Published in final edited form as:

Cancer Immunol Immunother. 2011 April ; 60(4): 547–558. doi:10.1007/s00262-010-0963-5.

Targeting NKT cells and PD-L1 pathway results in augmented anti-tumor responses in a melanoma model

Kevin Durgan,

Research Division, Puget Sound Blood Center, Seattle, WA 98104, USA

Mohamed Ali,

Research Division, Puget Sound Blood Center, Seattle, WA 98104, USA

Paul Warner, and

Research Division, Puget Sound Blood Center, Seattle, WA 98104, USA

Yvette E. Latchman

Research Division, Puget Sound Blood Center, Seattle, WA 98104, USA. Department of Medicine, Division of Hematology, University of Washington, Seattle, WA 98195, USA

Yvette E. Latchman: yvettel@psbcresearch.org

Abstract

Invariant or Type 1 NKT cells (iNKT cells) are a unique population of lymphocytes that share characteristics of T cells and natural killer (NK) cells. Various studies have shown that positive costimulatory pathways such as the CD28 and CD40 pathways can influence the expansion and cytokine production by iNKT cells. However, little is understood about the regulation of iNKT cells by negative costimulatory pathways. Here, we show that in vivo activation with α -GalCer results in increased cytokine production and expansion of iNKT cells in the absence of programmed cell death ligand-1 (PD-L1, B7-H1, and CD274). To study whether PD-L1 deficiency on NKT cells would enhance antigen-specific T-cell responses, we utilized CD8⁺ OT-1 OVA transgenic T cells. α -GalCer enhanced the expansion and cytokine production of OT-1 CD8⁺ cells after adoptive transfer into wild-type recipients. However, this expansion was significantly enhanced when OT-1 CD8⁺ T cells were adoptively transferred into PD-L1^{-/-} recipients. To extend these results to a tumor model, we used the B16 melanoma system. PD-L1^{-/-} mice given dendritic cells loaded with antigen and α -GalCer had a significant reduction in tumor growth and this was associated with increased trafficking of antigen-presenting cells and CD8⁺ T cells to the tumors. These data demonstrate that abrogating PDL1:PD-1 interactions during the activation of iNKT cells amplifies an anti-tumor response when coupled with DC vaccination.

Keywords

Costimulation; Programmed cell death-1; Programmed cell death ligand-1; Tumor; Invariant NKT cells; T cells

Introduction

Natural Killer T cells (NKT cells) are a unique population of lymphocytes that share characteristics of T cells and natural killer (NK) cells. The key features of NKT cells are a biased T-cell receptor (TCR) gene expression, restriction by CD1d molecules on antigen-presenting cells, and rapid production of a spectrum of cytokines following activation. In numerous murine models, NKT cells have been demonstrated to be important in: (1) protection against infectious pathogens and diseases, (2) immune surveillance of tumors, (3) induction and control of autoimmunity, and (4) asthma (reviewed in [1]). It has become apparent that both human and murine NKT cells represent a heterogeneous population of cells, with functional differences ascribed to differential expression of cell-surface markers such as CD4, CD8, and chemokine receptors [2]. It has also been shown that CD1d-restricted NKT cells can be subdivided into $V\alpha 14-J\alpha 18^+$ α -GalCer-reactive Type I NKT cells, termed invariant NKT cells (iNKT), and α -GalCer non-reactive Type II NKT cells (reviewed in [3]). Type II NKT cells produce IL-13 and may downregulate immune responses by inducing myeloid cells to produce TGF- β . Several factors may influence the activation and function of NKT cells: (1) cytokine environment, (2) interacting antigen-presenting cell, (3) subset of NKT cell or anatomical location, or (4) costimulatory interactions [1]. Thus, while all subsets of NKT cells are not well defined, the ability of these cells to influence the adaptive immune response is well accepted [1].

The rapid response to antigen by iNKT cells has led to the concept that these cells, unlike conventional T cells, may not require secondary signals to become activated. However, there is growing evidence that costimulation may play a role in the activation of iNKT cells. Murine and human iNKT cells constitutively express CD28 and rapidly upregulate CD40L (CD154) on activation [4, 5]. It also has been shown that blocking antibodies to B7, CD28, CD40, and CD154 inhibits the α -GalCer-induced proliferation of murine iNKT cells [6]. In addition, several studies have shown a role for CD40:CD40L and B7:CD28 pathways in enhancing cytokine production by iNKT cells in vitro and in vivo [4, 7, 8]. In contrast, other studies have shown no role for these pathways in the initial cytokine production by iNKT cells but costimulation was important for the expansion and subsequent systemic cytokine production by NKT cells [9, 10]. Recent studies indicate that the B7:CD28 pathway might be important in the development of iNKT cells [11, 12]. Newly discovered costimulatory molecules also have been implicated in the function of iNKT cells. Interestingly, blockade of the inducible costimulatory molecule (ICOS) has been shown to inhibit α -GalCer-induced cytokine production and anti-tumor effects of NKT cells [13]. Furthermore, iNKT cells from ICOS^{-/-} mice produced less IL-4 and IL-13 and failed to induce airway hyper-reactivity in a murine model of asthma [14]. The PD-1 pathway has been shown to be involved partially in anergy induced by α -GalCer, and the CD28 pathway is important in overcoming the PD-1 inhibition on iNKT cells [11, 15–17]. Therefore, similar to the activation of conventional T cells, it appears that iNKT cells have the same constraints for costimulation and negative regulatory signals.

The greatest challenge to an effective immune response against tumors is finding ways to overcome the immunosuppressive effect of the tumor itself. To address this factor, combination strategies to target tumors through the immune system have become increasingly prevalent. The knowledge that iNKT function can be modulated by secondary signals and their ability to influence adaptive immunity through cognate interactions with antigen-presenting cells has led us to examine whether the blockade of PD-L1:PD-1 pathways together with NKT cell activation will lead to an effective anti-tumor CD8⁺ T-cell response in a solid tumor model. In this study, we show that the PD-1 pathway negatively regulates iNKT expansion, and abrogation of the PD-1 pathway results in a further expansion of antigen-specific responses. Our results are the first to show that combining

NKT cell activation with inhibition of negative costimulation can affect the growth of B16 cells in a solid tumor model.

Materials and methods

Animals

C57BL6 and OT-II mice were purchased from Jackson Laboratory (Bar Harbor, ME). PD-L1-deficient ($^{-/-}$) mice were previously described [18]. OT-I CD45.1/2 heterozygous mice and *V α 14/J α 18*-deficient mice were kindly provided by Dr. Keith Elkon (University of Washington) and Dr. M Taniguchi (RIKEN Research Center for Allergy and Immunology), respectively. All animals were maintained under SPF conditions at the University of Washington in accordance with approved protocols and IACUC standards.

Antibodies and reagents

mCD1d/PBS57 and unloaded mCD1d tetramers were obtained from NIAID MHC tetramer core facility. α -GalCer (KRN7000) and vehicle were initially provided by Kirin Pharma Company and then obtained from Avanti Polar Lipids Inc (Alabaster, AL). Anti-PD-L1 (M1H5), anti-PD-1 (RMPI-30), anti-PD-L2 (TY25), anti-CD4 (L3T3), anti-NK1.1 (PK136), anti-CD19 (eBio1D3), anti-CD8 (53-6.7), anti-CD45.1 (A20), anti-CD28 (37.51), anti-CD3 (17A2), and anti-V α 2 TCR (B20.1) were obtained from eBiosciences (San Diego, CA). Anti-V β 5.1,5.2 TCR was obtained from BD Biosciences (San Diego, CA). All cytokine primary and detection antibodies for ELISA, ELISPOT, and intracellular staining were obtained from eBiosciences. OVA_{323–339} and OVA_{257–264} peptides were obtained from AnaSpec (San Jose, CA).

iNKT cell activation in vivo and in vitro

Mice were given 2 μ g α -GalCer or vehicle i.p. After 2 h, splenocytes were isolated and treated with $1\times$ RBC lysis buffer (eBiosciences). A total of 2×10^5 cells were resuspended in RPMI 1640 (Mediatech Herndon, VA) supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin 100 mg/ml, HEPES (10 mM), and 2-mercaptoethanol (50 mM) (all from Mediatech) and cultured in cytokine-coated ELI-SPOT plates for 18 h. For intracellular staining, splenocytes were isolated and cultured with monesin (eBiosciences) for 2 h. Cytokine analysis was preformed using Perm and Fix solutions from eBiosciences.

To study expansion of iNKT cells, mice were given 2 μ g α -GalCer or vehicle i.p. After 3 or 7 days, splenocytes were isolated and NKT cell expansion analyzed by flow cytometry using mCD1d/PBS57 tetramer, anti-CD4, and anti-CD19. Unloaded mCD1d tetramer was used as a control.

To analyze purified iNKT cells, splenocytes were depleted of B cells (anti-CD19) and CD8⁺ T cells (anti-CD8) using Biomag beads (Qiagen, Valencia, CA) and mCD1d/PBS57 tetramer⁺ cells were sorted. Purified iNKT cells (5×10^4) were cultured with 5×10^4 dendritic cells and 100 ng/ml α -GalCer. Supernatants were harvested after 5 days and analyzed for cytokines.

Dendritic cell isolation

Bone marrow-derived dendritic cells were prepared by depletion of bone marrow cells with anti-CD3, anti-CD19, and Gr-1. Cells were grown in GM-CSF and IL-4 for 8 days, and CD11c⁺ cells were isolated by anti-CD11c beads and magnetic-activated cell sorting separation (MACS) columns (Miltenyi Biotec, Auburn, CA) or cell sorting. For cytokine production, mice were given 2 μ g α -GalCer i.p. After 8 h, spleens were isolated and treated

with collagenase. CD11c⁺ cells were isolated from spleens using anti-CD11c beads as above. CD11c⁺ cells were cultured for 48 h and IL-12 production analyzed by ELISA.

Adoptive transfer studies

Purified iNKT cells were isolated as above. A total of 2×10^5 NKT cells were adoptively transferred into *Vα14-Jα18*-deficient mice (NKT type I-deficient mice). Two days later, mice were given α -GalCer or vehicle i.p and cytokine production analyzed by ELISPOT.

For antigen-specific studies, OT-I or OT-II cells were isolated by positive selection using anti-CD8 or anti-CD4 beads, respectively; 1×10^6 OT-I or OT-II cells were adoptively transferred into mice. The following day, mice were given OVA peptide (50 μ g/ml) together with 2 μ g α -GalCer or vehicle i.v. Four days later, splenocytes were isolated and stained with anti-CD45.1, anti-V α 2, and anti-V β 5.1 antibodies. Splenocytes were restimulated with 1 μ g/ml of OVA peptide and supernatants analyzed by ELISA.

Tumor studies

CD11c⁺ cells were loaded with 200 ng/ml α -GalCer or vehicle overnight and further cultured for 2 h with TRP-2_{181–188} peptide. A total of 5×10^5 dendritic cells were injected into their respective host, i.e., C57BL/6 dendritic cells into C57BL/6 mice or PD-L1^{−/−} dendritic cells into PD-L1^{−/−} mice. For the metastasis model, mice were challenged with 5×10^5 B16-F1 cells i.v (ATCC) 2 days later. After 14 days, mice were killed and lungs removed and placed in formalin. Tumor nodules were counted by light microscopy. For effector function, splenocytes were expanded for 7 days with TRP-2_{181–188} peptide and IL-2 and then cultured with B16 cells for a further 24 h. IFN- γ production was measured by ELISA. In the subcutaneous model, 2×10^5 B16-F1 melanoma cells were injected subcutaneously (s.c) in the right flank of mice and tumor growth was measured by calipers and analyzed by diameter (length $\times$ width) every 7 days. Mice were killed after 3 weeks or before the tumor on an individual mouse reached a diameter of 20 mm², and results were expressed as mean $\pm$ SD.

Results

The interaction of dendritic cells and iNKT cells and the subsequent cytokine release can be one of the features that bridge the gap between innate and adaptive immunity. We hypothesized that control of the interaction of dendritic cells and iNKT cells is crucial and governed by inhibitory pathways expressed on iNKT cells. To begin to address this hypothesis, we examined the expression of PD-1 and its ligands on iNKT cells derived from spleen, liver, and thymus of wild-type mice. PD-L1 was constitutively expressed on iNKT cells in spleen, liver, and thymus (Supplementary Fig. 1 and [16, 17]). PD-1 or PD-L2 was not expressed on freshly isolated iNKT cells. However, PD-1 and PD-L1 but not PD-L2 expression was upregulated with α -GalCer and IL-2 and in vivo after α -GalCer treatment (Supplementary Fig. 1A,B). To investigate whether PD-L1 deficiency influenced the development of iNKT cell, lymphocytes were isolated from liver, spleen, and thymus of C57BL/6 and PD-L1^{−/−} mice and stained with CD1d-tetramer (Fig. 1a). The percentage and number of iNKT cells was similar in the spleen, thymus, and liver of PD-L1^{−/−} mice when compared to wild-type mice (Fig. 1b). iNKT cells are subdivided according to their CD4 and NK1.1 expression. CD4⁺ and CD4[−] subsets of NKT cells were similar in PD-L1^{−/−} and wild-type mice (Fig. 1a).

The PD-1 pathway has been shown to be important in negatively regulating the activation of conventional T cells. To investigate the PD-1 pathway in NKT activation, splenocytes from PD-L1^{−/−} and wild-type mice were cultured with 100 ng/ml of α -GalCer or vehicle in

ELISPOT plates for 36 h. PD-L1^{-/-} iNKT cells produced increased IFN- γ and IL-4, compared to iNKT cells from wild-type mice (Supplementary data Fig. 1C). In splenocyte cultures, NKT cells are able to activate other cell types to produce cytokines, in particular NK cells. To study whether the cytokine production was intrinsic to the PD-L1^{-/-} iNKT cells, we sorted CD1d tetramer⁺ NKT cells from the livers of wild-type and PD-L1^{-/-} mice and cultured them with their respective dendritic cells, i.e., PD-L1^{-/-} CD1d tetramer⁺ NKT cells were cultured with PD-L1^{-/-} dendritic cells. PD-L1^{-/-} iNKT cells cultured with PD-L1^{-/-} DC and α -GalCer produced more IL-4 and IFN- γ indicating that this effect was intrinsic to the NKT cell (Fig. 2a). This was similar to the results seen with conventional T cells where PDL1^{-/-} T cells produced more IFN- γ than wild-type T cells [18]. Several studies have shown that NK1.1⁺ iNKT cells produce IL-17 (reviewed in [2]). Interestingly, PD-L1^{-/-} iNKT cells also produced increased levels of IL-17 (Fig. 2a). Our data imply that PD-L1 expression is necessary for negatively regulating NKT cells.

To study iNKT function in vivo, α -GalCer (2 μ g) or vehicle was administrated i.p. to PD-L1^{-/-} or wild-type mice. After 2 or 4 h, splenocytes were isolated and cytokine production was analyzed by ELISPOT. After α -GalCer activation in vivo, there was an increase in the number of cells producing IFN- γ , IL-4, and IL-17 in PD-L1^{-/-} mice when compared to wild-type mice (Fig. 2b). These results suggest that blocking the PD-L1 pathway influences Th1-, Th2-, and Th17-producing subsets of NKT cells. In addition, the ability of PD-L1^{-/-} NKT cells to activate dendritic cells in vivo was investigated. PD-L1^{-/-} and wild-type mice were treated with α -GalCer for 8 h and CD11c⁺ cells were isolated and IL-12 production analyzed by ELISA 48 h later. As shown in Fig. 2c, PD-L1^{-/-} DC produced more IL-12 compared to wild-type DC after α -GalCer treatment of iNKT cells. To study the intrinsic role of PD-L1 deficiency on NKT cells in vivo, PD-L1^{-/-} or wild-type NKT cells were sorted and adoptively transferred to iNKT-deficient (*Ja18* deficient) mice. After 2 days, recipient mice were given α -GalCer i.p. and 2 h later, splenocytes were isolated and analyzed. As shown in Fig. 2d, adoptively transferred PD-L1^{-/-} iNKT cells produced more cytokines than wild-type iNKT cells. iNKT cells are unique in their ability to rapidly produce cytokines after TCR engagement. This is due in part to the fact that NKT cells store preformed mRNA for various cytokines (reviewed in [19, 20]). To investigate whether PD-L1 deficiency affects the initial cytokine burst by iNKT cells, mice were given α -GalCer for 1 h and splenocytes were isolated and intracellular staining was preformed. There was no difference in cytokine production between PD-L1 and wild-type iNKT cells at this early time point indicating that the PD-L1 pathway had no role in the initial cytokine production by iNKT cells (Fig. 2e).

It has been previously shown that the B7:CD28 pathway is involved in the expansion of NKT cells [10]. As cytokine production by PD-L1^{-/-} iNKT cells was increased at later time points, it prompted us to study the role of the PD-L1 pathway in iNKT cell expansion. PD-L1^{-/-} and wild-type mice were immunized with 2 μ g α -GalCer or vehicle i.p. Mice were killed after 72 h and splenic and hepatic lymphocytes were isolated and stained to enumerate the percentage and total number of NKT cells and subsets (Fig. 3a). The percentage of CD1d tetramer⁺ NKT cells was significantly increased after in vivo activation in PD-L1^{-/-} mice when compared to wild-type mice (7.5% $\pm$ 0.45 in PD-L1^{-/-} mice vs. 5.2% $\pm$ 0.7 in wild-type mice; $P=0.03$). There was an even more significant increase in the number of CD1d tetramer⁺ NKT cells in PD-L1^{-/-} mice (Fig. 3b). This in vivo increase in CD1d tetramer⁺ NKT cells in PD-L1^{-/-} mice was due to an increase in both CD4⁺ and CD4⁻ subsets. This result also was reflected in the expansion of iNKT cells in the liver (Fig. 3c). PD-L1 deficiency had no effect on the normal contraction of iNKT cells seen at Day 7 (Supplementary Fig. 2A). These data indicate that the PD-1 pathway is involved in counteracting the expansion of NKT cells induced by signals through the TCR and CD28 pathway.

NKT activation can cause bystander activation of other cell types, assessed by an increase in CD69 expression [21]. Wild-type and PD-L1^{-/-} B cells, CD8⁺ T cells, and NK cells, but not CD4⁺ T cells upregulated CD69 expression after α -GalCer treatment (Supplementary Fig. 2B). PD-L1^{-/-} B cells expressed higher levels of CD69 after α -GalCer treatment compared to wild-type B cells (Supplementary Fig. 2B). It has been shown that NK cells are rapidly activated after α -GalCer treatment of iNKT cells [21]. A hallmark of this activation is IFN- γ production by NK cells. As PD-L1 and PD-1 are expressed on NK cells after activation (Supplementary data Fig. 2C), we investigated the transactivation of NK cells in PD-L1^{-/-} and wild-type mice after in vivo administration of α -GalCer. NK cells were intracellularly stained for IFN- γ production directly ex vivo. As previously shown, wild-type NK cells produced IFN- γ after α -GalCer treatment. Surprisingly, there was a slight but significant decrease in IFN- γ production by PD-L1^{-/-} NK cells (Supplementary Fig. 2D).

Several studies have shown that iNKT can enhance T-cell responses through dendritic cell maturation and CD40 stimulation [22–26]. To study whether PD-L1 deficiency on iNKT cells would enhance antigen-specific T-cell responses, we utilized CD8⁺ OT-1 OVA transgenic T cells. A total of 1×10^6 OT-1 cells were adoptively transferred into PD-L1^{-/-} or wild-type recipients. The following day, mice were given 25 μ g SINNFEEKL peptide plus α -GalCer or vehicle i.v. Groups of mice were left untreated. Four days later, splenocytes were analyzed for the expansion of OT-1 cells and cells were restimulated in vitro with SINNFEEKL peptide. α -GalCer enhanced the expansion and cytokine production of OT-1 CD8⁺ cells after adoptive transfer into wild-type recipients (Fig. 4a, b). This was significantly enhanced when OT-1 CD8⁺ T cells were adoptively transferred into PD-L1^{-/-} recipients (Fig. 4a, b). PD-L1 deficiency in the recipient also increased expansion and cytokine production of OT-1 CD8⁺ T cells with peptide alone.

To study the effect of iNKT activation on CD4⁺ T-cells responses, we adoptively transferred OT-II CD4⁺ T cells into wild-type and PD-L1^{-/-} mice, and injected the mice with OVA peptide and α -GalCer or control vehicle. There was a significant expansion when α -GalCer was used with OVA peptide in wild-type and PD-L1^{-/-} mice, compared to mice injected with vehicle plus OVA. While wild-type recipients given OVA peptide and α -GalCer had a higher average expansion of OT-II CD4⁺ T cells, compared to PD-L1^{-/-} mice treated in the same manner, it was not statistically significant (Fig. 4c). However, OT-II CD4 T cells activated with α -GalCer and OVA peptide from PD-L1^{-/-} recipients produced more IFN- γ than when activated in their wild-type counterparts (Fig. 4d). This indicates that PD-L1 deficiency on iNKT cells can result in an enhanced antigen-specific response, although there appears to be a more profound effect on CD8⁺ T cells.

Our results with enhanced CD8⁺ T-cell responses lead us to investigate whether this effect would result in clearance of tumors in B16 tumor model. There was no difference in the size of the tumors of PD-L1^{-/-} mice when compared to controls (Fig. 5a). In addition, the onset of tumor growth was similar in wild-type and PD-L1^{-/-} mice (data not shown). To determine whether there was an immune response to B16 tumors, splenocytes were isolated from PD-L1^{-/-} mice and wild-type mice immunized with tumor cells. Splenocytes were cultured with tyrosinase-related protein-2 peptide (TRP_{2181–188}) and IL-2 for 7 days. Viable cells were recovered and effector cells were cultured with B16 cells and IFN- γ production was measured. There was an increase in IFN- γ production by effector cells from PD-L1^{-/-} mice when compared to wild-type mice (Fig. 5b). In a metastases model, B16 cells were injected i.v into PDL1^{-/-} or wild-type mice, and we showed comparable results to our subcutaneous model (Fig. 5c, d). These results indicate that although PD-L1^{-/-} mice have a more efficient response to B16 tumors than wild-type mice, this effector response was insufficient to clear poorly immunogenic B16 tumors.

The adoptive transfer of dendritic cells loaded with tumor antigens has become an attractive immunotherapy for cancer (reviewed in [27]). Various methods have been used to increase the efficacy of dendritic cell vaccination protocols including gene transfection, modulation of costimulatory pathways, and manipulation of iNKT cells [28–33]. To explore whether we could overcome the immunosuppressive effects of B16 tumors, we adoptively transferred wild-type or PD-L1^{-/-} dendritic cells preloaded with TRP-2 and α -GalCer or vehicle, 2 days prior to B16 inoculation i.v (metastasis model). Dendritic cells generated from wild-type and PD-L1^{-/-} mice upregulated costimulatory molecules in a similar fashion after α -GalCer stimulation (Supplementary Fig. 3A). The adoptive transfer of PD-L1^{-/-} dendritic cells preloaded with TRP-2/vehicle into PD-L1 recipients resulted in a 50% reduction in tumor nodules in the lung. However, wild-type mice cleared B16 tumors when dendritic cells were loaded with TRP-2 and α -GalCer. PD-L1 deficiency did not decrease or accelerate the clearance of the tumors (Supplementary Fig. 3B). This result is consistent with the published data showing that α -GalCer-pulsed dendritic cells can inhibit tumor growth mainly through NK-dependent killing, and in Supplementary Fig. 3D, we show no difference in the transactivation of NK cells [34]. It has been reported that dendritic cells loaded with α -GalCer did not inhibit tumor growth in a subcutaneous B16 model; therefore, we investigated whether antigen and α -GalCer-pulsed dendritic cells would influence the growth of B16 tumors s.c. Mice were immunized with dendritic cells loaded with TRP-2 and α -GalCer or vehicle or α -GalCer only, 1 day prior to B16 challenge. There was no effect on B16 tumor growth whether wild-type mice were given dendritic cells loaded with TRP-2 and α -GalCer or TRP-2 and vehicle (Fig. 6a). However, PD-L1^{-/-} mice given PD-L1^{-/-} dendritic cells loaded with TRP-2 and α -GalCer had a significant reduction in tumor growth (Fig. 6a).

To investigate the mechanism by which NKT activation and lack of PD-1 interactions results in decreased tumor growth, we analyzed the tumor-infiltrating lymphocytes (TIL) by flow cytometry. Tumors were excised and weighed, and there was a significant reduction in the weight of tumors from PD-L1^{-/-} mice given dendritic cells loaded with TRP-2 compared to wild-type mice given the same treatment (Fig. 6b). There was significant increase in IFN- γ -producing cells in the tumors of PD-L1^{-/-} mice given TRP-2 and α -GalCer-pulsed dendritic cells compared to wild-type mice (Fig. 6c). However, there was no difference between the number of IFN- γ -producing cells in the tumors of PD-L1^{-/-} mice given dendritic cells loaded with TRP-2 and vehicle or TRP-2 and α -GalCer, suggesting that IFN- γ production is not the mechanism behind decreased tumor load in PD-L1^{-/-} mice treated with α -GalCer (Fig. 6c). On investigation of the cell types within tumors, there was a significant increase in dendritic cells (CD11c^{high} cells), macrophages (CD11b⁺), NK cells (NK1.1⁺), and CD8⁺ T cells in PD-L1^{-/-} mice, in contrast to wild-type mice (Fig. 6d). Data are presented as the number of cells per gram of tumor tissue [35]. Surprisingly, there also was a significant increase in CD4⁺ Foxp3⁺ T-regulatory cells (Tregs) and this may account for the incomplete clearance of tumors in PD-L1^{-/-} mice. To test the efficacy of NKT cell activation and PDL1 abrogation in a therapeutically relevant setting, mice were given dendritic cells loaded with TRP-2 and α -GalCer 2 or 4 days after the B16 inoculation. PD-L1^{-/-} mice given dendritic cells 4 days after B16 inoculation had a small but significant decrease in tumor growth (Fig. 6e). Although there was an increase in antigen-presenting cells, there was not an increase in CD8⁺ T cells necessary for the clearance of the tumor (Fig. 6f). This data imply that in a therapeutic setting, as with other models of combination therapy, Treg depletion may be required [35].

Discussion

Several studies have established that the PD-L1:PD-1 pathway is involved in negatively regulating conventional T cells. However, PD-L1 is expressed on a variety of innate

lymphocytes and the examination of the function of this pathway on innate cells has not been well characterized. In this study, we clearly demonstrate that the PD-L1 pathway inhibits the expansion and the cytokine production of iNKT cells. In addition, deficiency of PD-L1 on iNKT cells influenced an antigen-specific response even with intact PD-1 molecules on CD8⁺ T cells (Fig. 4). Furthermore, tumor-specific T cells were enhanced in the presence of NKT cell activation and antigen in PD-L1-deficient mice. These results indicate that negative pathways on innate cells play an important role in controlling a subsequent adaptive immune response and therefore warrant further investigation.

It has been shown previously that PD-1 pathway is involved in anergy induction of iNKT cells after repeated α -GalCer stimulation [16, 17]. These studies did not specifically address the expansion of iNKT after initial α -GalCer treatment; however, data presented by Parekh et al. showed a marginal increase in iNKT numbers by α -GalCer treatment in PD-1-deficient mice. This discrepancy might be due to the fact that PD-L1 deficiency removes two interactions, between PD-L1 and PD-1 as well as CD80, therefore eliminating the negative PD-1 pathway and allowing the engagement of CD80 and CD28. As the balance of signals through PD-L1/PD-1 and CD28 pathways are counterbalanced in the expansion of iNKT cells, blocking of PD-L1 rather than PD-1 would be more effective [15]. The study of iNKT cells in PD-1/B7 double-deficient mice, PD-L1-, and PD-1-deficient mice will give a clearer understanding of the contribution of the PD-1 pathway in regulating the expansion of iNKT cells [36]. Previous studies have revealed that PD-L1^{-/-} mice have enhanced T-cell responses in the presence of the second ligand for PD-1, PD-L2 (B7-DC, CD273) [18, 37]. Both PD-L1 and PD-L2 (B7-DC, CD273) are upregulated by α -GalCer on dendritic cells (Supplementary Fig. 3A and data not shown). Therefore, these results would indicate that the lack of PD-L1 on iNKT cells is important for heterotypic interactions between PD-L1 and PD-1 on iNKT cells as seen in conventional T cells. This is supported by the fact that adoptive transfer of PD-L1^{-/-} iNKT cells into iNKT-cell-deficient recipients, where PD-L1 is only deficient on the iNKT cells, leads to increased cytokine production compared to the adoptively transferred wild-type NKT cells. We are the first to show that the PD-L1 pathway negatively regulates IL-17 production by iNKT cells and therefore blocking the PD-L1 pathway not only has the potential to manipulate Th1 and Th2 subsets but also Th17-producing cells.

The idea that iNKT cells can bridge the gap between the innate and adaptive immune response is well accepted although the exact mechanism by which this may occur is not clear. α -GalCer treatment results in the maturation of dendritic cells as well as the release of various cytokines from iNKT cells. In our study, we do not show any difference in the maturation of dendritic cells as defined by the expression of CD80, CD86, and CD40, between wild-type and PD-L1^{-/-} mice. However, PD-L1^{-/-} dendritic cells and NKT cells produce more cytokines than wild-type dendritic cells and iNKT cells and this factor might account for the increased expansion of OVA-specific CD8⁺ T cells. Interestingly, there was an increase in the bystander activation of B cells but not NK cells when PD-L1^{-/-} mice were given α -GalCer in the context of antigen presentation. The ability of iNKT cell activation to target B cells as well as dendritic cells might be an important factor in the adjuvant effect of α -GalCer.

Blockade of negative regulators of the CD28 superfamily is a potent strategy for the design of therapeutic cancer vaccines due to powerful inhibitory role they play on the activation of naïve and effector T cells. It was originally shown in mice that some tumors expressing B7 were rejected with anti-CTLA-4 blockade [38, 39]. Since regression of tumors with anti-CTLA-4 was not as successful with poorly immunogenic tumors such as B16, combination therapy was employed to facilitate a robust immune response. Clinical trials for melanoma and prostate cancer are ongoing but autoimmune adverse reactions have been associated

with clinical response with anti-CTLA-4 treatment. In our studies, PD-L1^{-/-} mice had reduced tumor burden when dendritic cells were activated with α -GalCer and preloaded with antigen. It has been reported that dendritic cells loaded with α -GalCer inhibited tumor growth in a metastasis but not a subcutaneous B16 model [34]. Our data shows that combining abrogation of the PD-L1 pathway together with iNKT activation can overcome the immunosuppressive effects of B16-F1 cells. B16-F1 cells express PD-L1 (Supplementary data Fig. 4) and therefore our combination therapy reduced tumor growth even in the presence of PD-L1 on the tumor. In addition, PD-L1-deficient mice did not show signs of autoimmune depigmentation suggesting that PD-L1 blockade did not lead to an autoimmune response. The lack of autoimmune disease also has been observed when PD-L1 blockade with combined with Gvax or Fvax [40, 41]. This would imply that blocking the PD-L1 pathway in therapeutic setting would result in fewer autoimmune side effects. This is further evidence for independent and non-overlapping roles that the CTLA-4 and PD-L1 pathways may play regulating the cells within the immune system. It is still unclear whether these pathways may target T cells at various effector phases.

We observed increased Tregs within the TIL of PD-L1^{-/-} mice that have reduced tumor growth and there was no difference in the CD8⁺T cell:Treg ratio of wild-type and PD-L1-deficient mice. This result is somewhat surprising and counterintuitive, as two groups have shown that the PD-L1 pathway induces Tregs in vitro [42, 43]. However, a recent study showed that PD-L1 blockade increased Tregs in a mesothelioma tumor model and required CD4⁺ T-cell removal plus anti-PD-L1 treatment to reduce tumor growth [44]. In the tumor setting with high TGF- β concentrations within the tumors, Tregs may be induced more readily by PD-L1 blockade. Notably, Fvax in combination with anti-PDL1 blockade increased Tregs in TIL whereas Fvax plus anti-PD-L1 and anti-CTLA-4 decreases Tregs in TIL [40], showing that the PD-1 and CTLA pathways target Tregs in different ways. Inclusion of an anti-Treg regime with NKT cell activation may result in further clearance of tumors in PD-L1-deficient mice. We detected increased antigen-presenting cells in the TIL of PD-L1 mice which may be due to α -GalCer treatment, as there was a non-significant increased number of antigen-presenting cells in the TIL of PD-L1^{-/-} mice given dendritic cells activated with α -GalCer compared to wild-type mice treated the same (data not shown). Collectively, these results gives supporting evidence that the adjuvant effects of α -GalCer together with antigen and PD-L1 blockade can augment tumor responses. Furthermore, in a clinical setting, the outcome of PD-L1 blockade might result in fewer side effects, compared to the anti-CTLA-4 blockade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Pei Fan for her excellent technical support. This study was funded by National Institute of Health 5R21A1066135 (YEL and PW) and Leukemia and Lymphoma society 3321-03 (YEL).

References

1. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol.* 2004; 4:231–237. [PubMed: 15039760]
2. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol.* 11:197–206. [PubMed: 20139988]
3. Berzofsky JA, Terabe M. A novel immunoregulatory axis of NKT cell subsets regulating tumor immunity. *Cancer Immunol Immunother.* 2008; 57:1679–1683. [PubMed: 18369622]

4. Hayakawa Y, Takeda K, Yagita H, Van Kaer L, Saiki I, Okumura K. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J Immunol.* 2001; 166:6012–6018. [PubMed: 11342617]
5. DelaRosa O, Tarazona R, Casado JG, Alonso C, Ostos B, Pena J, Solana R. Valpha24+ NKT cells are decreased in elderly humans. *Exp Gerontol.* 2002; 37:213–217. [PubMed: 11772506]
6. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* 1997; 278:1626–1629. [PubMed: 9374463]
7. Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, Sato M, Takeda K, Okumura K, Van Kaer L, Kawano T, Taniguchi M, Nakui M, Sekimoto M, Koda T. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol.* 2000; 12:987–994. [PubMed: 10882410]
8. Ikarashi Y, Mikami R, Bendelac A, Terme M, Chaput N, Terada M, Tursz T, Angevin E, Lemonnier FA, Wakasugi H, Zitvogel L. Dendritic cell maturation overrules H-2D-mediated natural killer T (NKT) cell inhibition: critical role for B7 in CD1d-dependent NKT cell interferon gamma production. *J Exp Med.* 2001; 194:1179–1186. [PubMed: 11602646]
9. Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, Mohrs M, Locksley RM, Kronenberg M. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc Natl Acad Sci USA.* 2003; 100:8395–8400. [PubMed: 12829795]
10. Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, Bouillet P, Strasser A, Smyth MJ, Godfrey DI. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J Immunol.* 2005; 175:3092–3101. [PubMed: 16116198]
11. Zheng X, Zhang H, Yin L, Wang CR, Liu Y, Zheng P. Modulation of NKT cell development by B7-CD28 interaction: an expanding horizon for costimulation. *PLoS One.* 2008; 3:e2703. [PubMed: 18628995]
12. Williams JA, Lumsden JM, Yu X, Feigenbaum L, Zhang J, Steinberg SM, Hodes RJ. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. *J Immunol.* 2008; 181:907–917. [PubMed: 18606642]
13. Kaneda H, Takeda K, Ota T, Kaduka Y, Akiba H, Ikarashi Y, Wakasugi H, Kronenberg M, Kinoshita K, Yagita H, Okumura K. ICOS costimulates invariant NKT cell activation. *Biochem Biophys Res Commun.* 2005; 327:201–207. [PubMed: 15629449]
14. Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, DeKruyff RH. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol.* 2008; 180:5448–5456. [PubMed: 18390727]
15. Wang J, Cheng L, Wondimu Z, Swain M, Santamaria P, Yang Y. Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *J Immunol.* 2009; 182:6644–6647. [PubMed: 19454657]
16. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, Kumar V, Wu L, Kaer LV. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol.* 2009; 182:2816–2826. [PubMed: 19234176]
17. Chang WS, Kim JY, Kim YJ, Kim YS, Lee JM, Azuma M, Yagita H, Kang CY. Cutting edge: programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J Immunol.* 2008; 181:6707–6710. [PubMed: 18981087]
18. Latchman YE, Liang SC, Wu Y, Chernova T, Sobel RA, Klemm M, Kuchroo VK, Freeman GJ, Sharpe AH. PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci USA.* 2004
19. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol.* 2003; 21:483–513. [PubMed: 12543936]
20. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol.* 2005; 23:877–900. [PubMed: 15771592]

21. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, Bendelac A. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol.* 1999; 163:4647–4650. [PubMed: 10528160]
22. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med.* 2004; 199:1607–1618. [PubMed: 15197224]
23. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med.* 2003; 198:267–279. [PubMed: 12874260]
24. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, Sato M, Takeda K, Okumura K, Van Kaer L, Kawano T, Taniguchi M, Nishimura T. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med.* 1999; 189:1121–1128. [PubMed: 10190903]
25. Vincent MS, Leslie DS, Gumperz JE, Xiong X, Grant EP, Brenner MB. CD1-dependent dendritic cell instruction. *Nat Immunol.* 2002; 3:1163–1168. [PubMed: 12415264]
26. Gumperz JE. CD1d-restricted “NKT” cells and myeloid IL-12 production: an immunological crossroads leading to promotion or suppression of effective anti-tumor immune responses? *J Leukoc Biol.* 2004; 76:307–313. [PubMed: 15123775]
27. Gilboa E. DC-based cancer vaccines. *J Clin Invest.* 2007; 117:1195–1203. [PubMed: 17476349]
28. Zitvogel L, Mayordomo JI, Tjandrawan T, DeLeo AB, Clarke MR, Lotze MT, Storkus WJ. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med.* 1996; 183:87–97. [PubMed: 8551248]
29. Eggert AA, Schreurs MW, Boerman OC, Oyen WJ, de Boer AJ, Punt CJ, Figdor CG, Adema GJ. Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res.* 1999; 59:3340–3345. [PubMed: 10416590]
30. Kaplan JM, Yu Q, Piraino ST, Pennington SE, Shankara S, Woodworth LA, Roberts BL. Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. *J Immunol.* 1999; 163:699–707. [PubMed: 10395660]
31. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol.* 2002; 3:867–874. [PubMed: 12154358]
32. Yamano T, Kaneda Y, Huang S, Hiramatsu SH, Hoon DS. Enhancement of immunity by a DNA melanoma vaccine against TRP2 with CCL21 as an adjuvant. *Mol Ther.* 2006; 13:194–202. [PubMed: 16112911]
33. Begley J, Vo DD, Morris LF, Bruhn KW, Prins RM, Mok S, Koya RC, Garban HJ, Comin-Anduix B, Craft N, Ribas A. Immunosenitization with a Bcl-2 small molecule inhibitor. *Cancer Immunol Immunother.* 2008
34. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med.* 2007; 204:2641–2653. [PubMed: 17923500]
35. Quezada SA, Peggs KS, Simpson TR, Shen Y, Littman DR, Allison JP. Limited tumor infiltration by activated T effector cells restricts the therapeutic activity of regulatory T cell depletion against established melanoma. *J Exp Med.* 2008; 205:2125–2138. [PubMed: 18725522]
36. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity.* 2007; 27:111–122. [PubMed: 17629517]
37. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med.* 2006; 203:883–895. [PubMed: 16606670]
38. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* 1996; 271:1734–1736. [PubMed: 8596936]

39. Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, Burg MB, Allison JP. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci USA*. 1997; 94:8099–8103. [PubMed: 9223321]
40. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci USA*.
41. Li B, VanRoey M, Wang C, Chen TH, Korman A, Jooss K. Anti-programmed death-1 synergizes with granulocyte macrophage colony-stimulating factor-secreting tumor cell immunotherapy providing therapeutic benefit to mice with established tumors. *Clin Cancer Res*. 2009; 15:1623–1634. [PubMed: 19208793]
42. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci USA*. 2008; 105:9331–9336. [PubMed: 18599457]
43. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009; 206:3015–3029. [PubMed: 20008522]
44. Currie AJ, Prosser A, McDonnell A, Cleaver AL, Robinson BW, Freeman GJ, van der Most RG. Dual control of antitumor CD8 T cells through the programmed death-1/programmed death-ligand 1 pathway and immunosuppressive CD4 T cells: regulation and counterregulation. *J Immunol*. 2009; 183:7898–7908. [PubMed: 20007574]

Fig. 2.

Increased cytokine production by PD-L1^{-/-} CD1d tetramer⁺ cells. **a** CD1d tetramer⁺ cells were sorted from the livers of wild-type or PD-L1^{-/-} mice. A total of 5×10^4 CD1d tetramer⁺ cells were cultured with 5×10^4 of their respective bone marrow-derived CD11c⁺ cells and 100 ng/ml α -GalCer. Supernatants were taken at Day 5 and analyzed by ELISA. **b** Mice were given 2 μ g α -GalCer or vehicle i.p. After 2 h, splenocytes were isolated and cultured in ELISPOT plates. The number of cytokine-producing cells was analyzed by ELISPOT at 18 h. ($n = 9$; 3 independent experiments). **c** Mice were given 2 μ g α -GalCer or vehicle i.p. After 8 h, spleens were treated with collagenase and splenocytes isolated. CD11c⁺ cells were isolated and cultured for a further 48 h and IL-12 production analyzed by ELISA. **d** NKT cells were sorted with CD1d tetramer from wild-type or PD-L1^{-/-} and 2×10^5 CD1d tetramer⁺ cells were adoptively transferred to V α 14J α 281-deficient host. Two days later, recipients were given 2 μ g of α -GalCer or vehicle i.p. After 2 h, splenocytes were isolated and analyzed by ELISPOT. **e** WT and PD-L1^{-/-} mice were given 2 μ g α -GalCer or vehicle i.p. After 2 h, splenocytes were isolated and cultured with monesin for 2 h. Intracellular staining was performed using a Fix and Perm Kit (ebiosciences). These data are representative of 3 or more independent experiments. * $P = 0.01$; ** $P < 0.005$

Fig. 3. Greater Expansion of CD1d tetramer⁺ cells in PD-L1^{-/-} mice compared to wild-type mice. **a** Representative flow cytometry data from PD-L1^{-/-} and wild-type mice. Wild-type and PD-L1^{-/-} were injected i.p with 2 μ g of α -GalCer or vehicle 3 days later, **b** splenocytes, and **c** liver lymphocytes were isolated and stained with CD1d tetramer and anti-CD4 and analyzed by flow cytometry ($n = 11$ mice/group; 4 independent experiments). * $P < 0.02$; ** $P < 0.001$

Fig. 4. PD-L1 deficiency on NKT cells results in an enhanced antigen-specific response. **a** 1×10^6 CD8⁺ OT-1 T cells were adoptively transferred into wild-type or PD-L1^{-/-} mice. One day later, mice were given 25 μ g SIINFEKL peptide together with α -GalCer or vehicle for four days. Splenocytes were isolated and stained with anti-CD45.1, anti-V α 2, and V β 5.1. Graph depicts the number of OT-1 CD8⁺ T cells/spleen ($n = 9$; 3 independent experiments). **b** Splenocytes were restimulated with 1 μ g/ml of SIINFEKL peptide and supernatants analyzed by ELISA. This graph is representative of 3 independent experiments (3 mice/group). **c** 1×10^6 CD4⁺ OT-II T cells were adoptively transferred into wild-type or PD-L1^{-/-} mice. One day later, mice were given 50 μ g OVA₃₂₃₋₃₃₉ peptide together with α -GalCer or vehicle for four days. Splenocytes were isolated and stained with anti-CD4, anti-V α 2 and V β 5.1. Graph depicts the number of OT-II CD4⁺ T cells/spleen ($n = 6$; 2 independent experiments). **d** Splenocytes were restimulated with 1 μ g/ml of OVA₃₂₃₋₃₃₉ peptide and supernatants analyzed by ELISA. This graph is representative of 2 independent experiments (3 mice/group) * $P < 0.05$; ** $P < 0.001$

Fig. 5.

B16 melanoma tumor growth in PD-L1-deficient mice **a** 2×10^5 B16 melanoma cells were injected s.c. in the right flank ($n = 30,6$ individual experiments) of mice. After 3 weeks, tumor were measured length $\times$ width and results expressed mean $\pm$ SD. **b** Effector cells were expanded for 7 days with TRP-2₁₈₁₋₁₈₈ peptide, and cultured with B16 cells for a further 24 h. IFN- γ production measured by ELISA. **c** 2×10^5 B16 melanoma cells were injected i.v., and 14 days later the mice, were killed and lungs removed and placed in formalin ($n = 7$; two independent experiments). Tumor nodules were counted in double-blind fashion. **d** Effector cells were expanded for 7 days with TRP-2₁₈₁₋₁₈₈ peptide, and cultured with B16 cells for a further 24 h and IFN- γ production measured. * $P = 0.02$

Fig. 6.

Reduced tumor growth in PD-L1^{-/-} after dendritic cell vaccination. **a** 5×10^5 dendritic cells were injected into their respective recipients. One day later, 1×10^5 B16-F1 tumor cells were injected subcutaneously into the right flank of mice. Tumor growth was monitored weekly. Tumor size was calculated width $\times$ diameter ($n = 9/\text{group}$; three independent studies. * $P < 0.01$). **b** Tumors were excised and weighed. **c** Tumors were excised after 21 days and cut into small pieces and digested with collagenase and DNase for 30 min. The resulting suspensions were filtered and placed on Ficoll to recover the lymphocytes. Cells were restimulated with PMA and ionomycin and IFN- γ -producing cells were detected by intracellular staining. **d** Cells were isolated as in **c**, stained, and analyzed by flow cytometry. * $P = 0.05$; ** $P < 0.01$. **e** 5×10^5 DC were injected per mouse and 4 days after the mice were challenged with 5×10^5 B16 cells subcutaneously * $P < 0.05$. **f** Cells were isolated as in **c**, stained, and analyzed by flow cytometry