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B7-DC-Ig enhances vaccine effect by a novel mechanism dependent on PD-1 expression level on T cell subsets

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Abstract

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 IFNγ 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 extracellular domain of murine B7-DC, fused to Fc portion of murine IgG2a. Here we demonstrate that B7-DC-Ig can enhance therapeutic efficacy of vaccine when combined with cyclophosphamide (CPM). We show that this combination significantly enhances antigen-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 than direct blocking of the PD-L1/PD-1 interaction. Here, 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-1^{high} CD4 T cells, leading to significant decrease in the level of these cells, which are enriched for Tregs, within the tumor. In addition, it also leads to the decrease in PD-1^{high} CD8 T cells, tipping the balance towards non-exhausted functional PD-1^{low} 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.

Keywords

PD-1 targeting; vaccine; combinational immunotherapy

Introduction

A significant barrier to tumor regression in the setting of a successful immune response is tumor-mediated immunosuppression. Programmed death receptor-1 (PD-1) is an important

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signaling molecule and in the context of the tumor microenvironment may inhibit T cell immunity when bound to its ligands by inducing T cell apoptosis and anergy (1). PD-1 has two known ligands, PD-L1 and PD-L2, both members of the B7 family, and while they share a high degree of structural and genetic homology, they have been found to have unique functional properties (2–5). PD-1 is expressed on the surface of activated T cells and B cells, in addition to activated myeloid cells (6). Its cognate ligand, PD-L1, is expressed on activated T cells, B cells, dendritic cells and macrophages, in addition to a wide array of non-hematopoietic cells (7). Several human malignancies upregulate PD-L1, and this has been shown to correlate inversely with survival in many types of cancer (8–13). The expression of PD-L2, was believed to be much more restricted, and limited to the surface of activated dendritic cells and macrophages (14), although recently its expression was shown in activated human T cells and various tumor cells (15, 16).

It has been shown that when PD-L1 binds PD-1, T cell receptor (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 monoclonal antibodies, it has been shown that tumor eradication can be enhanced by PD-L1/PD-1 blockade (22–27).

T regulatory (Treg) cells 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 Treg cells has been actively pursued, in order to enhance antitumor immunotherapies. Recently we demonstrated that the combination of CT-011 anti-PD-1 antibody with vaccine and low-dose CPM, which reduces the number and function of Treg cells (33–38), significantly enhances antigen-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.

Here 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. Interestingly, we found that B7-DC-Ig, that composed of the extracellular domain of murine B7-DC, fused to the Fc portion of murine IgG2a, is unable to block PD-L1/PD-1 interaction, but instead decreases tumor-infiltrated Treg cells and reduces the percentage of tumor-infiltrating exhausted $PD\text{-}1^{high}$ 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 weeks old 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 co-transfection of human papillomavirus strain 16 (HPV16) early proteins 6 and 7 (E6 and E7) and activated ras oncogene to primary C57BL/6 mouse lung epithelial cells were obtained from ATCC (Manassas, VA), and cells were grown in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin (100 U/ml each) and L-glutamine (2 mM) at 37°C with 5% CO_2. HPV16 $E7_{49–57}$, a 9-mer peptide (RAHYNIVTF) was purchased from Celtek Bioscience (Nashville, TN). $E7_{49–57}$ was used as a model vaccine (100 μg/mouse), along with anti-CD40 (20 μg/mouse, BioLegend, San Diego, CA), GM-CSF (5 μg/mouse, Peprotech, Rocky Hill, NJ), and IFA (50 μg/mouse, Sigma, St. Louis, MO) in all studies (subcutaneous (s.c.) injection). GM-CSF and anti-CD40 have been previously shown to synergize with peptide vaccine (39). Cyclophosphamide (CPM) was obtained from Baxter Healthcare Corporation (Deerfield, IL) and injected intraperitoneally (i.p.) at a dose of 1 mg/mouse. B7-DC-Ig recombinant PD-L2 protein fused with the Fc portion of mouse IgG2a was obtained from Amplimmune Inc. (Gaithersburg, MD) and injected i.p. at 300 μg/mouse. Anti-CD4 (clone GK1.5) and anti-CD8a (clone 53.6.72) monoclonal antibodies (mAb) used for *in vivo* assays were purchased from BioXCell (West Libanon, 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 antibodies and appropriate isotype controls used for flow cytometry were purchased from BD Biosciences (San Jose, CA) or eBiosciences (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, while 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 formulation buffer (Amplimmune Inc) i.p. Mice were treated with vaccine and B7-DC-Ig a total of three times (on days 8, 15 and 22 after tumor implantation). Tumors were measured every 3–4 days using digital calipers, and tumor volume was calculated using the formula $V= (W^2xL)/2$, whereby V is volume, L 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 six days after the second treatment, on day 21. Spleens and tumors were isolated and analyzed for antigen-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 antigen-specific cellular immune responses

ELISPOT was used to detect IFNγ production in E7-restimulated (10 μg/ml) splenocyte cultures from vaccinated and control mice, as suggested by the manufacturer (BD Biosciences, San Jose, CA). A CTL Immunospot Analyzer (Cellular Technology Ltd., Shaker Heights, OH) was used to analyze spots. The number of spots from irrelevant peptide (hgp $100_{25–33}$-KVPRNQDWL-Celtek Bioscience, Nashville, TN) re-stimulated 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 effector: target (E:T) ratios of 50:1, 25:1 and 10:1. After a 3 hour co-incubation, the E:T mixtures 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, San Jose, CA).

Detection of tumor-infiltrated T cells

In experiments with immunologic endpoints, tumor tissue was harvested 6 days 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 $CD8^+$,$CD8^+PD\text{-}1^{high}$, $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells were analyzed using the previously described flow cytometry assay and the following fluorochrome antibodies: anti-mouse CD8, anti-mouse CD4, anti-mouse PD-1 (BD Biosciences, San Jose, CA) and anti-mouse Foxp3 (eBioscoences, San Diego, CA) mAb and appropriate isotype control Ab (BD Biosciences, San Jose, CA). The level of $CD4^+FoxP3^+$ cells (Treg cells) was also evaluated in spleens 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 antibody, purified and 48h stimulated CD4 T cells were pre-incubated with B7-DC-Ig, anti-PD-1 antibody (BD Biosciences) or irrelevant IgG prior to staining with PE-labeled antibody at different ratios. Samples were analyzed using FACSCalibur 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 carboxyfluorescein diacetate, succinimidyl ester (CFSE)-based suppression assay. The $CD4^+CD25^-$ T (Tconv) cells were purified from the spleens of naïve mice using the Miltenyi Biotec MACS T cell purification kit as suggested by the manufacturer (Miltenyi Biotec, Auburn, CA). 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 co-incubated with TC-1 cells at a 1:2 ratio for four days, alone or in the presence of 20 μg/ml concentrations of B7-DC-Ig, CT-011 anti-PD-1 antibody (Curetech Ltd, Israel), anti-PD-1 mAb (BD Biosciences) or irrelevant IgG. Samples were washed and evaluated for T cell expansion via CFSE dye dilution using a FACScan Flow Cytometer and CellQuest software (BD Biosciences).

The same CFSE assay was used in experiments investigating the B7-DC-Ig effect on proliferation of Treg, Tconv and CD8 T cells. All three subsets were purified from the spleens of naïve mice using the appropriate Miltenyi Biotec MACS purification kit as suggested by the manufacturer. After CFSE-labeling and stimulation, cells were cultured for three days 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 FACSCalibur 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 non-stimulated Treg, Tconv and CD8 T cells, as well as after 24, 48 and 72 hours 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 naïve mice, stained with APC-labeled 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, Fishers, IN). Mean Fluorescence Intensity (MFI) in the FL-2 channel (PE) was calculated using a FACSCalibur 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 pS6

Non-stimulated Treg, Tconv and CD8 T cells, as well as cells stimulated for 24, 48 and 72 hours were incubated at 37°C for 10 min in the presence of 20 μg/mL of B7-DC-Ig or irrelevant IgG. Cells were stained with APC-CD4 or APC-CD8 mAb, fixed and permeabilized using cytofix/cytoperm solution (BD Biosciences San Jose, CA) and stained with FITC-FoxP3 and anti-phospho S6 antibody (Cell Signaling Technology, Danvers, MA), followed by anti-rabbit biotin-conjugated secondary antibody and streptavidin-PE. MFI in the FL-2 channel (PE) was calculated using FACSCalibur Flow Cytometer and CellQuest software (BD Biosciences). In addition, pS6 levels were analyzed in lyzates of Treg, Tconv and CD8 T cells stimulated for 48h and treated with B7-DC-Ig or irrelevant IgG as described above using pS6 ELISA kit per manufacturer’s suggestions (Cell Signaling Technology, Danvers, MA).

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 with Tukey's multiple comparison post-test ($P<0.05$ was considered statistically significant).

Results

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

We have previously demonstrated that inhibition of Tregs with CPM combined with an anti-PD-1 antibody significantly enhances the antitumor effect of vaccine (26). In addition, Marshall et al. also demonstrated that inhibition of Tregs with CPM combined with B7-DC-Ig significantly enhanced anti-tumor responses (Marshall SA et al. submitted 2011). 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 antigen-specific IFNγ-producing cells and direct killing of tumor cells by splenocytes from treated tumor-bearing mice. We used a subcutaneous TC-1 tumor model based on E7-expressing lung epithelial cells and CTL epitope from HPV16 E7 antigen was used as a model vaccine. We chose a delayed treatment schedule and implanted a high number of tumor cells in order to minimize the effect of vaccine alone. Mice were injected with TC-1 cells on day 0, and on day 7 with CPM or PBS, then vaccine or PBS and B7-DC-Ig or formulation buffer on days 8 and 15. For immune response analysis, mice were sacrificed six days after the second treatment and IFNγ 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 IFNγ-producing cells, but combining vaccine with both CPM and B7-DC-Ig led to a significant increase of IFNγ-producing cells, compared to all other groups ($P<0.001$) (Figure 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 effector:target ratios of 50:1, 25:1 and 1:10 ($P<0.001$ compared to E7, E7/B7-DC-Ig, and $P<0.05$ compared to 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 above can lead to anti-tumor effect, we tested various combination treatments on tumor growth in the same model. As above, mice were implanted with 50,000 TC-1 cells subcutaneously 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) (Figure 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 (Figure 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 (Figure 2B). Also, this combination led to a significant prolongation in survival (Figure 2C), compared to 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 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 above. Animals were depleted of either CD4 T cells or CD8 T cells using anti-CD4 or anti-CD8 monoclonal antibody. As expected, given that $E7_{49–57}$ 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 to non-treated animals (Figure 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 antibody, CT-011 that 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 $CD4^+FoxP3^-$ (Tconv) or $CD8^+$ T cells, and PD-L1 on TC-1 tumor cells (26) in an immune suppression assay. While co-incubation of Tconv with TC-1 tumor cells almost completely suppressed the proliferation of stimulated T cells, addition of either CT-011 or blocking anti-PD-1 antibody (BD Biosciences) partially rescued tumor-mediated suppression of T cell proliferation (proliferation was significantly increased in the presence of both antibodies compared to irrelevant IgG). In contrast, B7-DC-Ig was unable to rescue the proliferation of stimulated Tconv (Figure 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 antibody the mechanism of action for B7-DC-Ig is not a direct blocking of PD-L1/PD-1 interaction.

B7-DC-Ig decreases PD-1^{high} expressing $CD8^+$T cells within the tumors

To further determine the mechanism by which combining 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 post tumor implantation in mice treated as described above. 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 to 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 (Figure 4A). To further understand this surprising finding, we assessed whether B7-DC-Ig impacts 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). Interestingly, when we compared the percent of tumor-infiltrated PD-1^{high} CD8 T cells amongst 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 two-fold lower percent of PD-1^{high} CD8 T cells compared to groups that did not receive the B7-DC-Ig (Figure 4B). Thus, when we plotted the numbers of tumor-infiltrated CD8^{+}PD-1^{low} T cells, we found that Vaccine/B7-DC-Ig/CPM induced significantly higher levels of non-exhausted CD8^{+}PD-1^{low}T cells compared to other treatments ($P<0.05$) (Figure 4C). To confirm that in vivo treatment with B7-DC-Ig does not affect the staining with PD-1 antibody, in a separate experiment we co-incubated PD-1 expressing 48h stimulated CD4 T cells with B7-DC-Ig or anti-PD-1 antibody prior to staining with PE-labeled anti-PD-1 antibody (BD Biosciences). We showed that while pre-incubation with anti-PD-1 antibody at 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 (Figure 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 is not altering total number of tumor-infiltrated CD8^{+} T cells, addition of B7-DC-Ig to Vaccine/CPM leads to a significantly higher number of non-exhausted functional CD8^{+} T cells.

B7-DC-Ig decreases tumor-infiltrated FoxP3^{+}CD4^{+} Tregs and FoxP3^{-}CD4^{+} T cells

To further explore the immune mechanisms by which B7-DC-Ig enhances the effect of vaccine we tested the effect of the combination on CD4^{+} T cells. Accordingly, tumor-infiltrated CD4^{+}FoxP3^{+} Treg and CD4^{+}FoxP3^{-} T cells were tested on day 21 after tumor implantation from mice treated as described above. Interestingly, we found that in contrast to CT-011 PD-1 blocking antibody that required the presence of CPM to decrease the level of tumor-infiltrated Treg cells (26), B7-DC-Ig treated mice, either alone or in combination with vaccine, CPM, or both had significantly lower numbers of tumor-infiltrated Treg cells, compared to other groups on day 21 after tumor-implantation (Figure 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 (Figure 5B).

We previously demonstrated that CPM led to a short-term decrease of splenic Tregs, with the nadir at 4 days after the administration of CPM and returning to post-treatment levels on day 10. Additionally, we have shown that the combination of CPM treatment with CT-011 anti-PD-1 antibody resulted in maintaining the low level of splenic Tregs (26). Here 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 (Figure 5C).

Thus, we demonstrate that although CPM is required to decrease the level of peripheral Tregs at early stages and to allow an effective antigen-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 Treg and Tconv, 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 simulated 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 (Figure 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 (Figure 6B) but does not affect the proliferation of CD8 T cells (Figure 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

Since B7-DC-Ig is a PD-1 binding agent and it is differentially suppressing 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 Treg, Tconv and CD8 T cells at different time points after stimulation (0h, 24h, 48h and 72h).

We showed for the first time, that upon stimulation with anti-CD3/anti-CD28/IL-2, both Treg and Tconv cells express similar numbers of PD-1 receptors at all time-points, reaching a maximum of over 10^5 receptors per cell after 48 hours of stimulation (Figure 7A). We found that at 24 and 48 hours post stimulation, CD8 T cells express significantly lower levels of PD-1 compared to both Treg and Tconv cells. At the 72 hour time-point, the number of PD-1 receptors on CD8 T cells reaches a level similar to Treg and Tconv (below 8×10^4 receptors/cell) (Figure 7A).

Next, we investigated the effect of B7-DC-Ig on Treg, Tconv and CD8 T cell activation at various time-points, by analyzing the level of phosphorylated S6 (pS6). We didn’t observe a significant change in the levels of pS6 in non-stimulated cells, at 24 hours, or at 72 hours post-stimulation. However, at 48 hours post-stimulation, when the levels of PD-1 were maximal for Treg and Tconv, we found that B7-DC-Ig significantly decreased the levels of pS6 in Treg and Tconv, but not in CD8 T cells (Figures 7B and 7C). We also confirmed the effect of B7-DC-Ig on different T cell subsets using a pS6 sandwich ELISA method. Similar to phospho-flow cytometry data, the level of pS6 after 48h of activation was significantly decreased in Tconv and Treg but not CD8 T cell lysates (Figure 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 antibody CT-011 which blocks the PD-L1/PD-1 interaction, with low dose CPM and vaccine significantly enhanced antigen-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 (Marshall SA et al. submitted 2011).

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 underlining mechanisms responsible for these effects. We showed that B7-DC-Ig fusion protein synergizes with Treg suppression by a single, low dose of cyclophosphamide, 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 antigen-specific IFNγ-producing cells compared to 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 antigen-specific immune responses. However, interestingly we 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). Here, however, we found that unlike blocking anti-PD-1 antibody, B7-DC-Ig is unable to block PD-L1/PD-1 interaction. In order to understand the role of B7-DC-Ig in contributing to effective outcome of the treatment that is leading to enhancement of antigen-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 non-exhausted functional $CD8^+$ T cells ($PD\text{-}1^{low}$) revealed that the addition of B7-DC-Ig led to a significant increase in $PD\text{-}1^{low}CD8^+$ T cells within the tumors. While the effect of B7-DC-Ig on antigen specific versus non-specific CD8 T cells remains to be determined, we believe that this effect is solely mediated by B7-DC-Ig, since $CD8^+$T cells expressing high levels of PD-1 were decreased in all groups treated with B7-DC-Ig. Thus we concluded, that CPM is required to increase the total number of $CD8^+$ T cell infiltration into the tumor since 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 $PD\text{-}1^{high}$ $CD8^+$ T cells.

We and others have previously shown that the low dose CPM selectively ablates Treg cells, 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 while PD-1 blocking antibody 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 though, here 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 Treg cells compared to 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 is significantly inhibiting the proliferation of Treg 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 the 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, Tonv 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 which needs to be considered to favorably alter the tumor microenvironment when designing PD-1 targeting immune therapies.

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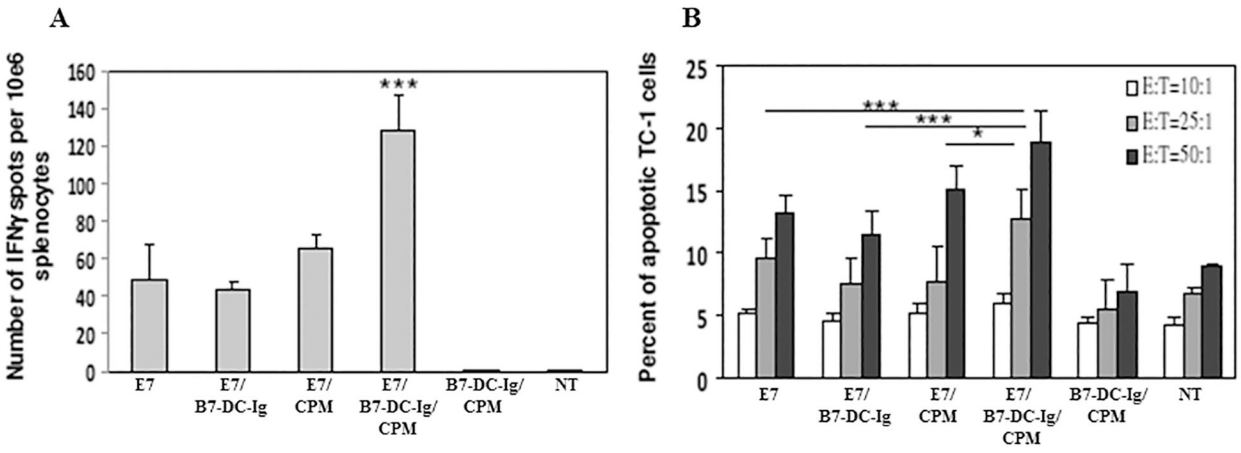

Figure 1.

The combination of B7-DC-Ig, CPM and vaccine induces significant antigen-specific immune responses. C57BL/6 mice (n=5) were injected s.c. in the right flank with 5×10^4 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. IFNγ activity in the presence of $E7_{49–57}$ peptide vs. irrelevant peptide control was assayed by ELISPOT. Values presented as number of spots from $E7_{49–57}$ re-stimulated culture minus irrelevant antigen re-stimulated culture per million splenocytes ± SD (A). Freshly isolated lymphocytes were co-incubated with TC-1 cells for 3h at effector:target ratios of 50:1, 25:1, and 10:1 ± SD. Percentage of caspase-3 positive TC-1 cells after co-incubation was measured by flow cytometry (B). *P<0.05, ***P<0.001. Data shown are representative of two independent experiments.

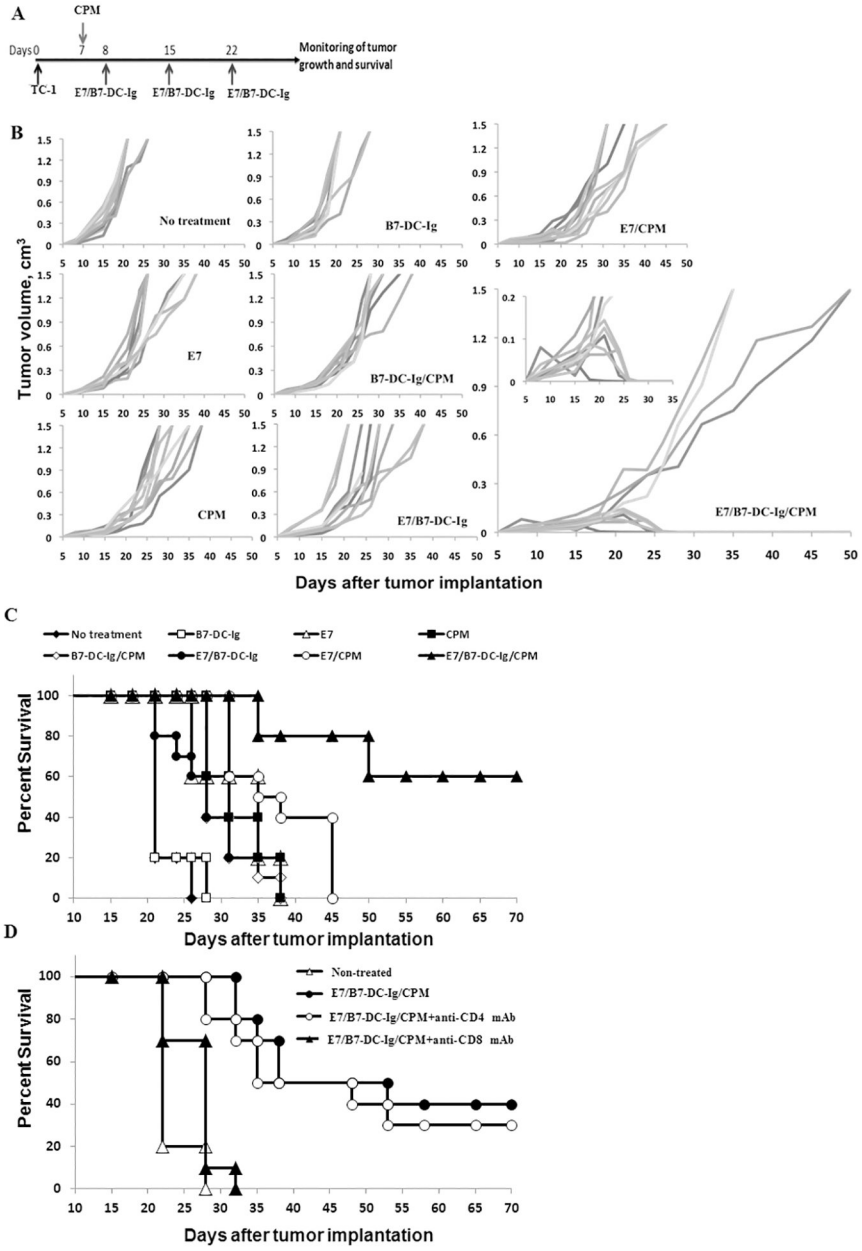

Figure 2.

B7-DC-Ig and CPM synergize with vaccine to promote tumor rejection in a CD8 dependant manner. C57BL/6 mice (n= 10) were injected s.c. in the right flank with 5×10^4 TC-1 cells. On day 7 CPM or PBS was injected. On days 8, 15, and 22 of the therapeutic study mice were injected with vaccine and/or B7-DC-Ig or PBS and formulation buffer (A). Tumor sizes were monitored periodically. Plots show tumor progression of individual mice per group (B). (C) Kaplan-Meier plot depicting overall survival. (D) C57BL/6 mice (n=10) were treated as described in Material and Methods. The Kaplan-Meier plot depicts overall survival after CD8 or CD4 T cell depletion. Similar results were obtained from three independent experiments.

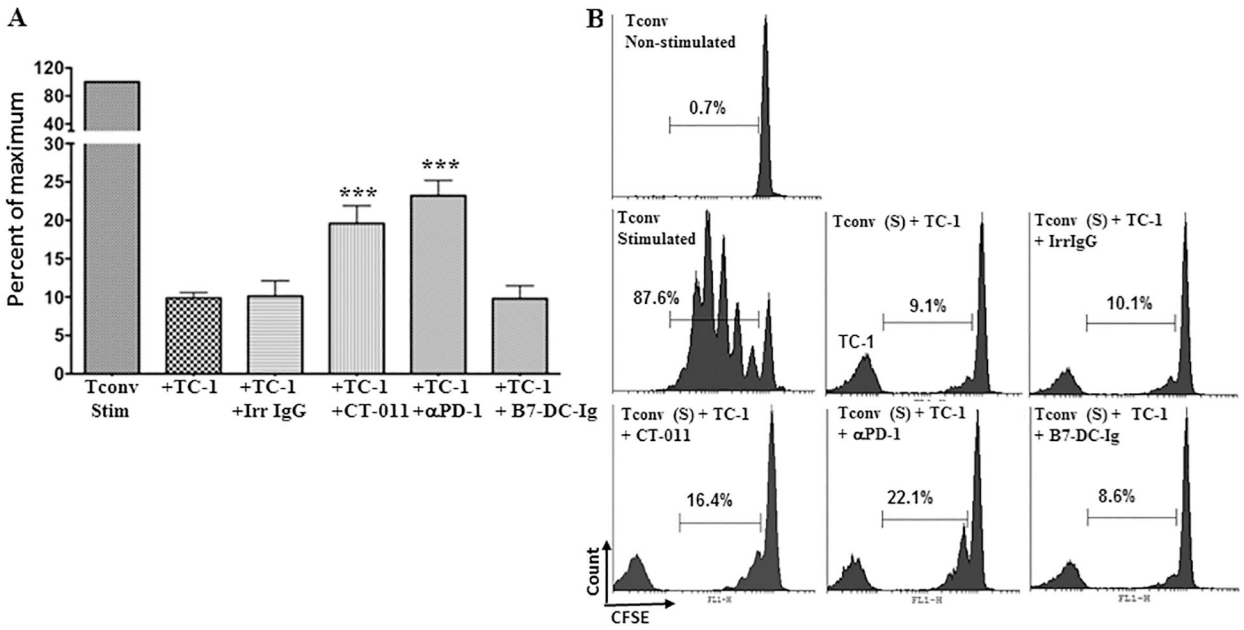

Figure 3.

B7-DC-Ig is unable to block PD-L1/PD-1 interaction. Purified CFSE-labeled $CD4^{+}CD25^{-}$ (Tconv) cells when stimulated either alone or co-cultured with TC-1 cells and in the presence or absence of B7-DC-Ig, CT-011, Irr IgG or anti-PD-1 mAb. Proliferation of Tconv cells was analyzed by CFSE dilution assay. Percents of proliferating T cells co-cultured with TC-1 cells and compounds were standardized over proliferation of stimulated Tconv cells alone. Proliferation of Tconv cells was analyzed by CFSE dilution assay (A). Results presented are the average and SD from three experiments and representative histograms shown (B). ***P<0.001.

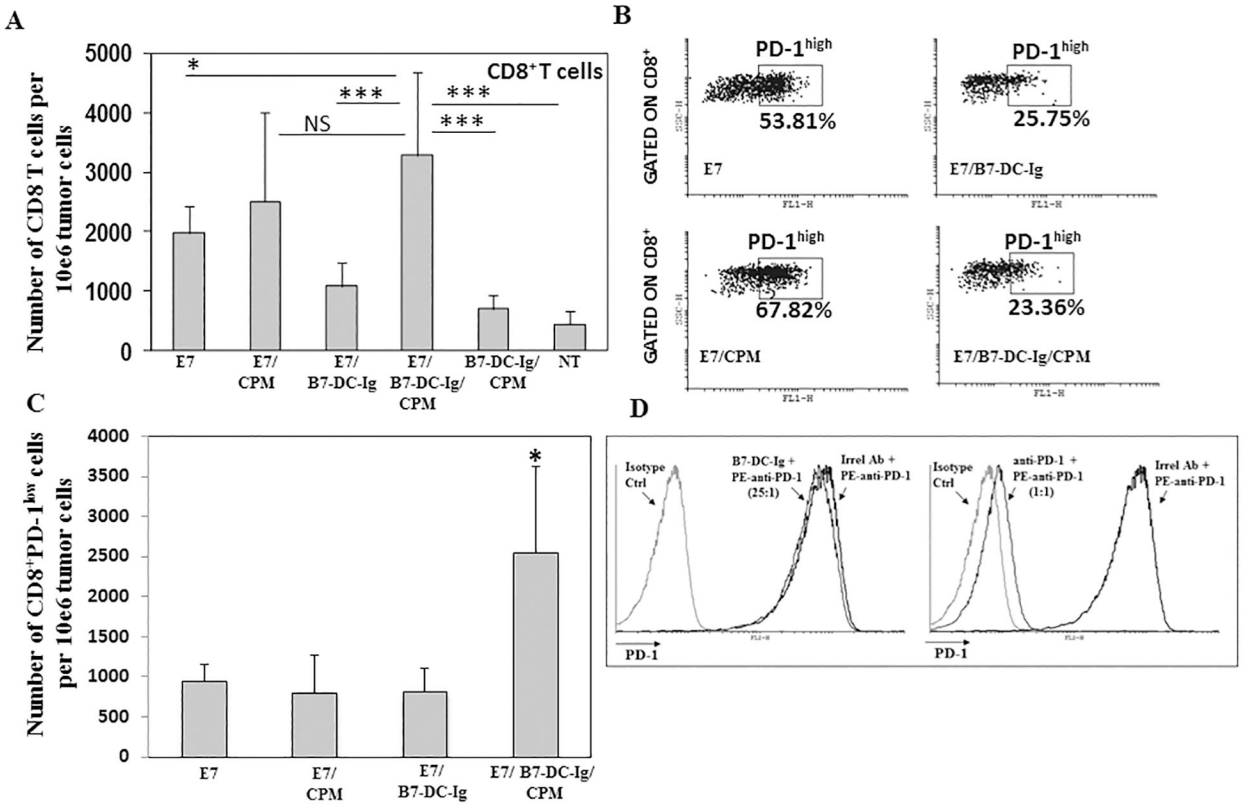

Figure 4.

Addition of B7-DC-Ig to Vaccine/CPM treatment decreases the percent of PD-1^{high} CD8^{+}T cells within the tumors. C57BL/6 mice (n=5) were treated as described in Figure 1. On day 21, mice were sacrificed and tumor-infiltrated CD8 T cells were analyzed in tumor homogenates by flow cytometry. The numbers of infiltrated cells were standardized per 1×10^{6} of total tumor cells and presented as mean values ± SD (A). Representative dot plots of PD-1 expression in CD8 gated population from different groups (B). The numbers of tumor-infiltrated PD-1^{low}CD8^{+}T cells were standardized per 10e6 of total tumor cells ± SD (C). Expression of PD-1 on purified and 48h stimulated CD4 T cells pre-incubated with irrelevant IgG, B7-DC-Ig or anti-PD-1 antibody (D). *P<0.05, **P<0.01, ***P<0.001. Similar results were obtained from two independent experiments.

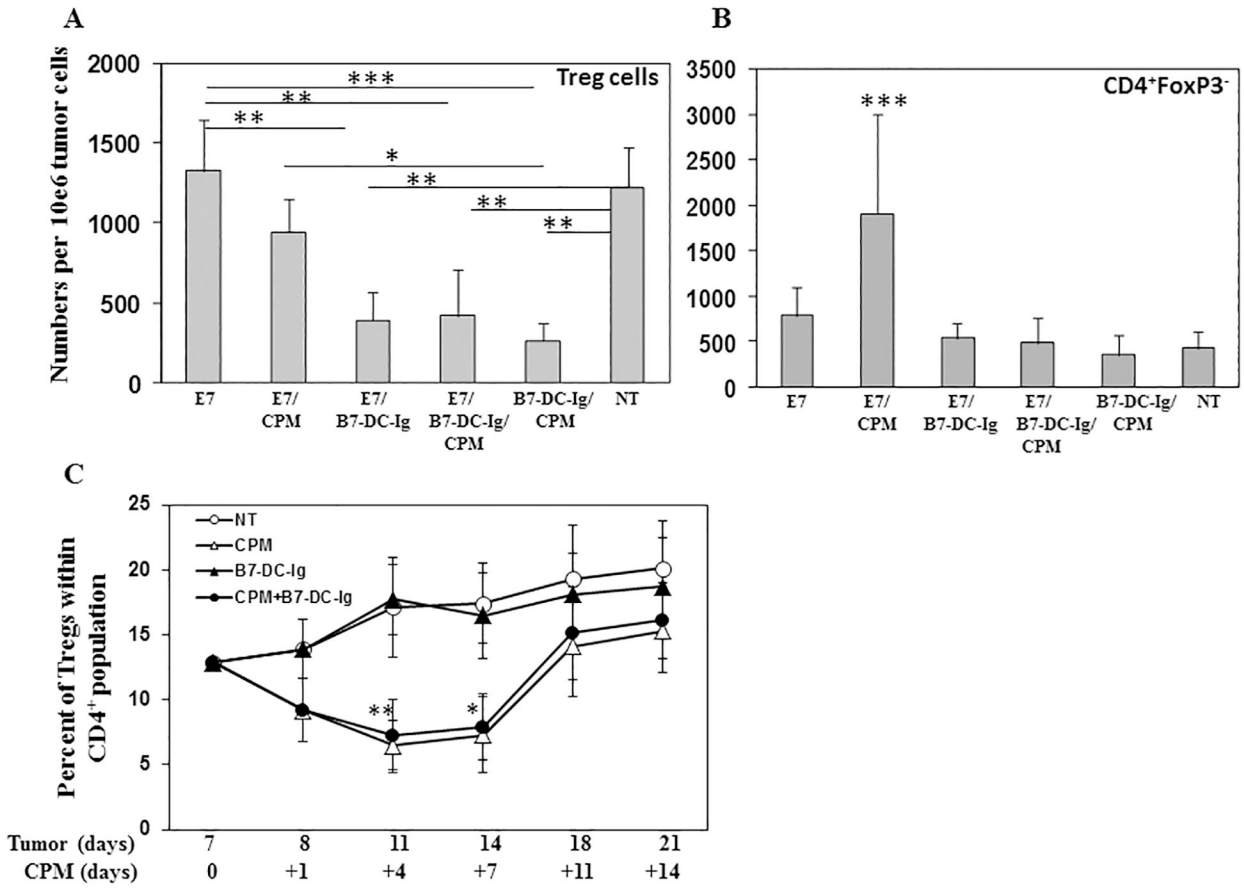

Figure 5.

B7-DC-Ig decreases tumor-infiltrated Tregs and $CD4^+FoxP3^-$ T cells. C57BL/6 mice (n=5) were treated as described in Figure 1. On day 21, mice were sacrificed and tumor-infiltrated $CD4^+FoxP3^+$ Treg (A) and $CD4^+FoxP3^-$ T cells were analyzed in tumor homogenates by flow cytometry. The numbers of infiltrated cells were standardized per 1×10^6 of total tumor cells and presented as mean values ± SD. (C) C57BL/6 mice (n=5) were treated with CPM, B7-DC-Ig or combination of two as described above. Dynamics of splenic Treg level changes presented as the percentage of $CD4^+FoxP3^+$ cells within $CD4^+$ cell population detected at different time-points. *P<0.05, **P<0.01, ***P<0.001. Similar results were obtained from two independent experiments.

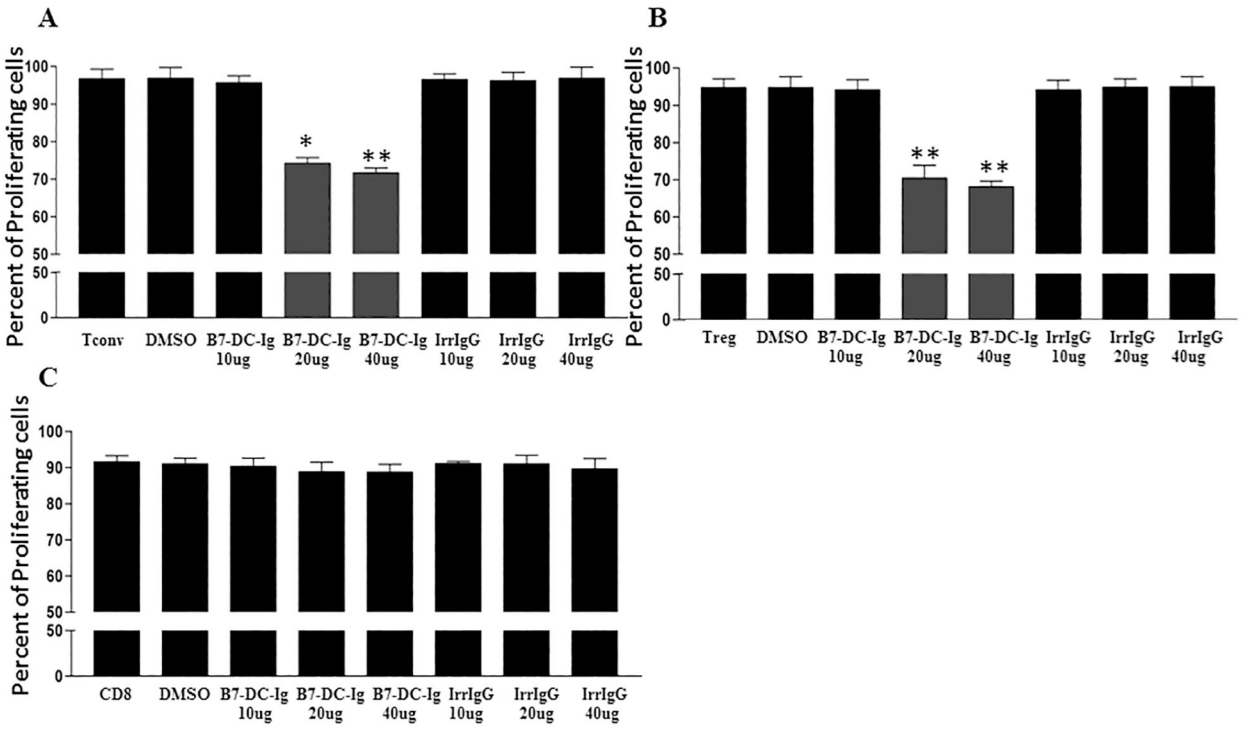

Figure 6.

B7-DC-Ig inhibits the proliferation of Tconv and Treg but does not affect CD8 T cell proliferation. Purified CFSE-labeled $CD4^{+}CD25^{-}$ (Tconv), Treg and CD8 T cells were stimulated either alone or in the presence of different concentrations of B7-DC-Ig or Irr IgG. The effect of different doses of B7-DC-Ig or Irr IgG on proliferation of CFSE-labeled Tconv (A), Treg (B) and CD8 (C) T cells was analyzed by flow cytometry using a CFSE dilution assay. Percents of proliferating T cells are presented. *P<0.05, **P<0.01, ***P<0.001. Results presented are the average and SD from three experiments.

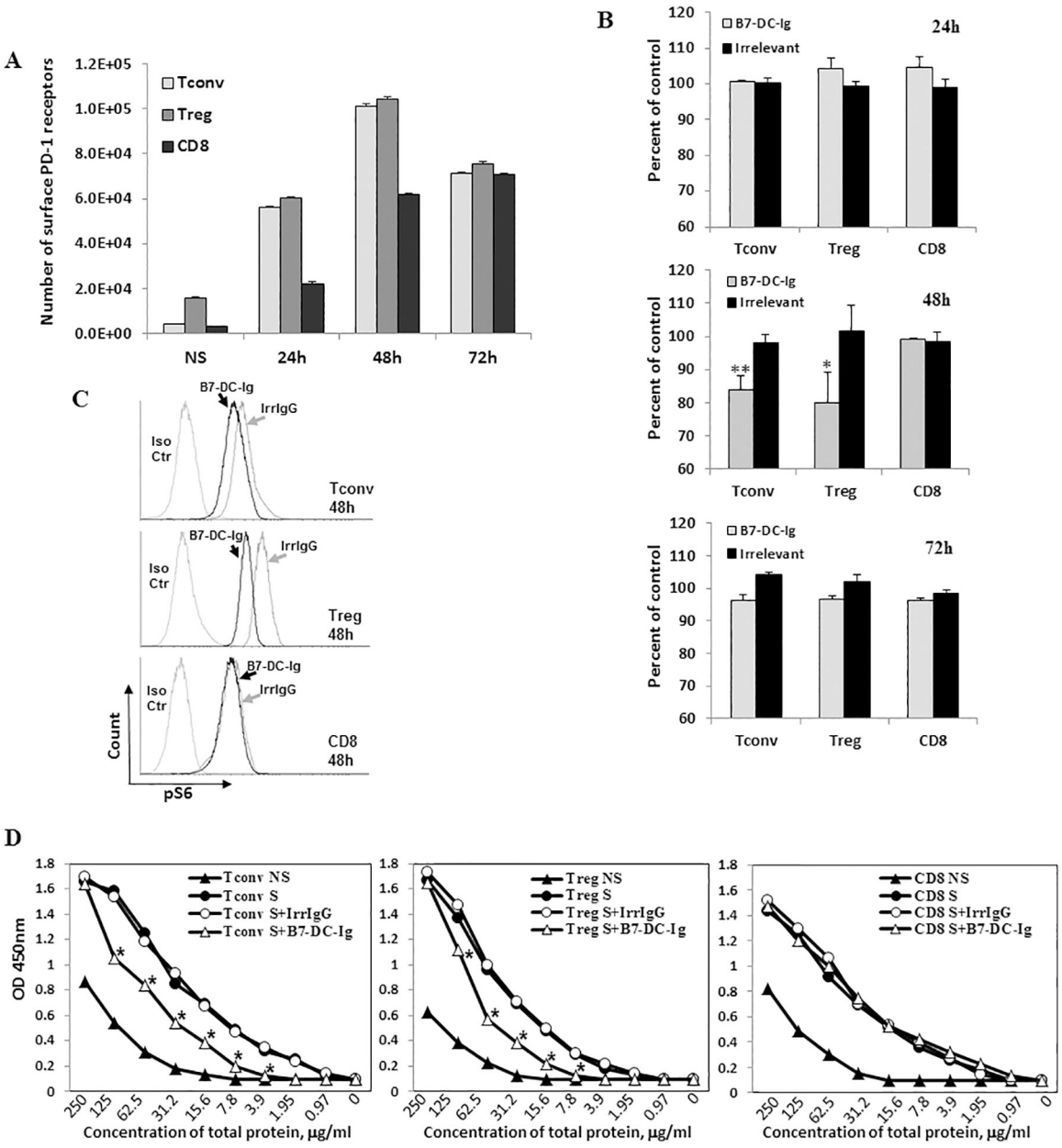

Figure 7.

Number of surface PD-1 receptors differs among different T cell subsets and B7-DC-Ig differentially affects these subsets. (A) Purified Tconv, Treg and CD8 T cells were stimulated with anti-CD3, anti-CD28 and IL-2 for 0, 24, 48 and 72hours. MFIs after anti-PD-1-PE staining were calculated and the numbers of surface PD-1 molecules calculated based on calibration curve from standard beads are presented. (B) Levels of pS6 after 24, 48 and 72 hours of stimulation for Tconv, Treg and CD8 T cells co-incubated with B7-DC-Ig or Irr IgG (10min) calculated as percents over pS6 MFI in T cell subsets without addition of compounds. (C) Representative flow cytometry histograms of pS6 in T cell subsets after 48 hours of stimulation and treatment with B7-DC-Ig or irrelevant IgG. (D) Levels of pS6 in lyzates of different T cell subsets after 48 hours activation and treatment with B7-DC-Ig or

irrelevant IgG analyzed by pS6 sandwich ELISA kit, presented as OD450 over total protein concentrations. *P<0.05, **P<0.01. Results presented are the average and SD from three experiments.