Blockade of Programmed Death Ligand 1 Enhances the Therapeutic Efficacy of Combination Immunotherapy against Melanoma

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*J Immunol* 2010; 184:3442-3449; Prepublished online 1 March 2010; doi: 10.4049/jimmunol.0904114

http://www.jimmunol.org/content/184/7/3442
Blockade of Programmed Death Ligand 1 Enhances the Therapeutic Efficacy of Combination Immunotherapy against Melanoma

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

Inhibition of antitumor 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 (DCs) and B16 melanoma. In this study, in vitro blockade of PD-L1 interaction on DCs led to enhanced IFN-γ production and cytotoxicity by Ag-specific T cells. In vivo, the systemic administration of anti–PD-L1 Ab plus melanoma peptide-pulsed DCs 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. Although the addition of 600 rad of total body irradiation delayed tumor growth, further adoptive transfer of Ag-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 Ab 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. The Journal of Immunology, 2010, 184: 3442–3449.

Certain immunotherapeutic strategies have been designed to activate and expand tumor-reactive T cells. For example, tumor Ag(s)-loaded dendritic cells (DCs) 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 antitumor 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 DCs 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 APCs, including DCs. 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 patients with melanoma, tumor-infiltrating CD8⁺ T cells express high levels of PD-1 that correlate with impaired effector cell function (10).

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

Studies have shown that active vaccination during homeostatic proliferation skews the T cell repertoire toward self- or tumor-associated Ags (16–18). Transfer of naive or activated T cells in a lymphopenic setting is an effective means of generating antitumor 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 antitumor 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). B6.fv.l5.2Cr 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 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 U/ml penicillin, 50 μg/ml gentamycin, 0.5 μg/ml fungizone (all from Life
Technologies, Rockville, MD), and 0.05 mM 2-ME (Sigma-Aldrich, St. Louis, MO). The B16 melanoma is a tumor of spontaneous origin. A poorly immunogenic, highly metastatic subclone of B16-D5, herein denoted B16-D5, 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, Boston, MA). M05 cells were maintained by serial in vitro passages in CM supplemented with 0.8 mg/ml G418.

**Generation of bone marrow-derived DCs and Ag pulsing**

Erythrocyte-depleted bone marrow cells were cultured in CM supplemented with 20 mg/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 pipetting 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, DCs were resuspended at 1 × 10^7 cells/ml in PBS and incubated with 10 μg/ml normal rat IgG (NrIgG) or rat anti-mouse PD-L1 Ab (anti–PD-L1, clone 10F9G2; Bio-Xcell, West Lebanon, NH). DCs were washed one time and resuspended at 1 × 10^6 cells/ml CM. For Ag pulsing, DCs were pulsed overnight with 10 μg/ml of hgp10025–33, OVA257–264 (OVASINFEKL), or OVA323–339 peptide (Invitrogen, Carlsbad, CA).

**In vitro T cell stimulation and [51Cr] assay**

Splenocytes from OT-I or OT-II mice were incubated with DCs pulsed with OVA peptide at a 10:1 ratio. Supernatants were then collected, and IFN-γ secretion was measured in an ELISA assay. One of two representative experiments is shown. EL4 cells were pulsed with gp100 peptide-pulsed DCs on days 3 and 4 after the final immunization. The percentage of CD8+, OVA tetramer-positive cells was measured by flow cytometry. In addition, T cells were plated either alone or cocultured with DCs pulsed with OVA257–264 peptide at a 10:1 ratio for 48 h. Supernatants were then collected, and IFN-γ secretion was measured by ELISA.

**In vitro treatment model**

A total of 1 × 10^6 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 Abs on day 3. Additional treatments were given every 3 to 4 d until the end of the experiment. In some experiments, mice also received injections of 1 × 10^6 gp100 peptide-pulsed DCs 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 (600 cGy) of total body irradiation (TBI) administered by a [137Cs] γ radiation source. For adoptive transfer experiments, T cells from the spleens of naïve pmel mice or C57BL/6 mice vaccinated three to four times with DC-OVA257–264 were enriched on a T cell column (R&D Systems). On day 4 following tumor injection, T cells (1 × 10^7) with labeled EL4 target cells at various E:T ratios in a 96-well plate for 5 h. 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 1-wk intervals with DCs pulsed with OVA257–264. Mice also received 20 mg/kg NrIgG or anti–PD-L1 Ab i.p. beginning on the first day of immunization and continuing every 3 to 4 d thereafter. Splenocytes were then prepared from these mice 1 wk after the final immunization. The percentage of CD8+, OVA tetramer-positive cells was measured by flow cytometry. In addition, T cells were plated either alone or cocultured with DCs pulsed with OVA257–264 peptide at a 10:1 ratio for 48 h. Supernatants were then collected, and IFN-γ secretion was measured by ELISA.

**FIGURE 1.** Peptide-loaded DCs pretreated with anti–PD-L1 Ab enhance stimulation of a specific T cell response. A. After 5 d of in vitro culture, bone marrow-derived DCs were stained with anti–IAβ and PD-L1 Abs and analyzed by flow cytometry. Filled histogram indicates anti–PD-L1; open histogram indicates isotype control. B, DCs were pulsed with OVA257–264 or gp10025–33 peptide and treated with either 10 μg/ml NrIgG or anti–PD-L1 Ab for 24 h. These DCs were then cocultured with OT-I splenocytes at a 1:10 ratio for 48 h. C, DCs were pulsed with OVA323–337 peptide and treated with either 10 μg/ml NrIgG or anti–PD-L1 Ab for 24 h. These DCs were cocultured with OT-II splenocytes at a 1:10 ratio for 48 h. Supernatants were collected and tested in an IFN-γ ELISA assay. One of two representative experiments is shown. *p < 0.001; **p < 0.01.
were transferred i.v., and mice were also immunized s.c. with 1 × 10^6 gp100 or OVA257–264-pulsed DCs. 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 Ab starting on day 4 and continuing every 3 to 4 d for 60 d or until the end of the experiment.

Isolation of tumor infiltrating lymphocytes

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

Proliferation assay

Splenocytes were collected at various time points after adoptive T cell transfer and cocultured with 10 μg/ml gp100 peptide in 96-well plates for 48 h. [3H]thymidine (1 μCi per well) was added for the final 18 h of incubation. Cells were harvested, and the amount of thymidine incorporated was measured on a Trilux cubation. Cells were harvested, and the amount of thymidine incorporated was measured on a Trilux Scintillation Counter (Wallac, Finland).

Abs and flow cytometric analysis

For FACS analyses, DCs were stained with FITC-conjugated anti-1A6 and PE-conjugated anti-PD-L1 mAbs postincubation with purified anti-CD16/32Fc blocking Ab. Splenocytes and tumor infiltrating lymphocytes (TILs) were stained with FITC-conjugated anti-CD8 and APC-conjugated anti-CD45.2 mAbs postincubation with purified anti-CD16/32Fc blocking Ab. All Abs were purchased from BD Pharmingen (San Diego, CA). For tetramer analysis, splenocytes were stained with PE-conjugated anti-CD8 mAb and anti-Kb OVA tetramer (Beckman Coulter, Fullerton, CA). Data were acquired on an FACSCalibur and analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA).

Statistical analysis

A Mann-Whitney U test (unpaired) or a Student paired t test was used to compare between two treatment groups. All statistical evaluations of data were performed using GraphPad Prism software (GraphPad, La Jolla, CA). Statistical significance was achieved at p < 0.05.

Results

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

After 5 d of culture, ∼75% of bone marrow-derived DCs expressed high levels of PD-L1 (Fig. 1A). We first examined IFN-γ production by T cells after coculturing with DCs treated with anti–PD-L1 Ab. Coculture of OVA257–264 peptide-pulsed DCs pretreated with NrIgG resulted in the production of IFN-γ by CD8+ OT-I T cells (2535 ± 55 pg/ml; Fig. 1B). Coculture after pretreatment of DCs with anti–PD-L1 Ab resulted in increased production of IFN-γ by CD8+ OT-I T cells (6445 ± 116 pg/ml; p < 0.001 compared with DCs treated with NrIgG). OT-I T cells did not produce IFN-γ in response to gp100 peptide-pulsed DCs pretreated with NrIgG or anti–PD-L1 Ab. Coculture of OVA253–339 peptide-pulsed DCs pretreated with anti–PD-L1 Ab led to enhanced stimulation of CD4+ OT-II T cells (1063 ± 76 pg/ml; Fig. 1C) compared with NrIgG pretreated DCs (498 ± 21 pg/ml; p < 0.01).

Using a second experimental system, we also examined cytotoxicity of pmel T cells poststimulation with gp100 peptide-pulsed DCs pretreated with NrIgG or anti–PD-L1 Ab. After 5 d of coculture, we assessed the ability of pmel T cells to kill gp100-loaded target cells. As shown in Fig. 2, pmel T cells stimulated in vitro with DC-gp100-anti–PD-L1 Ab demonstrated enhanced killing of EL4-gp100 compared with 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 DCs in vitro could enhance T cell effector functions.

Immunization with peptide-pulsed DCs and administration of anti–PD-L1 Ab

To examine the efficacy of peptide-pulsed DC immunization in combination with anti–PD-L1 Ab to enhance T cell responses in vivo, we immunized mice three times with DCs pulsed with OVA peptide. Mice also received NrIgG or anti–PD-L1 Ab beginning on the first day of immunization and continuing every 3 to 4 d. Splenocytes were collected 1 wk after the final immunization. The percentage of CD8+, OVA tetramer-positive T cells was measured by flow cytometry. As shown in Fig. 3A, a higher percentage of OVA-specific CD8+ T cells was measured in the splenocytes of mice that received anti–PD-L1 Ab in combination with DC-OVA peptide immunization (p < 0.01 compared with NrIgG-treated mice). Re-stimulation with OVA peptide for 48 h resulted in higher production of IFN-γ by splenocytes of anti–PD-L1 Ab-treated immunized mice.
(2164 ± 200 pg/ml; Fig. 3B) compared with splenocytes of NrIgG Ab-treated immunized mice (1348 ± 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 Ab and peptide-pulsed DCs**

PD-L1 is expressed by B16 melanoma (MH 173; Fig. 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 Ab alone or in combination with DC pulsed with gp100 peptide (DC-gp100). Mice received NrIgG or anti–PD-L1 Ab every 3 to 4 d 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 Ab alone or in combination with DC-gp100 immunization was unable to induce a significant delay in the growth of B16 tumor or to improve mouse survival.

**Treatment of lymphopenic mice with anti–PD-L1 Ab**

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-Ags (27). We examined whether treatment with anti–PD-L1 Ab during homeostatic proliferation could lead to a reduction in the growth of B16 melanoma. Mice were treated with 600 rad TBI 3 d postinjection of B16 tumor cells. On the following day, mice were immunized with DC-gp100 alone or in combination with anti–PD-L1 Ab treatment. An additional DC-gp100 immunization was given 7 d later. Anti–PD-L1 Ab (or NrIgG) treatments were given i.p. every 3 to 4 d until the end of the experiment. As shown in Table II, combination therapy with DC-gp100 and anti–PD-L1 Ab was able to delay the growth of B16 in lymphopenic mice and enhance survival (p < 0.05 compared with all other groups); however, all mice eventually succumbed to tumor growth.

**Adoptive transfer of melanoma-specific T cells in combination with anti–PD-L1 Ab 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 postinjection of B16 tumor cells, mice were treated with 600 rad of TBI. On day 4, mice received an i.v. injection of 1 × 10⁷ 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 Ab starting on day 4 and continuing every 3 to 4 d for 60 d. An additional DC-gp100 treatment was given on day 11. As shown in Fig. 5A, tumors in mice treated with pmel T cells, DC-gp100, and anti–PD-L1 Ab demonstrated a significantly slower growth rate. Moreover, 40% of mice treated with pmel T cells, DC-gp100, and anti–PD-L1 Ab went on to show prolonged survival past 150 d (Fig. 5B).

**Persistence of melanoma-specific T cells in mice treated with anti–PD-L1 Ab**

We next examined whether pmel T cells persisted for a longer duration in mice treated with anti–PD-L1 Ab. Ly5.2 (CD45.1+) mice were treated with TBI. Mice received 1 × 10⁷ pmel T cells (CD45.2+) 1 d later in combination with either NrIgG or anti–PD-L1 Ab. Abs were given every 3 to 4 d for 4 wk. Splenocytes were collected at various time points, and the percentage of CD45.2+ T cells was measured by flow cytometry. As shown in Fig. 6A, mice treated with anti–PD-L1 Ab 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-γ by splenocytes from mice treated with pmel T cells and anti–PD-L1 Ab at day 30 (Fig. 6B; p < 0.05 compared with NrIgG-treated mice). In addition, splenocytes from mice treated with anti–PD-L1 Ab demonstrated enhanced proliferation against gp100 peptide at day 30 (Fig. 6C; 10,264 ± 1,538) compared with NrIgG-treated mice (2970 ± 1017 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 Ab.

** Trafficking of melanoma-specific T cells to tumor posttreatment with anti–PD-L1 Ab**

We next examined the percentage of pmel T cells present in the TILs of mice treated with anti–PD-L1 Ab. Mice were injected s.c. with B16 cells and received 600 rad of TBI on day 11. On day 12, mice received 1 × 10⁷ pmel T cells i.v. in combination with NrIgG or anti–PD-L1 Ab. Ab treatment was continued every 3 to 4 d 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 Fig. 7, mice treated with anti–PD-L1 Ab had a higher percentage of CD45.2+ T cells in TIL (6.6% compared with 3% in mice treated with NrIgG; p < 0.05).

**Table I. Treatment of B16 melanoma with peptide-pulsed DCs and anti–PD-L1 Ab**

<table>
<thead>
<tr>
<th>Treatment* (n = 6)</th>
<th>Mean Tumor Area (mm²)⁺</th>
<th>Mean Survival (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NrIgG</td>
<td>392 ± 8</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>Anti–PD-L1</td>
<td>347 ± 53</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>DC-gp100 plus NrIgG</td>
<td>333 ± 67</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>DC-gp100 plus anti–PD-L1</td>
<td>268 ± 72</td>
<td>31 ± 15</td>
</tr>
</tbody>
</table>

* Mice were treated beginning on day 3 after s.c. injection of B16 cells.
⁺ Measurements recorded on day 28.

**Table II. Treatment of B16 melanoma with TBI, peptide-pulsed DCs, and anti–PD-L1 Ab**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Mean Tumor Size (mm²)</th>
<th>Survival (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NrIgG</td>
<td>325 ± 4</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Anti–PD-L1</td>
<td>371 ± 46</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>DC-gp100 plus NrIgG</td>
<td>315 ± 52</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>DC-gp100 plus anti–PD-L1</td>
<td>175 ± 41*</td>
<td>46 ± 9*</td>
</tr>
</tbody>
</table>

* 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 Ab every 3 to 4 d beginning on day 4.
⁺ Tumor measurements were recorded on day 32.

*p < 0.05 compared with all other groups.
Adoptive transfer of effector T cells in combination with anti–PD-L1 Ab treatment

We next examined whether treatment with anti–PD-L1 Ab could enhance the therapeutic efficacy of adoptively transferred T cells isolated from mice previously immunized with DCs pulsed with OVASINFEKL (DC-OVA). Fig. 8A shows that PD-L1 is expressed by M05 melanoma cells (MFI 31.5). Mice were treated with TBI on day 3 postinjection of M05 cells. On day 4, mice received $1 \times 10^7$ T cells isolated from the spleens of DC-OVA immunized donor mice. In addition, DC-OVA immunizations were given s.c. to the mice each week. A. Tumor growth. B. Survival. The results are representative of two similar experiments. $^{*}p < 0.05$ compared with pmel plus DC-gp100 plus NrlgG-treated mice.

**Adoptive transfer of effector T cells in combination with anti–PD-L1 Ab treatment**

We next examined whether treatment with anti–PD-L1 Ab could enhance the therapeutic efficacy of adoptively transferred T cells isolated from mice previously immunized with DCs pulsed with OVAASINFEKL (DC-OVA). Fig. 8A shows that PD-L1 is expressed by M05 melanoma cells (MFI 31.5). Mice were treated with TBI on day 3 postinjection of M05 cells. On day 4, mice received $1 \times 10^7$ T cells isolated from the spleens of DC-OVA immunized donor mice. In addition, DC-OVA immunizations were given s.c. to the mice each week. A. Tumor growth. B. Survival. The results are representative of two similar experiments. $^{*}p < 0.05$ compared with pmel plus DC-gp100 plus NrlgG-treated mice.
recipients on days 4 and 11 along with NrIgG or anti–PD-L1 Ab beginning on day 4 and continuing every 3 to 4 d until the end of the experiment. Mice treated with the adoptive transfer of OVA-immune T cells in combination with anti–PD-L1 Ab displayed a significant delay in the growth of M05 tumor (Fig. 8; p < 0.05 compared with all other groups).

Discussion

DC-based vaccines are an effective means to induce antitumor 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 antitumor T cell responses. PD-L1 binds to PD-1 on activated T cells to induce and maintain peripheral tolerance (5). Pulko et al. (28) have shown that DCs generated from the bone marrow of PD-L1 knockout mice are superior to wild-type DCs at inducing both Ag-specific CD8+ T cell responses and tumor suppression. In our current study, blocking PD-L1 expression on DCs led to enhanced secretion of IFN-γ and cytotoxicity by Ag-specific T cells. In addition, vaccination with OVA peptide-pulsed DCs in combination with anti–PD-L1 Ab treatment induced more OVA-specific CD8+ T cells and enhanced IFN-γ secretion. Despite these positive effects, treatment of mice bearing B16 tumors with gp100 peptide-pulsed DCs in combination with anti–PD-L1 Ab 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 Ab and administered peptide-pulsed DCs in a lymphopenic environment. In this setting, the opportunity

![Image 1](https://example.com/image1.png)

**FIGURE 7.** Higher numbers of pmel T cells infiltrate B16 tumor in mice treated with anti–PD-L1 Ab. 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 Ab 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. This experiment was repeated two times with similar results. *p < 0.05 compared with NrIgG-treated mice.

![Image 2](https://example.com/image2.png)

**FIGURE 8.** Adoptive transfer of OVA-immune T cells enhances the antitumor response in M05-bearing mice treated with anti–PD-L1 Ab and peptide-pulsed DCs. A, Expression of PD-L1 on the surface of M05 cells. Filled histogram indicates anti–PD-L1; open histogram indicates 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 SINSFEKL. Beginning on day 4 and continuing every 3 to 4 d until day 60, mice received i.p. injections of either NrIgG or anti–PD-L1 Ab. Mice also received DCs pulsed with OVA SINSFEKL peptide s.c. on days 4 and 11 after B16 injection. Tumors were measured twice each week. The results are representative of two experiments. *p < 0.05 compared with T cells plus DC-OVA plus NrIgG-treated mice.
exists to educate reconstituting T cells during homeostasis-driven proliferation. Vaccination after the induction of lymphopenia has been shown to enhance the activation and expansion of antitumor 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 Ab alone after the induction of lymphopenia had no effect on tumor growth. Combination therapy with anti–PD-L1 Ab 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 pml6 T cells in mice treated with NrIgG or PD-L1 Ab (data not shown). However, pml6 T cells persisted longer in mice treated with anti–PD-L1 Ab. By day 30 after adoptive transfer, the number of pml6 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 antitumor 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 antitumor memory T cell responses (34). In our current study, adoptive transfer of pml6 T cells in combination with DC vaccination led to a delay in tumor growth in mice receiving either NrIgG or anti–PD-L1 Ab, but eventually all of the NrIgG-treated mice grew tumors and died. In contrast, some mice treated with anti–PD-L1 Ab 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 Ab 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.

Acknowledgments
We thank the Ocala Royal Dames for Cancer Research for their generous support. We also thank the Flow Cytometry Core Facility at the H. Lee Moffitt Cancer Center and Research Institute for its contribution to this work.

Disclosures
The authors have no financial conflicts of interest.

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10. Ahsanullah, M. L., A. J. Winn, T. A. Heemsker, and M. E. Dudley. 2002. B7-H1 ligands mediate positive selection in the thymus control T cell survival and immune stimulation cytokines, such as IL-15 and IL-7 (29), and the expression of PD-L1 late in antitumor 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 antitumor memory T cell responses (34). In our current study, adoptive transfer of pml6 T cells in combination with DC vaccination led to a delay in tumor growth in mice receiving either NrIgG or anti–PD-L1 Ab, but eventually all of the NrIgG-treated mice grew tumors and died. In contrast, some mice treated with anti–PD-L1 Ab 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 Ab 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.

Acknowledgments
We thank the Ocala Royal Dames for Cancer Research for their generous support. We also thank the Flow Cytometry Core Facility at the H. Lee Moffitt Cancer Center and Research Institute for its contribution to this work.

Disclosures
The authors have no financial conflicts of interest.

References


