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Blockade of PD-L1 Enhances the Therapeutic Efficacy of Combination Immunotherapy Against Melanoma[1]

Shari Pilon-Thomas[*], **Amy Mackay**[*], **Nasreen Vohra**[*,†], and **James J. Mulé**[*]

[*]H. Lee Moffitt Cancer Center and Research Institute, University of South Florida School of Medicine

[†]Department of Translational Science, University of South Florida School of Medicine

Abstract

Inhibition of anti-tumor T cell responses can be mediated by the productive interaction between the programmed death-1 (PD-1) receptor on T cells and its ligand PD-L1. PD-L1 is highly expressed on both murine bone marrow-derived dendritic cells (DC) and B16 melanoma. In this study, in vitro blockade of PD-L1 interaction on DC led to enhanced IFN-gamma production and cytotoxicity by antigen-specific T cells. In vivo, the systemic administration of anti-PD-L1 antibody plus melanoma peptide-pulsed DC resulted in a higher number of melanoma peptide-specific $CD8^+$ T cells but this combination was insufficient to delay the growth of established B16 melanoma. While the addition of 600 rad of total body irradiation (TBI) delayed tumor growth, further adoptive transfer of antigen-specific $CD8^+$ T cells was needed to achieve tumor regression and long-term survival of the treated mice. Lymphopenic mice treated with anti-PD-L1 antibody demonstrated increased activation and persistence of adoptively transferred T cells, including a higher number of $CD8^+$ T cells infiltrating the tumor mass. Together, these studies support the blocking of PD-L1 signaling as a means to enhance combined immunotherapy approaches against melanoma.

INTRODUCTION

Certain immunotherapeutic strategies have been designed to activate and expand tumor-reactive T cells. For example, tumor antigen(s)-loaded dendritic cells (DC) have been shown to induce specific $CD8^+$ T cell responses against a variety of distinct tumor types (1–3). It is evident that even in the face of strongly induced anti-tumor T cell responses there are distinct mechanisms that allow tumors to escape immune destruction. Among these mechanisms, tumor expression of Programmed Death Ligand 1 (PD-L1, B7-H1) may contribute to the downregulation of immune responses by limiting the expansion or survival of effector T cells (4). PD-L1 is normally expressed on resting B cells, T cells, myeloid cells, and DC and is important for the maintenance of peripheral tolerance (5,6). Many tumors express PD-L1, including lung, ovarian, melanoma, and pancreatic tumors (4,7). Several receptors have been identified that bind to PD-L1, including PD-1, expressed on activated T cells, and CD80, expressed on antigen presenting cells, including DC. Ligation of PD-L1 with its binding partners results in T cell apoptosis, downregulation of proliferative responses, and decreased cytokine secretion (4,8,9). In melanoma patients, tumor-infiltrating $CD8^+$ T cells express high levels of PD-1 that correlates with impaired effector cell function (10).

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Corresponding Author: Shari Pilon-Thomas, Phone: 813-745-1540, Fax: 813-745-6188, shari.pilon-thomas@moffitt.org.

Strategies to block PD-L1 signaling and enhance anti-tumor $CD8^+$ T cell responses have yielded promising results. Blocking PD-L1 on myeloid DC isolated from ovarian cancer patients leads to enhanced T cell activation *in vitro* (11). In murine tumor models, PD-L1 blockade enhances tumor-specific cytotoxic T lymphocyte (CTL) killing (12). In some murine tumor models, sole administration of specific antibody recognizing PD-1 or PD-L1 leads to successful treatment of established tumors (12–14). In other models, combination treatment with anti-PD-L1 antibody improves immunotherapeutic approaches to induce tumor regressions (12,15).

Studies have shown that active vaccination during homeostatic proliferation skews the T cell repertoire towards self- or tumor associated-antigens (16–18). Transfer of naïve or activated T cells in a lymphopenic setting is an effective means of generating anti-tumor immunity and is successful at inducing tumor regression in both murine cancer models and human clinical trials (19–22). Active vaccination in combination with lymphodepletion and adoptive T cell transfer can further enhance anti-tumor immunity (23,24). Our studies have shown that DC-based vaccination following total body irradiation and bone marrow transplant is an effective means to induce regression of established tumors and long-term survival of mice (25). In this study, we show that blocking PD-L1 signaling following lymphodepletion in melanoma-bearing mice enhances the efficacy of adoptively transferred T cells in combination with a DC-based vaccine.

MATERIALS AND METHODS

Animals

Six- to eight-week-old C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). B6Ly5.2Cr mice expressing CD45.1, were obtained from the National Institutes of Health-Frederick Cancer Research and Development Center (Frederick, MD). Pmel, OT-I, and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and housed at the Animal Research Facility of the H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida, Tampa, FL.

Culture Medium and Tumor Cell Lines

Complete medium (CM) consisted of RPMI 1640 supplemented with 10% heat inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamycin, 0.5 μg/ml fungizone (all from Life Technologies, Inc. Rockville, MD) and 0.05 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO.). The B16 melanoma is a tumor of spontaneous origin. A poorly immunogenic, highly metastatic subclone of B16-D5, herein denoted B16, has been previously characterized. B16-D5 expresses a low level of MHC class I molecules and no detectable MHC class II molecules (26). EL4 is a T cell thymoma of C57BL/6 origin. B16 and EL4 cells were maintained by serial *in vitro* passages in CM. M05 tumor was generated by transfection of B16 melanoma with pAc-neo-OVA plasmid and was provided by Dr. Kenneth Rock (Dana-Farber Cancer Institute). M05 cells were maintained by serial *in vitro* passages in CM supplemented with 0.8 mg/ml G418.

Generation of Bone Marrow-Derived DC and Antigen Pulsing

Erythrocyte-depleted mouse bone marrow cells were cultured in complete medium supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN) as described previously (1). On day 5, cells were harvested by gentle pipeting and layered onto an Optiprep gradient (Axis-Shield, Oslo, Norway). The low-density cell

interface was collected and washed twice. For PD-L1 blockade experiments, DC were resuspended at 1×10^7 cells/ml in PBS and incubated with 10 ug/ml normal rat IgG (NrIgG) or rat anti-mouse PD-L1 antibody (anti-PD-L1, clone 10F.9G2, BioXcell, West Lebanon, NH). DC were washed one time and resuspended at 1×10^6cells/ml CM. For antigen-pulsing, DC were pulsed overnight with 10 μg/ml of either $hgp100_{25–33}$, $OVA_{257–264}$ ($OVA_{SIINFEKL}$), or $OVA_{323–339}$ peptide (Invitrogen, Carlsbad, CA).

In Vitro T cell Stimulation and ^{51}Cr Assay

Splenocytes from OT-I or OT-II mice were incubated with DC pulsed with OVA peptide at a 10:1 ratio. Supernatants were collected after 48 hours and IFN-gamma was measured in an ELISA assay. To measure cytotoxicity, splenocytes from pmel mice were co-cultured for 5 days with NrIgG or anti-PD-L1 pre-treated DC pulsed with $gp100_{25–33}$ peptide at a 10 to 1 ratio. On day 5, pmel cells were collected and cell cytotoxicity was measured in a ^{51}Cr assay. EL4 cells pulsed with $hgp100_{25–33}$ or $OVA_{257–264}$ peptide were labeled with ^{51}Cr for 90 min at 37 °C (100 μCi / 2×10^6 cells). Pmel effector cells were co-cultured with labeled EL4 target cells at various effector to target ratios in a 96 well plate for 5 hours. Percent specific lysis was calculated as 100 [(experimental CPM –spontaneous CPM)/(maximal CPM - spontaneous CPM)]. Labeled target cells cultured in CM alone or in 10% SDS were used to determine the spontaneous and maximum release, respectively.

In Vivo Immunizations

C57BL/6 mice were immunized three times at one week intervals with DC pulsed with $OVA_{257–264}$. Mice also received 20 mg/kg NrIgG or anti-PD-L1 antibody i.p. beginning on the first day of immunization and continuing every 3–4 days thereafter. Splenocytes were then prepared from these mice one week after the final immunization. The percentage of $CD8^+$OVA tetramer$^+$ cells was measured by flow cytometry. In addition, T cells were plated either alone or co-cultured with DC pulsed with $OVA_{257–264}$ peptide at a 10:1 ratio for 48 hours. Supernatants were then collected and IFN-gamma secretion was measured by ELISA.

In Vivo Treatment Model

A total of 1×10^5 B16 tumor cells were injected s.c. in the left flank of either C57BL/6 or Ly5.2 mice. Mice received i.p. injections of 20 mg/kg NrIgG or anti-PD-L1 antibodies on day 3. Additional treatments were given every 3–4 days until the end of the experiment. In some experiments, mice also received injections of 1×10^6 gp100 peptide-pulsed DC on days 3 and 7 after injection of tumor cells. Mice were humanely euthanized when tumors exceeded 1.5 cm in diameter, appeared necrotic, or interfered with locomotion.

Lymphopenia Model

A total of 1×10^5 B16 or 3×10^5 M05 tumor cells were injected s.c. in the left flank of C57BL/6 or Ly5.2 mice. Three days later, mice received a sublethal dose (600cGy) of total body irradiation (TBI) administered by a ^{137}Cs γ radiation source. For adoptive transfer experiments, T cells from the spleens of either naïve pmel mice or C57BL/6 mice vaccinated 3–4 times with DC-$OVA_{257–264}$ were enriched on a T cell column (R&D Systems). On day 4 following tumor injection, T cells (1×10^7) were transferred i.v. and mice were also immunized s.c. with 1×10^6 gp100 or $OVA_{257–264}$ pulsed DC. A second DC treatment was given on day 11. In addition, mice were treated i.p. with 20 mg/kg NrIgG or anti-PD-L1 antibody starting on day 4 and continuing every 3–4 days for 60 days or until the end of the experiment.

Isolation of Tumor Infiltrating Lymphocytes (TIL)

Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in HBSS (Life Technologies, Inc.) containing 1 mg/ml collagenase, 0.1 mg/ml DNAse I, and 2.5 units/ml of hyaluronidase (all from Sigma) with constant stirring for 2 hours at room temperature. The resulting suspension was passed through a 70 μm cell strainer, washed once with HBSS, and resuspended in PBS + 3% BSA to a concentration of 1×10^6 cells/ml for flow cytometric analysis.

Proliferation Assay

Splenocytes were collected at various timepoints after adoptive T cell transfer and co-cultured with 10 μg/ml gp100 peptide in 96 well plates for 48 hours. ^{3}H-thymidine (1μCi per well) was added for the final 18 hours of incubation. Cells were harvested and the amount of thymidine incorporated was measured on a Trilux, Beta Scintillation Counter (Wallac, Finland).

Antibodies and Flow Cytometric Analysis

For FACS analyses, DC were stained with FITC conjugated anti-IA^b and PE-conjugated anti-PD-L1 monoclonal antibodies (mAbs) after incubation with purified anti-CD16/32 Fc blocking antibody. Splenocytes and TIL were stained with FITC conjugated anti-CD8 and APC conjugated anti-CD45.2 mAbs after incubation with purified anti-CD16/32 Fc blocking antibody. All antibodies were purchased from BD Pharmingen (San Diego, CA). For tetramer analysis, splenocytes were stained with PE conjugated anti-CD8 mAb and anti-K^b OVA tetramer (Beckman Coulter Inc., Fullerton, CA). Data were acquired on a FACSCalibur and analyzed using CellQuest Pro software (BD Bioscience, San Jose, CA).

Statistical Analysis

A Mann-Whitney test (unpaired) or a Student's paired t-test was used to compare between two treatment groups. All statistical evaluations of data were performed using GraphPad Prism software. Statistical significance was achieved at $p<0.05$.

RESULTS

Expression of PD-L1 on DC and T cell stimulation in vitro

After five days of culture, approximately 75% of bone marrow-derived DC expressed high levels of PD-L1 (Figure 1A). We first examined IFN-γ production by T cells after co-culturing with DC treated with anti-PD-L1 antibody. Co-culture of $OVA_{257–264}$ peptide-pulsed DC pre-treated with NrIgG resulted in the production of IFN-γ by CD8$^+$ OT-I T cells (2,535 ± 55 pg/ml, Figure 1B). Co-culture after pre-treatment of DC with anti-PD-L1 antibody resulted in increased production of IFN-γ by CD8$^+$ OT-I cells (6,445 ± 116 pg/ml, $p<0.001$ compared to DC treated with NrIgG). OT-I T cells did not produce IFN-γ in response to gp100 peptide-pulsed DC pre-treated with NrIgG or anti-PD-L1 antibody. Co-culture of $OVA_{323–339}$ peptide-pulsed DC pre-treated with anti-PD-L1 antibody led to enhanced stimulation of CD4$^+$ OT-II T cells (1,063 ± 76 pg/ml, Figure 1C) compared to NrIgG pre-treated DC (498 ± 21 pg/ml, $p<0.01$).

Using a second experimental system, we also examined cytotoxicity of pmel T cells after stimulation with gp100 peptide-pulsed DC pre-treated with NrIgG or anti-PD-L1 antibody. After 5 days of co-culture, we assessed the ability of pmel T cells to kill gp100-loaded target cells. As shown in Figure 2, pmel T cells stimulated *in vitro* with DC-gp100-anti-PD-L1 antibody demonstrated enhanced killing of EL4-gp100 compared to pmel T cells stimulated with DC-gp100-NrIgG ($p<0.01$). Pmel T cells did not kill EL4 cells loaded with an

irrelevant peptide (not shown). Together, these experiments demonstrated that blocking PD-L1 on DC *in vitro* could enhance T cell effector functions.

Immunization with peptide-pulsed DC and administration of anti-PD-L1 antibody

To examine the efficacy of peptide-pulsed DC immunization in combination with anti-PD-L1 antibody to enhance T cell responses *in vivo*, we immunized mice three times with DC pulsed with OVA peptide. Mice also received NrIgG or anti-PD-L1 antibody beginning on the first day of immunization and continuing every 3–4 days. Splenocytes were collected one week after the final immunization. The percentage of $CD8^+$, OVA tetramer positive T cells was measured by flow cytometry. As shown in Figure 3A, a higher percentage of OVA-specific $CD8^+$ T cells was measured in the splenocytes of mice that received anti-PD-L1 antibody in combination with DC-OVA peptide immunization ($p<0.01$ compared to NrIgG treated mice). Restimulation with OVA peptide for 48 hours resulted in higher production of IFN-γ by splenocytes of anti-PD-L1 antibody treated, immunized mice (2,164 ± 200 pg/ml, Figure 3B) compared to splenocytes of NrIgG antibody treated, immunized mice (1,348 ± 389 pg/ml, $p<0.05$). These studies demonstrated that blocking PD-L1 *in vivo* can enhance T cell activation induced by peptide-pulsed DC immunization.

Treatment of B16 melanoma with anti-PD-L1 antibody and peptide-pulsed DC

PD-L1 is expressed by B16 melanoma (MFI 173, Figure 4), which could lead to the inhibition of B16-specific T cells. To test this possibility, we injected B16 melanoma cells s.c. into C57BL/6 mice. On day 3, mice received NrIgG or anti-PD-L1 antibody alone or in combination with DC pulsed with gp100 peptide (DC-gp100). Mice received NrIgG or anti-PD-L1 antibody every 3–4 days until the end of the experiment. In addition, mice received a second DC-gp100 immunization on day 10. As shown in Table I, treatment with anti-PD-L1 antibody alone or in combination with DC-gp100 immunization was unable to induce a significant delay in the growth of B16 tumor or to improve mice survival.

Treatment of lymphopenic mice with anti-PD-L1 antibody

It has been shown that PD-1 expression is downregulated on T cells undergoing acute homeostatic proliferation; however, by day 21, PD-1 is upregulated on T cells that are highly reactive to self antigens (27). We examined whether treatment with anti-PD-L1 antibody during homeostatic proliferation could lead to a reduction in the growth of B16 melanoma. Mice were treated with 600 rad TBI three days after injection of B16 tumor cells. On the following day, mice were immunized with DC-gp100 alone or in combination with anti-PD-L1 antibody treatment. An additional DC-gp100 immunization was given 7 days later. Anti-PD-L1 antibody (or NrIgG) treatments were given i.p. every 3–4 days until the end of the experiment. As shown in Table II, combination therapy with DC-gp100 and anti-PD-L1 antibody was able to delay the growth of B16 in lymphopenic mice and enhance survival ($p<0.05$ compared to all other groups); however, all mice eventually succumbed to tumor growth.

Adoptive transfer of melanoma-specific T cells in combination with anti-PD-L1 antibody and DC immunization in lymphopenic mice

As a means to enhance the effect of peptide-pulsed DC immunization in the setting of lymphopenia, we adoptively transferred melanoma-specific T cells isolated from the spleens of pmel mice. Three days after injection of B16 tumor cells, mice were treated with 600 rad of TBI. On day 4, mice received an i.v. injection of 1×10^7 pmel T cells in combination with s.c. injection of DC-gp100. In addition, mice were treated i.p. with NrIgG or anti-PD-L1 antibody starting on day 4 and continuing every 3–4 days for 60 days. An additional DC-gp100 treatment was given on day 11. As shown in Figure 5A, tumors in mice treated with

pmel T cells, DC-gp100, and anti-PD-L1 antibody demonstrated a significantly slower growth rate. Moreover, 40% of mice treated with pmel T cells, DC-gp100, and anti-PD-L1 antibody went on to show prolonged survival past 150 days (Figure 5B).

Persistence of melanoma-specific T cells in mice treated with anti-PD-L1 antibody

We next examined whether pmel T cells persisted for a longer duration in mice treated with anti-PD-L1 antibody. Ly5.2 ($CD45.1^+$) mice were treated with TBI. Mice received 1×10^7 pmel T cells ($CD45.2^+$) one day later in combination with either NrIgG or anti-PD-L1 antibody. Antibodies were given every 3–4 days for 4 weeks. Splenocytes were collected at various timepoints and the percentage of $CD45.2^+$ T cells was measured by flow cytometry. As shown in Figure 6A, mice treated with anti-PD-L1 antibody had a higher percentage of $CD45.2^+$ T cells remaining in the spleen at days 16 and 30 ($p<0.05$). This increased persistence correlated with an enhanced secretion of IFN-gamma by splenocytes from mice treated with pmel T cells and anti-PD-L1 antibody at day 30 (Figure 6B, $p<0.05$ compared to NrIgG treated mice). In addition, splenocytes from mice treated with anti-PD-L1 antibody demonstrated enhanced proliferation against gp100 peptide at day 30 (Figure 6c, 10,264 ± 1,538) compared to NrIgG treated mice (2,970 ± 1,017 cpm, $p<0.05$). Together, these data indicated that enhanced persistence and activation of pmel T cells in mice could be achieved by administration of anti-PD-L1 antibody.

Trafficking of melanoma-specific T cells to tumor after treatment with anti-PD-L1 antibody

We next examined the percentage of pmel T cells present in the tumor infiltration lymphocytes (TIL) of mice treated with anti-PD-L1 antibody. Mice were injected s.c. with B16 cells and received 600 rad of TBI on day 11. On day 12, mice received 1×10^7 pmel T cells i.v. in combination with NrIgG or anti-PD-L1 antibody. Antibody treatment was continued every 3–4 days until spleens and tumors were collected on day 19. $CD45.2^+$ T cells present in the spleen and TIL were analyzed by flow cytometry. As shown in Figure 7, mice treated with anti-PD-L1 antibody had a higher percentage of $CD45.2^+$ T cells in TIL (6.6% compared to 3% in mice treated with NrIgG, $p<0.05$).

Adoptive transfer of effector T cells in combination with anti-PD-L1 antibody treatment

We next examined whether treatment with anti-PD-L1 antibody could enhance the therapeutic efficacy of adoptively transferred T cells isolated from mice previously immunized with DC pulsed with $OVA_{SIINFEKL}$ (DC-OVA). Figure 8A shows that PD-L1 is indeed expressed by M05 melanoma cells (MFI 31.5). Mice were treated with TBI on day 3 after injection of M05 cells. On day 4, mice received 1×10^7 T cells isolated from the spleens of DC-OVA immunized donor mice. In addition, DC-OVA immunizations were given s.c. to the recipients on days 4 and 11 along with NrIgG or anti-PD-L1 antibody beginning on day 4 and continuing every 3–4 days until the end of the experiment. Mice treated with the adoptive transfer of OVA-immune T cells in combination with anti-PD-L1 antibody displayed a significant delay in the growth of M05 tumor (Figure 8B, $p<0.05$ compared to all other groups).

DISCUSSION

DC-based vaccines are an effective means to induce anti-tumor T cell responses. However, their use rarely translates into clinically meaningful responses in patients with cancer. The expression of PD-L1 on DC and tumor cells may limit the induction of robust anti-tumor T cell responses. PD-L1 binds to PD-1 on activated T cells to induce and maintain peripheral tolerance (5). Pulko et al. have shown that DC generated from the bone marrow of PD-L1 knock-out mice are superior to wild-type DC at inducing both antigen-specific $CD8^+$ T cell responses and tumor suppression. (28). In our current study, blocking PD-L1 expression on

DC led to enhanced secretion of IFN-gamma and cytotoxicity by antigen-specific T cells. In addition, vaccination with OVA peptide-pulsed DC in combination with anti-PD-L1 antibody treatment induced an increased number of OVA-specific $CD8^+$ T cells and enhanced IFN-gamma secretion. Despite these positive effects, treatment of mice bearing B16 tumors with gp100 peptide-pulsed DC in combination with anti-PD-L1 antibody did not result in tumor regression. This result suggests that in addition to expressing high levels of PD-L1, B16 melanoma employs additional operative mechanisms of immune evasion.

In an effort to improve immunity, we treated B16 melanoma bearing mice with anti-PD-L1 antibody and administered peptide-pulsed DC in a lymphopenic environment. In this setting, the opportunity exists to educate reconstituting T cells during homeostatis-driven proliferation. Vaccination after the induction of lymphopenia has been shown to enhance the activation and expansion of anti-tumor T cells (20), which may be due to an increased availability of immune stimulating cytokines, such as IL-15 and IL-7 (29), and the downregulation of inhibitory molecules on T cells during acute homeostatic proliferation (30). It has been shown that PD-1 expression is deficient on T cells undergoing acute homeostatic proliferation (27). Normal expression of PD-1 is restored by day 21 and is found to be highly expressed on autoreactive T cells, leading to the elimination of these cells (30). In our tumor model, treatment with anti-PD-L1 antibody alone after the induction of lymphopenia had no effect on tumor growth. Combination therapy with anti-PD-L1 antibody and peptide-pulsed DC immunizations resulted in a significant delay in B16 tumor growth. The addition of adoptively transferred, tumor-specific T cells enhanced this effect and led to an improved survival in mice bearing either B16 or the M05 melanoma.

The efficacy of T cell adoptive transfer in the setting of lymphopenia depends on the persistence of the transferred T cells (31). In viral models, blocking PD-L1 led to enhanced activation and persistence of viral-specific T cells (32). In the current study, there was no difference in the initial proliferative rate of adoptively transferred pmel T cells in mice treated with NrIgG or PD-L1 antibody (data not shown). However, pmel T cells persisted longer in mice treated with anti-PD-L1 antibody. By day 30 after adoptive transfer, the number of pmel T cells was significantly lower in the spleens of mice treated with NrIgG, which correlated inversely with the growth of B16 tumors.

The expression of PD-L1 late in anti-tumor immune responses may regulate and suppress long-term immunity. Upregulation of PD-L1 expression in the spleen following vaccination has been linked to lower numbers and impaired function of both memory and effector T cells (33). In mice bearing renal cell or lung carcinomas, upregulation of PD-L1 on splenocytes correlated with an IFN-γ dependent loss of $CD4^+$ T cells and impaired anti-tumor memory T cell responses (34). In our current study, adoptive transfer of pmel T cells in combination with DC vaccination led to a delay in tumor growth in mice receiving either NrIgG or anti-PD-L1 antibody, but eventually all of the NrIgG treated mice grew tumors and died. In contrast, some mice treated with anti-PD-L1 antibody went on to be tumor-free. This correlated with an enhanced number of T cells infiltrating the tumor and increased proliferation and IFN-γ production by tumor-specific T cells.

Collectively, our studies support the addition of anti-PD-L1 antibody to immunotherapeutic approaches that employ the adoptive transfer of T cells and active vaccination, particularly in the setting of lymphopenia. Blocking PD-L1 signaling allows longer persistence and enhanced infiltration of T cells into PD-L1-expressing tumor. This approach may be valuable as a means to enhance clinical responses in patients with melanoma.

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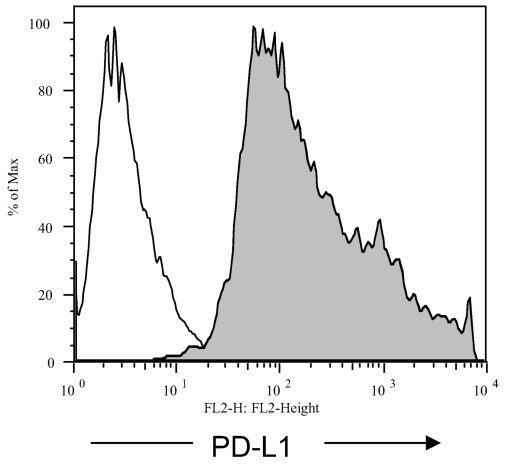
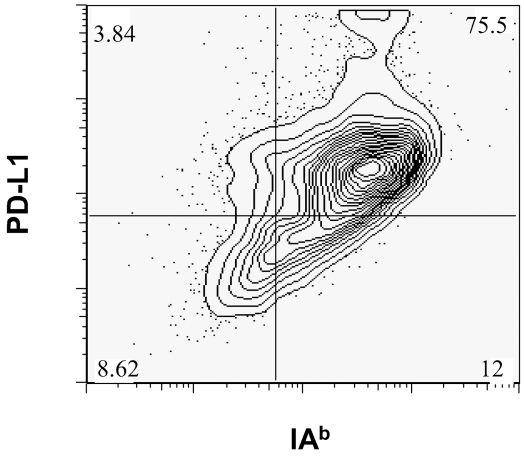
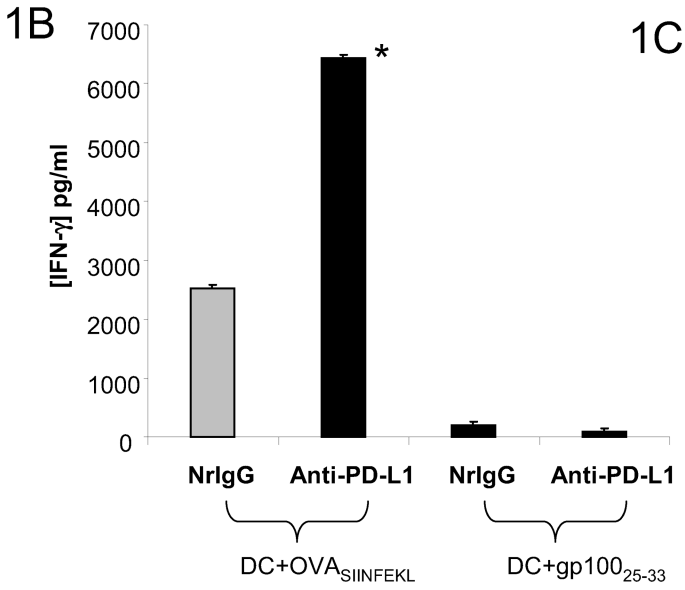
Figure 1A







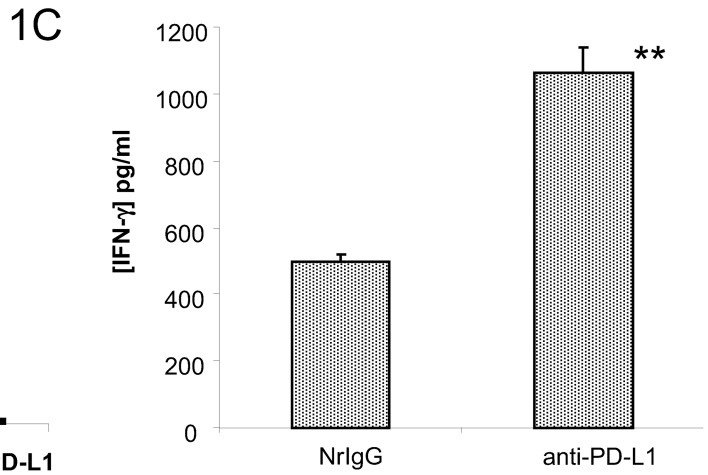


Figure 1.

Peptide-loaded DC pre-treated with anti-PD-L1 antibody enhance stimulation of a specific T cell response. (A) After 5 days of *in vitro* culture, bone marrow-derived DC were stained with anti-IA^b and PD-L1 antibodies and analyzed by flow cytometry. Filled histogram = anti-PD-L1, open histogram = isotype control. (B) DC were pulsed with $OVA_{257–264}$ or $gp100_{25–33}$ peptide and treated with either 10 μg/ml NrIgG or anti-PD-L1 antibody for 24 hours. These DC were then co-cultured with OT-I splenocytes at a 1:10 ratio for 48 hours. (C) DC were pulsed with $OVA_{323–337}$ peptide and treated with either 10 μg/ml NrIgG or anti-PD-L1 antibody for 24 hours. These DC were co-cultured with OT-II splenocytes at a 1:10 ratio for 48 hours. Supernatants were collected and tested in an IFN-gamma ELISA

assay. One of 2 representative experiments is shown. *indicates $p<0.001$, **indicates $p<0.01$.

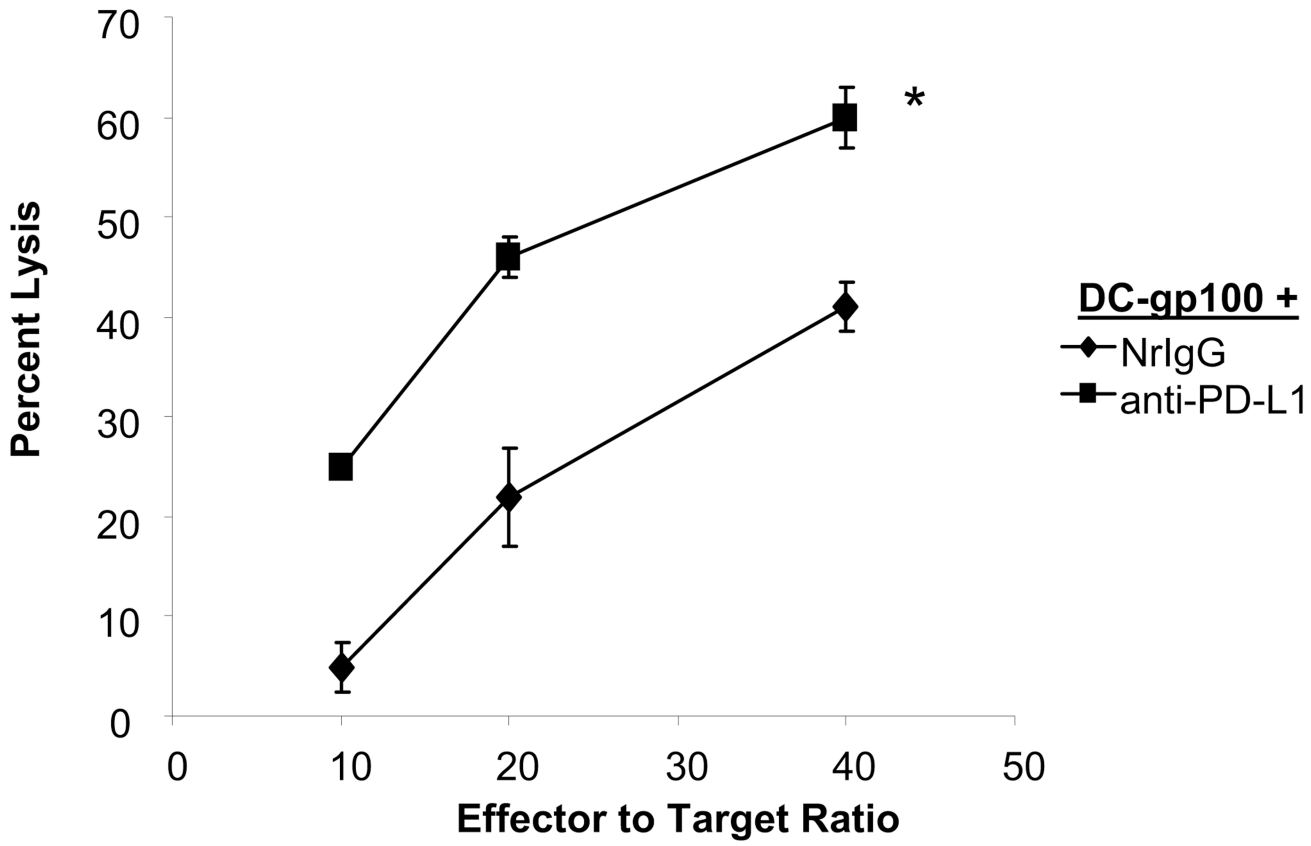
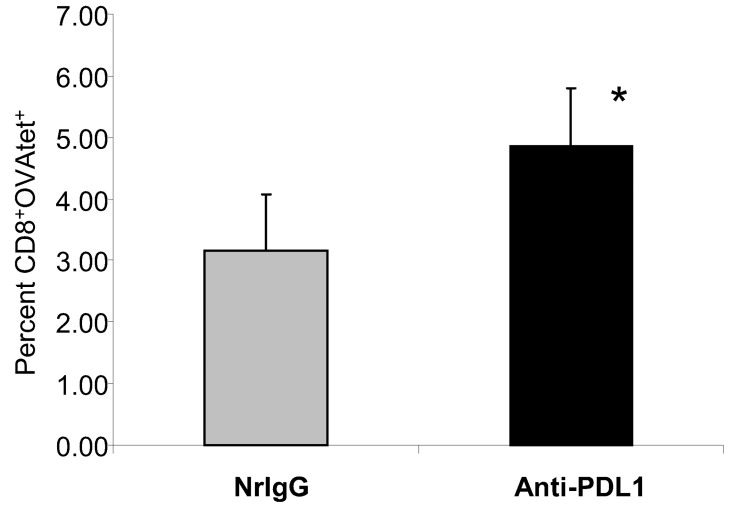


Figure 2.
Stimulation with anti-PD-L1 antibody-treated DC enhances T cell cytotoxicity. Purified pmel T cells were stimulated *in vitro* for 5 days with gp100-pulsed DC pre-treated with NrIgG or anti-PD-L1 antibody. Cell cytotoxicity against gp100-coated EL4 target cells was measured in a 5 hour ^{51}Cr release assay. *indicates $p<0.01$.

Figure 3A



3B

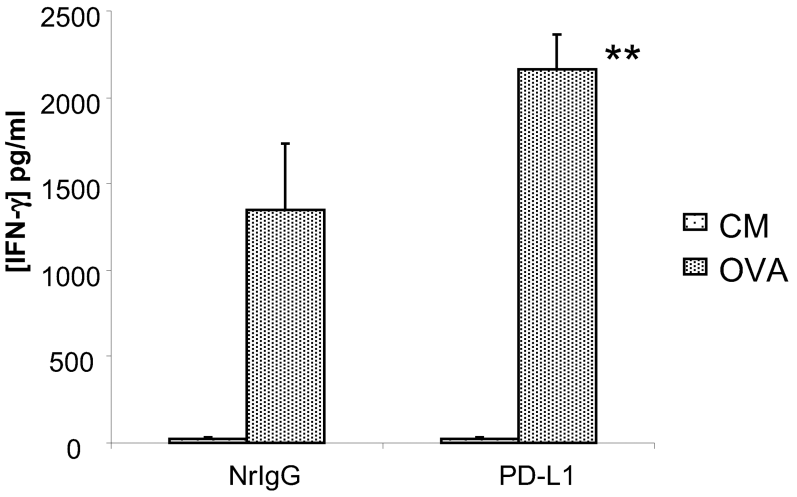


Figure 3.

Immunization with OVA peptide-pulsed DC in combination with anti-PD-L1 antibody treatment leads to enhanced $CD8^+$ T cell response. C57BL/6 mice (n=4) were immunized s.c. three times with DC pulsed with $OVA_{257–264}$ peptide at one week intervals. Mice also received NrIgG or anti-PD-L1 antibody every 3–4 days. Splenocytes were collected one week after the final peptide-pulsed DC immunization. (A) The percentage of $CD8^+$ and OVA $tetramer^+$ cells was measured by flow cytometry. * indicates $p<0.01$. (B) Splenocytes alone or co-cultured with 10 μg/ml $OVA_{257–264}$ peptide *in vitro* for 48 hours. Supernatants were collected and IFN-gamma was measured by an ELISA assay. This experiment was repeated twice with similar results. ** indicates $p<0.05$.

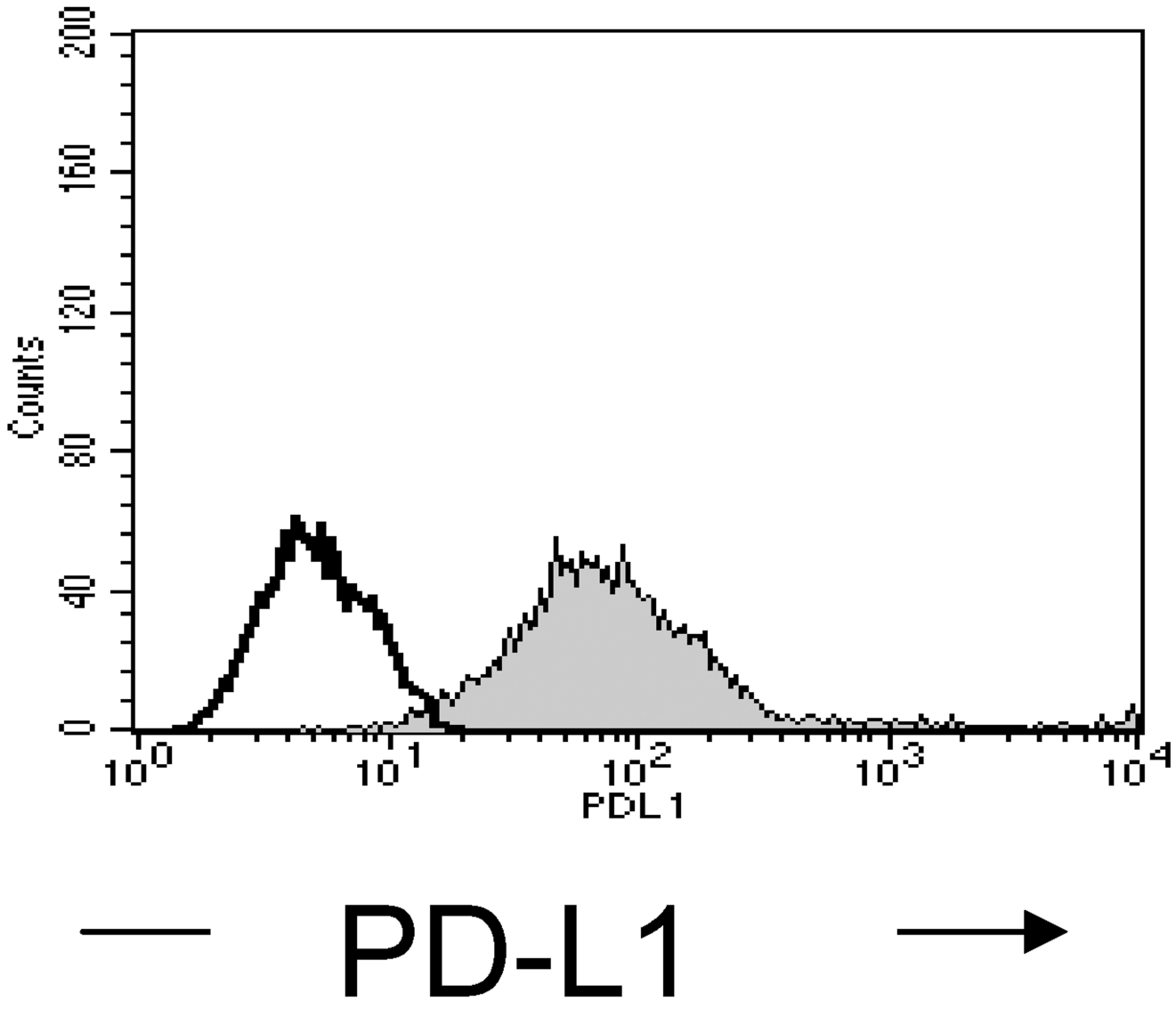


Figure 4.
Expression of PD-L1 on B16 melanoma cells. B16 cells were stained with anti-PD-L1 antibody. Expression was measured by flow cytometry. Open histogram = Isotype control; filled histogram: PD-L1 expression.

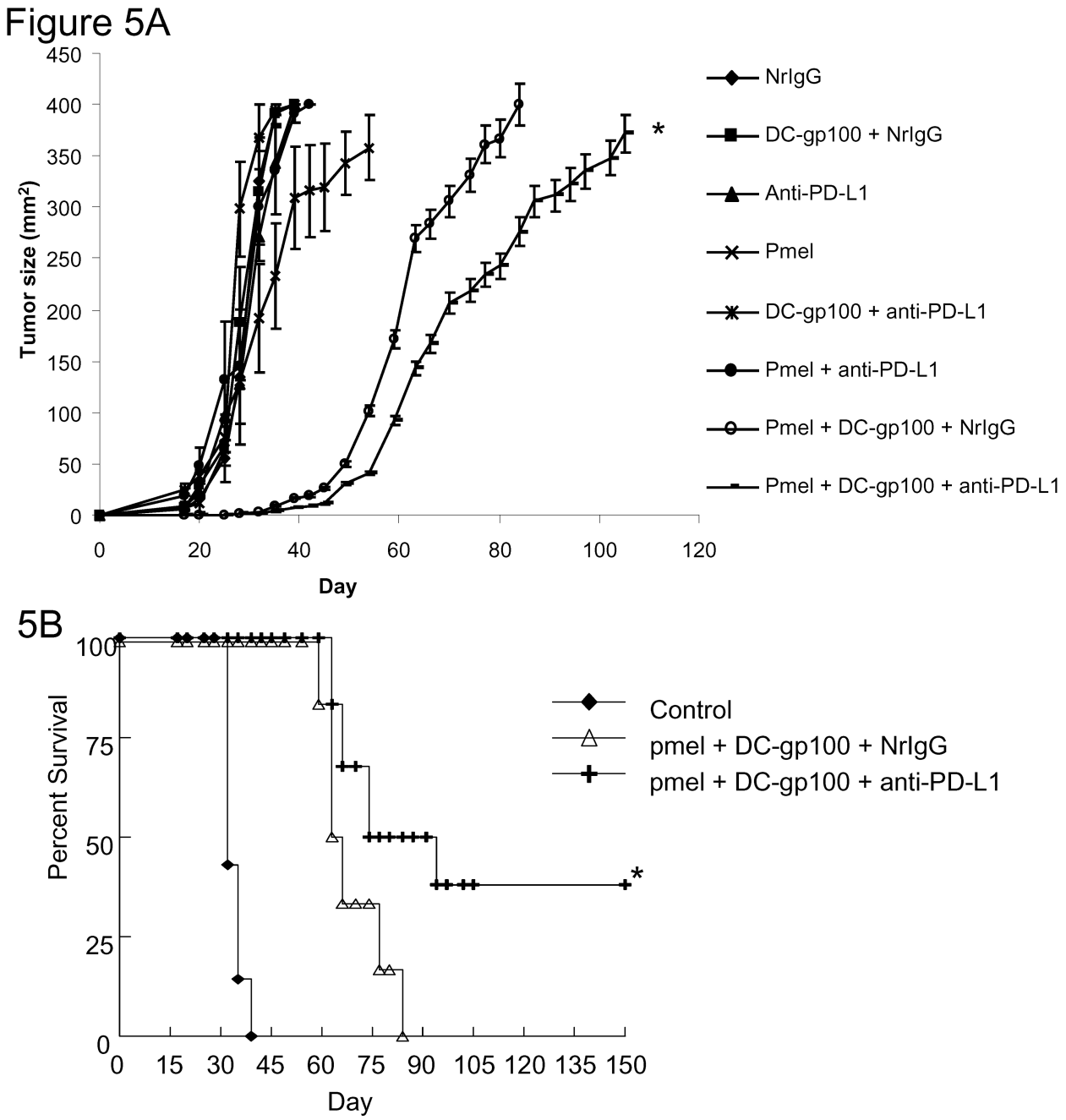


Figure 5.

Adoptive transfer of pmel T cells enhances the anti-B16 tumor response in tumor-bearing mice treated with anti-PD-L1 antibody and peptide-pulsed DC. C57BL/6 (n=6 per group) mice were injected s.c. with B16 cells. On day 3, mice received 600 rad of TBI. On day 4, mice received 1×10^7 pmel T cells i.v. Beginning on day 4 and continuing every 3–4 days until day 60, mice received i.p. injections of NrIgG or anti-PD-L1 antibody. Mice also received s.c. injections of DC pulsed with gp100 peptide on days 4 and 11 after B16 injection. Tumor sizes were measured twice each week. (A) Tumor growth. (B) Survival. The results are representative of two similar experiments. * indicates $p<0.05$ compared to pmel + DC-gp100 + NrIgG treated mice.

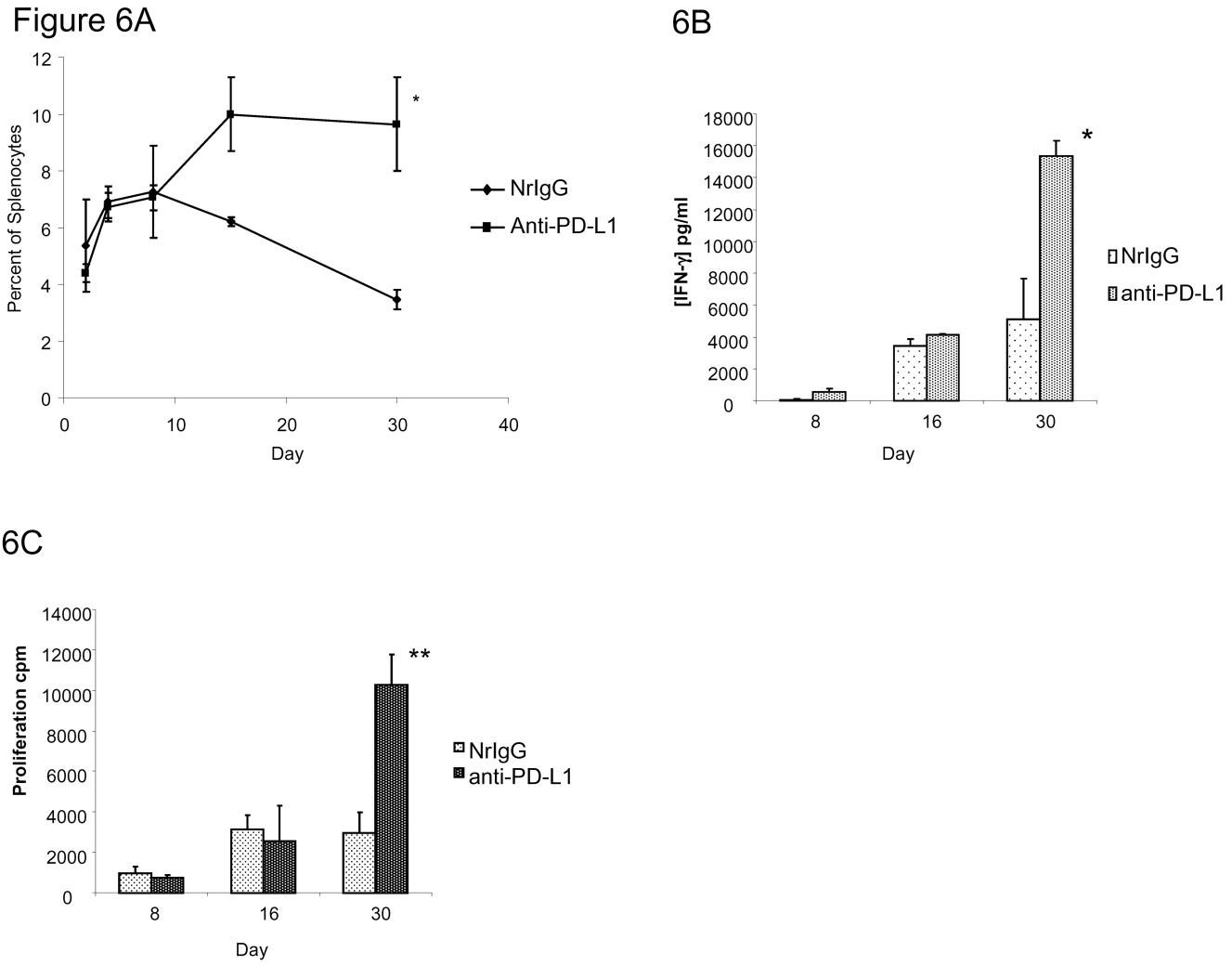


Figure 6.

Pmel T cells persist in mice treated with anti-PD-L1 antibody. Ly5.2 (CD45.1$^+$) mice (n=20) were treated with 600 rad of TBI. On day 3, mice received i.v. 1×10^7 pmel T cells. Beginning on day 3 and continuing every 3–4 days, mice received i.p. injections of either NrIgG or anti-PD-L1 antibody. Spleens (n=4) were collected at each time point and (A) the percentage of CD45.2$^+$ pmel T cells was measured by flow cytometry. Splenocytes were restimulated in vitro with gp100-peptide pulsed DC for 48 hours. (B) Supernatants were collected and IFN-gamma was measured by an ELISA assay. (C) ^{3}H-thymidine was added for an additional 18 hours. Cells were harvested and the amount of thymidine incorporation was measured. These experiments were repeated two times. * indicates $p<0.01$, **indicates $p<0.05$.

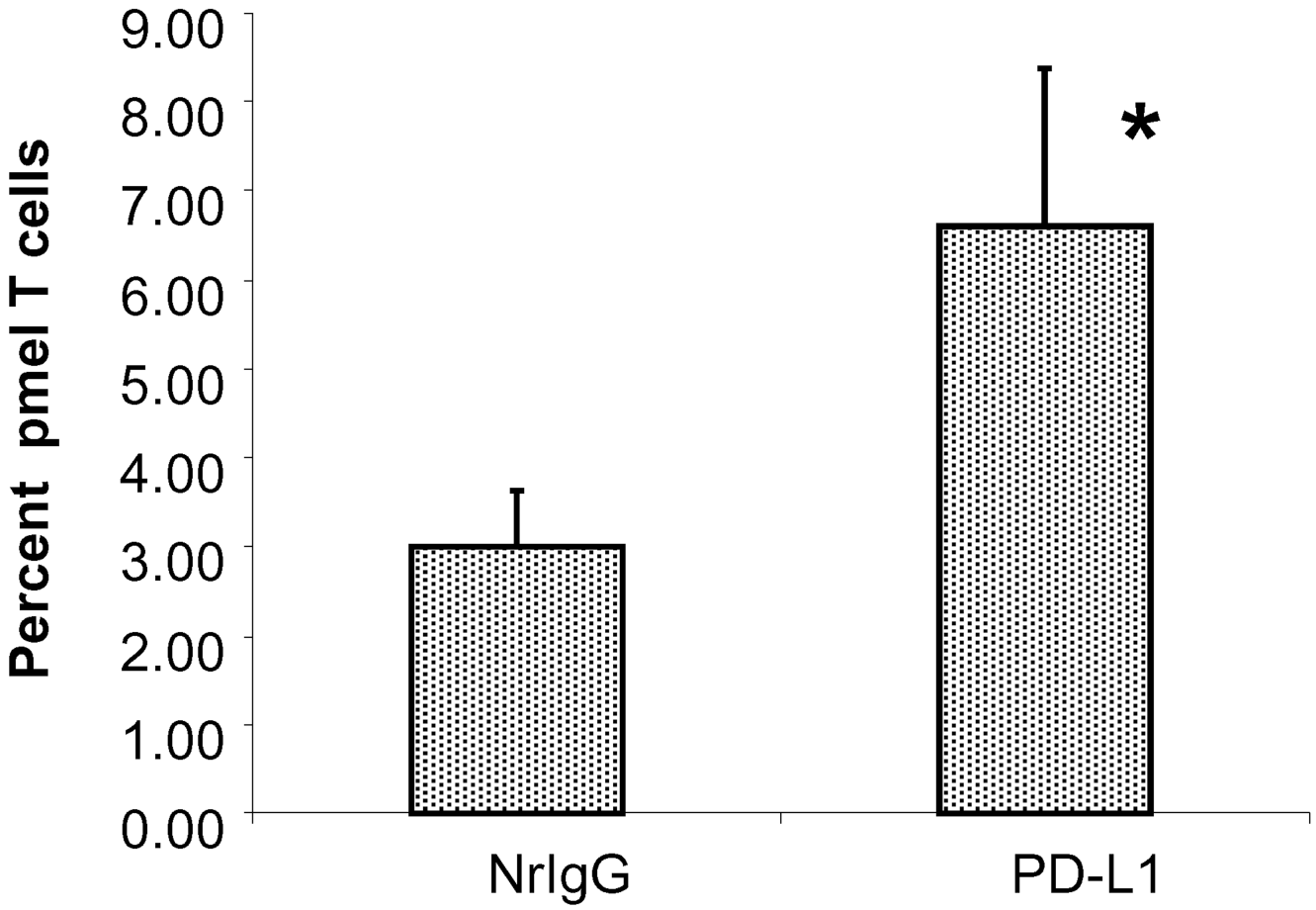
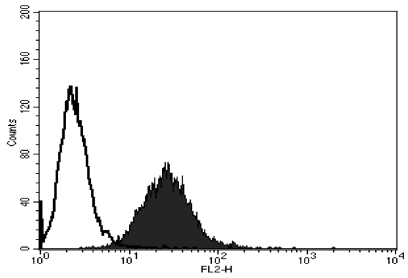


Figure 7.

Higher numbers of pmel T cells infiltrate B16 tumor in mice treated with anti-PD-L1 antibody. Ly5.2 mice (n=10) were injected s.c. with B16 tumor cells. On day 11, mice received 1×10^7 pmel T cells i.v. Mice also received i.p. injections of either NrIgG or anti-PD-L1 antibody on days 11, 14, and 17. Spleens and tumors were collected on day 19 and the percentage of $CD45.2^+$ pmel T cells was measured. * indicates $p<0.05$ compared to NrIgG treated mice. This experiment was repeated two times with similar results.

Figure 8A



8B

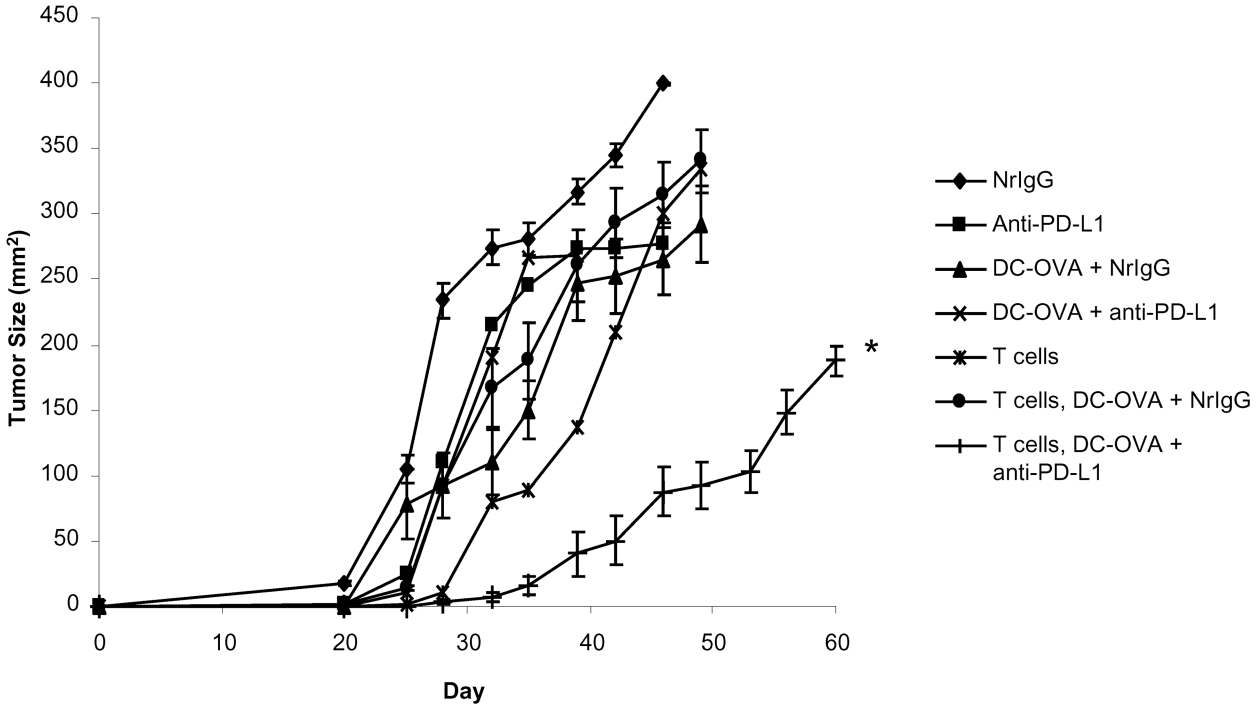


Figure 8.

Adoptive transfer of OVA-immune T cells enhances the anti-tumor response in M05-bearing mice treated with anti-PD-L1 antibody and peptide-pulsed DC. (A) Expression of PD-L1 on the surface of M05 cells. Filled histogram: anti-PD-L1, open histogram: NrIgG. (B) C57BL/6 (n=6 per group) mice were injected s.c. with M05 cells. On day 3, mice received 600 rad of TBI. On day 4, mice received 1×10^7 T cells i.v. that were isolated from mice immunized four times with DC-$OVA_{SIINFEKL}$. Beginning on day 4 and continuing every 3–4 days until day 60, mice received i.p. injections of either NrIgG or anti-PD-L1 antibody. Mice also received DC pulsed with $OVA_{SIINFEKL}$ peptide s.c. on days 4 and 11 after B16 injection. Tumors were measured twice each week. The results are representative of two experiments. * indicates $p<0.05$ compared to T cells + DC-OVA + NrIgG treated mice.

Table I

Treatment of B16 melanoma with peptide-pulsed DC and anti-PD-L1 antibody

Treatment[1] (n=6)	Mean Tumor Area (mm^2)[2]	Mean Survival (days)
NrIgG	392 ± 8	27 ± 1
anti-PD-L1	347 ± 53	24 ± 6
DC-gp100 + NrIgG	333 ± 67	26 ± 4
DC-gp100 + anti-PD-L1	268 ± 72	31 ± 15

[1] Mice were treated beginning on day 3 after s.c. injection of B16 cells

[2] Measurements recorded on day 28

Table II

Treatment of B16 melanoma with TBI, peptide-pulsed DC and anti-PD-L1 antibody

Treatment[1]	Mean tumor size (mm^2)[2]	Survival (days)
NrIgG	325 ± 4	33 ± 4
anti-PD-L1	371 ± 46	37 ± 3
DC-gp100 + NrIgG	315 ± 52	34 ± 4
DC-gp100 + anti-PD-L1	175 ± 41*	46 ± 9*

[1] Mice (n=6) were treated with TBI on day 3 after B16 injection. Mice received DC injections on days 4 and 11. Mice received NrIgG or anti-PD-L1 antibody every 3–4 days beginning on day 4

[2] Tumor measurements were recorded on day 32

* indicates $p<0.05$ compared to all other groups