combination with CPM leads to prolonged suppression of peripheral Tregs resulting in a decrease of tumor-infiltrating Tregs (26). Surprisingly, however, in this study we found that in all groups where mice received B7-DC-Ig, regardless of vaccine or CPM, there was a significant decrease of tumor-infiltrated Tregs compared with groups that had no B7-DC-Ig. Moreover, the addition of B7-DC-Ig also significantly decreased vaccine–CPM–induced CD4+Foxp3+ T cell infiltration into the tumor.

Further investigation of the mechanism of action revealed that B7-DC-Ig significantly inhibits the proliferation of Tregs and Tconv cells but does not affect the proliferation of CD8 T cells in vitro. Analysis of the expression levels of PD-1 receptors on T cells at different stimulation stages showed a strong correlation...
between the number of PD-1 receptors on the cell surface and the effect of B7-DC-Ig. More specifically, in addition to showing for the first time to our knowledge the difference in PD-1 expression levels on three stimulated T cell subsets (Treg, Tconv, and CD8) at different time points, we found that there is a specific "effect threshold" for B7-DC-Ig molecules.

In conclusion, besides presenting a promising anti-tumor immunotherapeutic approach, we also demonstrate that the level of PD-1 expression on different T cell subsets determines the "effect zone" for anti-PD-1 molecules. We believe that this represents an important parameter that needs to be considered to alter favorably the tumor microenvironment when designing PD-1-targeting immunotherapies.

Acknowledgments
We thank Katherine McKinnon from the National Cancer Institute FACS core facility for technical assistance with flow cytometry assays.

Disclosures
L.L. and S.L. are employees of Amplimmune, Inc., which provided B7-DC-Ig and input on study design. The other authors have no financial conflicts of interest.

References

