

PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells

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Abstract

Since metastasis is the major cause of death for cancer patients, there is an urgent need to develop new therapies to control hematogenous dissemination of cancer cells. Previously we and others demonstrated a novel mechanism that allows tumors to escape from the host immune response by expressing PD-L1 which can negatively regulate immune response through the interaction with PD-1, an immunoinhibitory receptor belonging to the CD28 family. In this study, we report that hematogenous spread of poorly immunogenic B16 melanoma cells to the liver was inhibited in PD-1-deficient mice. After inoculation to spleen, PD-L1 was induced on tumor cells, which did not express PD-L1 *in vitro*. As compared with wild-type mice, intrasplenic injection of B16 cells into PD-1-deficient mice showed enhanced induction of effector T cells in spleen, prolonged T cell proliferation and cytokine production, and augmented homing of effector T cells to tumor sites in the liver, resulting in accumulation of effector T cells in the tumor sites. PD-1 blockade by genetic manipulation or antibody treatment inhibited not only hematogenous dissemination of B16 melanoma cells to the liver on the C57BL/6 background, but also dissemination of CT26 colon cancer cells to the lung on the BALB/c background. These results suggest that PD-1 blockade may be a powerful tool for treatment of hematogenous spread of various tumor cells.

Introduction

Hematogenous dissemination of tumor cells may be an early event in metastasis. Since undetectable hematogenous dissemination of tumor cells may be already present at the time of diagnosis of the primary tumor or can occur during surgical and endoscopic treatments, many cancer patients die of metastatic disease despite the resection of primary tumors. It is therefore important to develop new therapies to control hematogenous tumor spread.

Although immunotherapy is one of the best choices for adjuvant therapy, many tumors are poorly immunogenic. The absence of costimulatory ligands, B7-1 and B7-2, has been suggested to be one of the reasons for the poor immunogenicity of tumors (1–5). Increased appreciation of the importance of costimulation, both positive and negative (6–9), in the regulation of T cell responses has allowed the development of new strategies for cancer immunotherapy (10,11). CTLA-4 is a T-cell counter-receptor for B7s that plays an inhibitory role in

T cell activation. CTLA-4 blockade has been demonstrated to enhance immune responses against a number of experimental cancers (12,13). However, CTLA-4 blockade as a single agent is not effective in the treatment of poorly immunogenic tumors such as B16 melanoma (14,15).

Recently, we and others demonstrated a novel mechanism by which tumors can escape from the host immune system by expressing PD-L1 (also called B7-H1) on their surface (16,17). PD-L1 is a B7 family member which negatively regulates T-cell immune responses by engagement with PD-1, an immunoinhibitory receptor belonging to the CD28 family (18,19). PD-1 is induced on T cells, B cells and myeloid cells *in vitro* (20), but is predominantly expressed on previously activated T cells *in vivo* (21). PD-L1 expression shows broad distribution both in lymphoid and non-lymphoid organs (18,22). Similar to B7 molecules, PD-L1 is upregulated upon activation on hematopoietic cells, especially on antigen-presenting cells (APCs)

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such as dendritic cells (DCs), macrophages/monocytes and B cells (18,23). PD-L1 is also expressed on activated T cells (24,25). In addition, unlike B7-1 and B7-2, PD-L1 protein is constitutively expressed on vascular endothelial cells in peripheral tissues and on liver non-parenchymal cells (LNPCs) such as liver sinusoidal endothelial cells (LSECs) and Kupffer cells (25–27). Furthermore, PD-L1 is expressed in various tumor cell lines and tumor tissues both in human and mouse (16,17,28,29).

Lines of evidence indicate that PD-1 plays a critical role in immune responses. Engagement of PD-1 by PD-L1 leads to the inhibition of T cell proliferation and cytokine production such as IL-2 and IFN- γ (18). PD-1 delivers a negative signal by the recruitment of SHP-2 to the phosphorylated tyrosine residue in the immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic region (30). PD-1 deficient (–/–) mice showed different types of autoimmune disease depending on genetic backgrounds. C57BL/6 (B6)-PD-1^{–/–} mice develop lupus-like arthritis and glomerulonephritis (31), while BALB/c-PD-1^{–/–} mice suffer from fatal dilated cardiomyopathy with deposition of anti-cardiac troponin I IgG in heart (32,33). These results indicate that PD-1 is involved in peripheral tolerance. Our recent study has shown that PD-1 deficiency enhances anti-viral immunity at the effector sites, resulting in rapid clearance of the virus (25). In contrast, CTLA-4 deficiency does not have this effect on viral infection (34,35). These results suggest that PD-1 blockade may have a stronger potency to augment host immune responses against virus than CTLA-4 blockade.

Our previous study has also shown that the PD-L1/PD-1 blockade is effective in the treatment of primary PD-L1-expressing tumors, which are moderately immunogenic (16). However, not all tumor cells express PD-L1. In this study, we show the therapeutic effect of PD-1 blockade on hematogenous spread of poorly immunogenic B16 melanoma which originally does not express PD-L1. This inhibition of tumor growth by PD-1 blockade appears to be mediated by enhanced recruitment of effector T cells at tumor sites, together with prolonged T cell proliferation and cytokine production. Furthermore, we demonstrate the effectiveness of the PD-1 blockade on hematogenous spread of CT26 colon cancer cells to the lung on the different genetic background, suggesting that the PD-1 blockade may be a powerful tool for treatment of hematogenous dissemination of various tumor cells.

Methods

Animals and cell lines

C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC. B6-PD-1^{–/–} and BALB/c-PD-1^{–/–} mice were prepared as previously reported (31,32). Murine PD-1 cDNA (2.0 kb) were cloned into the *SaI* site of the pAc-2 containing the chicken β -actin promoter (36). The purified *XhoI*–*Bam*HI fragment containing the PD-1 transgene was microinjected into B6 fertilized eggs to generate PD-1 transgenic (Tg) mice. All animals were maintained under specific pathogen-free conditions. B16 melanoma cells and CT26 colon carcinoma cells are of B6 and BALB/c origin, respectively. The cells were maintained in DMEM supplemented with 10 % FCS, 2 μ M L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Tumor cell inoculation

B16 cells (1×10^6) were subcutaneously injected into the right flank of B6 wt, B6-PD-1^{–/–} and B6-PD-1 Tg mice and tumor volumes were approximated using the ellipsoidal formula: length \times width \times height \times 0.52. For the hematogenous dissemination to the liver, B16 cells (1×10^6) were injected into the spleen of B6 wt and PD-1^{–/–} mice. For the hematogenous dissemination to the lung, CT26 cells (1×10^6) were intravenously injected into BALB/c wt and PD-1^{–/–} mice. For the treatment with antibody, 100 μ g/mouse of anti-mouse PD-1 mAb (clone J43), or normal hamster IgG as control, was intraperitoneally injected every 2 days after tumor injection.

Isolation of liver cells

Hepatocytes were isolated by the two-step collagenase perfusion method (37), and were centrifuged three times at 50 g for 1 min. Liver non-parenchymal cells (LNPCs) were isolated using the pronase E method as described previously (25,38). Liver CD11b⁺ cells and sinusoidal endothelial cells (LSECs) were purified from LNPCs by positive and negative selection using MACS, respectively. Intrahepatic lymphocytes (IHL) were isolated from livers as described previously (39).

Flow cytometry

The following reagents used for surface staining were purchased from BD Biosciences: anti-CD3–APC, anti-CD4–PE, anti-CD8–APC, anti-CD11b–APC, anti-CD11c–FITC, anti-CD19–FITC, anti-CD44–FITC, anti-CD62L–FITC and anti-CD54 (ICAM-1)–FITC. Streptavidin (SA)–FITC and SA–PE were purchased from DakoCytomation. Anti-PD-1 (J43), anti-PD-L1 (1-111) and their conjugates were generated in our laboratory (20,23). The cells were analyzed on a FACSCaliburTM and CellQuest software (Becton Dickinson) as described previously (25). T cells, B cells, macrophages and DCs were gated as CD3⁺, CD19⁺, CD11b⁺ and CD11c⁺ cells, respectively.

Transmigration assay

Transmigration assays were performed as described previously with some modifications (40,41). LSECs (3×10^5) were seeded on a collagen-coated transwell insert (5- μ m pore size, polycarbonate membrane, 24-well format, Corning Costar) and cultured for 1 or 2 days to make a confluent monolayer. T cells were isolated from the spleens of mice 4 days after intrasplenic tumor injection using T cell enrichment column (Genzyme). Then the T cells (2×10^5) were applied to the LSEC monolayer. After 3 h of incubation, the migrated cells were collected from the lower wells and counted with a FACScalibur flow cytometer as the flow-through cell number over a constant time period (60 s).

T cell proliferation and cytokine assay

Proliferation of B16 cells and liver CD11b⁺ cells was inhibited by incubation with mitomycin C (MMC, Sigma-Aldrich) at 50 μ g/ml for 30 min at 37°C. T cells were isolated from the spleens of mice 4 days after intrasplenic tumor injection, using T cell enrichment column (Genzyme). The T cells (1×10^5) were cultured with 3×10^3 MMC-treated B16 cells or liver CD11b⁺

cells for 60 h. Proliferation was determined by labeling of cultures for the last 12 h with BrdU using proliferation ELISA kit (Roche). Supernatants were collected at 48 h after initiation of cultures and IFN- γ concentration was determined using ELISA kit (Genzyme).

Cytotoxicity assay

CTL lysis of tumor cells was quantified by using the lactate dehydrogenase (LDH) release assay (42,43). As the effectors, we use splenocytes or T cells isolated from mice 4 days after intrasplenic injection of B16 cells. In some experiments, the T cells were subjected to a migration assay using LSECs, and the migrated T cells were harvested from the lower wells and used as the effectors. Target B16 or EL4 cells (1×10^4) were co-cultured with the effector cells at different effector/target (E:T) ratios. After 8 h of incubation, the levels of LDH in the culture supernatant were determined using a cytotoxicity detection kit (Roche) according to the manufacturer's instructions. We used 1% Triton X to determine maximum lysis. Percentage lysis was calculated according to a modified standard formula: $(OD_{\text{experimental}} - OD_{\text{spontaneous targets}} - OD_{\text{spontaneous effectors}}) / (OD_{\text{maximum}} - OD_{\text{spontaneous targets}}) \times 100$.

Histology

For histopathological studies, liver cryosections were fixed with 10% buffered formalin and stained with hematoxylin and eosin. For detection of PD-L1 and CD11b, liver cryosections (5 μ m) fixed with 3% paraformaldehyde were incubated with goat anti-mouse PD-L1 (Santa Cruz) in combination with biotinylated anti-CD11b (e-Bioscience) followed by anti-goat IgG-FITC in combination with Cy5-conjugated streptavidin. Nuclei were counterstained with propidium iodide. For detection of CD4 and CD8, acetone-fixed liver cryosections (5 μ m) were stained with anti-CD4 (RM4-5, BD Biosciences) or anti-CD8 (53-6.7, BD Biosciences) using alkaline phosphatase substrate kit (red; Vector Laboratories).

Results

B16 subcutaneous tumor grows rapidly in PD-1 transgenic mice

We examined the role of PD-1/PD-L1 interaction in the regulation of tumor growth of B16 melanoma, one of the most poorly immunogenic tumors. B16 cells were subcutaneously inoculated into the flank of syngenic B6 wild-type (wt), PD-1 $^{-/-}$, PD-1 transgenic (Tg) mice and tumor volume was measured (Fig. 1A). There were no differences in the tumor growth between PD-1 $^{-/-}$ and wt mice, in contrast to our previous report that growth of P815 subcutaneous tumor expressing PD-L1 was inhibited in PD-1 $^{-/-}$ mice (16). However, in PD-1 Tg mice, subcutaneous B16 tumors grew more rapidly than in PD-1 $^{-/-}$ or wt mice, suggesting that PD-L1/PD-1 signal may regulate immune responses even against poorly immunogenic tumors. In fact, PD-L1 expression on subcutaneous B16 tumors was induced *in vivo*, while no PD-L1 expression was detected on B16 melanoma cells cultured *in vitro* (Fig. 1B). The expression level of PD-L1 on the subcutaneous tumor in PD-1 $^{-/-}$ mice was higher than that in

wt mice. The PD-L1 expression on the subcutaneous tumor in PD-1 Tg mice was almost the same as that in wt mice (data not shown). A likely explanation for these differences between B16 and P815/PD-L1 tumors may be the expression level of PD-L1 and the inherent immunogenicity of the tumors. *In vivo* expression of PD-L1 on B16 cells (Fig. 1B) was orders of magnitude weaker than that on the P815/PD-L1 transfectants (16). In addition, the immunogenicity of B16 is much poorer than that of P815 tumors (44).

Hematogenous dissemination of B16 cells to the liver is inhibited in PD-1 $^{-/-}$ mice

Previously we reported that PD-L1 is constitutively expressed on vascular endothelial cells and LNPCs such as Kupffer cells and LSECs (25). Such cells lining blood vessels play an important role in hematogenous spread of tumors. To examine the involvement of PD-1 in tumor dissemination via the vascular system, B16 melanoma cells were injected into the spleen of B6-PD-1 $^{-/-}$ or wt mice to evaluate tumor formation in the liver. Compared with wt mice, tumor formation was extremely inhibited in the liver of PD-1 $^{-/-}$ mice. At day 12 after tumor injection, numerous tumor foci were macroscopically observed as dark spots in the liver of wt mice, while very few tumor foci were detected in PD-1 $^{-/-}$ mice (Fig. 1C). The average number of tumor foci per liver in PD-1 $^{-/-}$ mice was almost one-eighth of that in wt mice (wt, 786; PD-1 $^{-/-}$, 96) (Fig. 1D). At day 16, livers of wt mice were swollen with a large number of tumor nodules, whereas livers of PD-1 $^{-/-}$ mice kept normal size with very few tumor nodules (Fig. 1E). The weight of PD-1 $^{-/-}$ liver was almost one-fifth of that of wt liver (wt, 6.2 g; PD-1 $^{-/-}$, 1.3 g) (Fig. 1F).

PD-1/PD-L1 interaction can occur both in inductive and effector sites

To examine where PD-1/PD-L1 interaction takes place in the tumor-bearing environment, PD-1 and PD-L1 expression was analyzed both in the inductive site (spleen) and in the effector site (liver). After intrasplenic injection of B16 tumor cells, PD-1 expression was induced on a small fraction of T cells both in the spleen and in the liver (Fig. 2A). PD-1 expression was not observed on B cells, NK cells, NKT cells, macrophages and DCs in either spleen or liver (data not shown). PD-L1 expression was slightly induced both on the primary tumor in the spleen and on the disseminated tumor in the liver, while PD-L1 was not expressed on B16 cells cultured *in vitro* (Fig. 2B). In the liver, PD-L1 was strongly expressed on intrahepatic lymphocytes and CD11b $^{+}$ cells including Kupffer cells and macrophages, and weakly expressed on LSECs (Fig. 2C), in agreement with our previous report (25).

PD-L1 expression in tumor sites in the liver was analyzed by immunohistochemistry (Fig. 2D). Double staining with anti-PD-L1 and anti-CD11b showed that PD-L1 was strongly expressed on CD11b $^{+}$ cells around tumors. In the spleen, PD-L1 was highly expressed on DCs and macrophages and induced on T cells and B cells after tumor injection (Fig. 2E). Similar expression pattern of PD-1 and PD-L1 was observed from days 3 to 7 (data not shown). These results suggest that PD-1 on T cells can interact with PD-L1 not only on DCs and

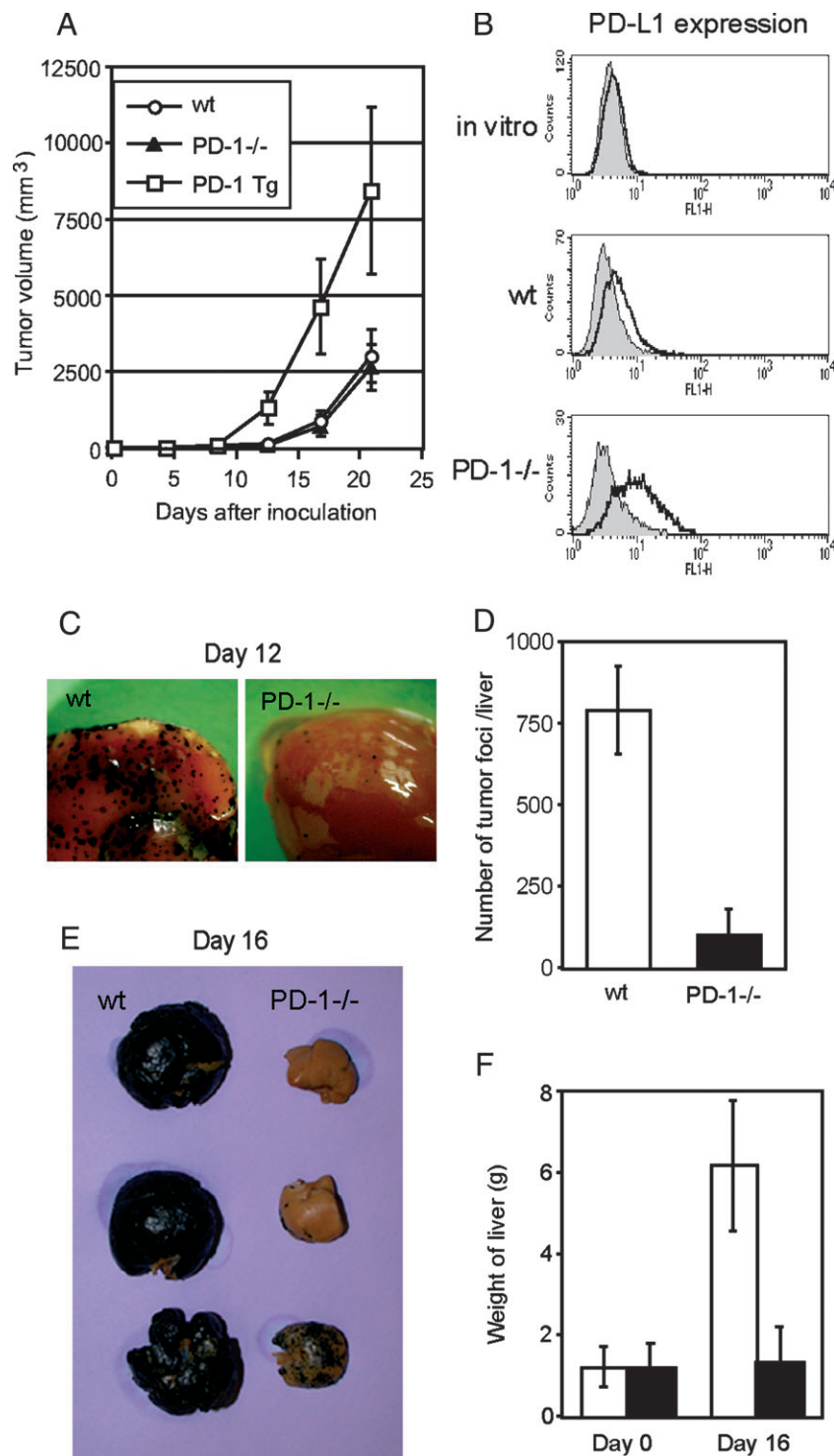


Fig. 1. Hematogenous spread of B16 melanoma cells to the liver was inhibited in PD-1^{-/-} mice. (A and B) B16 cells (1×10^6) were subcutaneously injected into the flank of syngenic C57BL/6 wt (B6), B6-PD-1^{-/-} or B6-PD-1 Tg mice. The growth of subcutaneous B16 tumor. Tumor volume was measured every 5 days. The results were expressed as mean \pm SD of tumor volume from five mice. PD-L1 expression on subcutaneous B16 tumors. B16 cells before injection or isolated from subcutaneous tumors in wt or PD-1^{-/-} mice 14 days after injection were analyzed by flow cytometry using anti-PD-L1 mAb. (C–F) B16 cells (1×10^6) were injected into the spleen of syngenic B6 wt or PD-1^{-/-} mice to examine tumor formation in the liver. Livers from wt or PD-1^{-/-} mice 12 days after tumor injection. Black spots represent melanin-laden tumor foci in the liver. The number of tumor foci in the liver from wt or PD-1^{-/-} mice 12 days after tumor injection. The number of tumor foci on the liver surface was counted. The results were expressed as mean \pm SD of five mice. Livers from wt or PD-1^{-/-} mice 16 days after tumor injection. The weight of liver from wt (open bar) or PD-1^{-/-} (filled bar) mice at day 0 and 16 after tumor injection. The results were expressed as mean \pm SD of five mice.

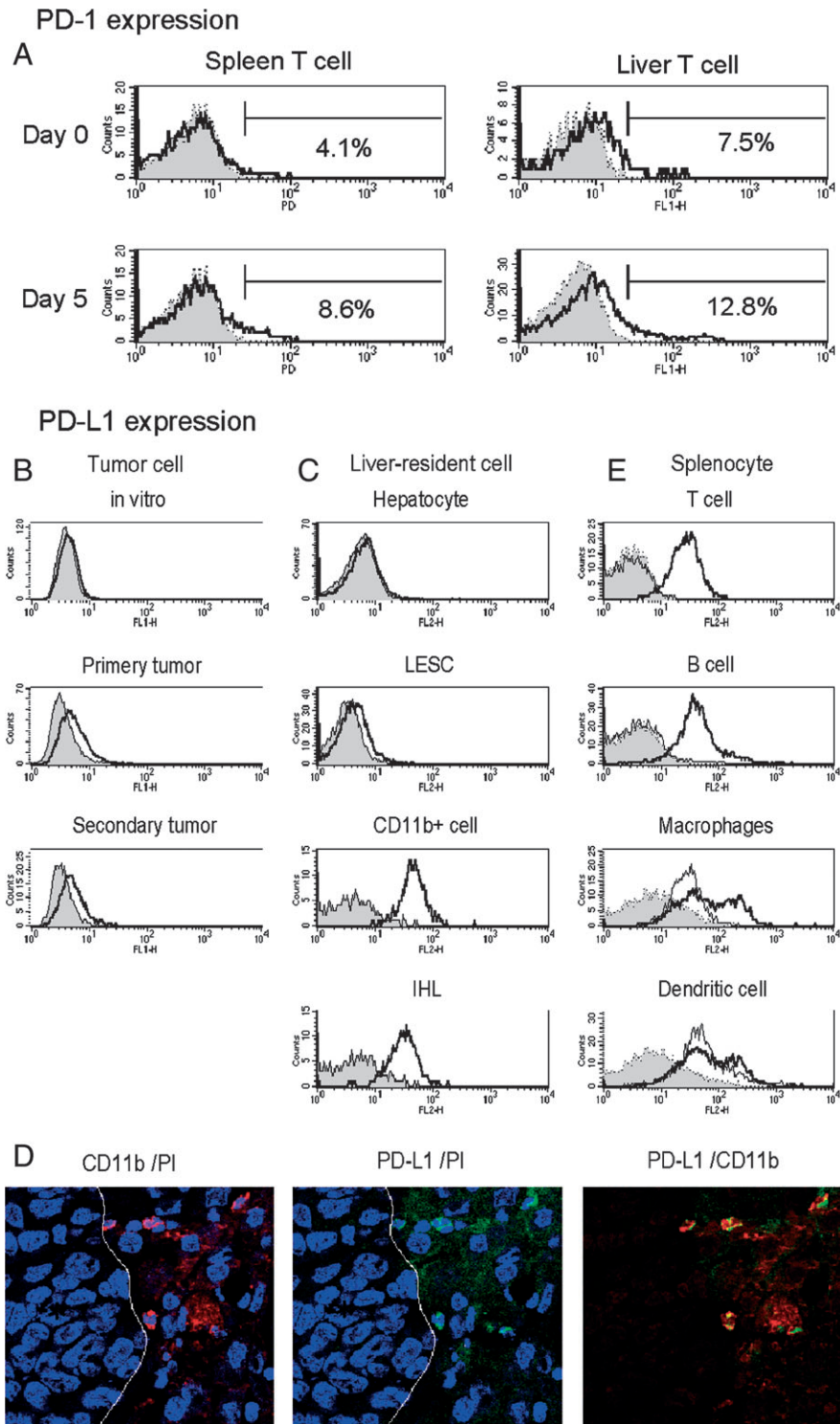


Fig. 2. PD-1 and PD-L1 expression in tumor-bearing environment. B16 cells (1×10^6) were injected into the spleen of syngenic B6 wt mice to examine tumor formation in the liver. PD-1 expression on T cells. T cells were isolated from spleen or liver at day 0 and 5 after tumor injection and stained with anti-PD-1 (open histograms) or control IgG (filled histograms). PD-L1 expression on primary tumors in the spleen and on secondary tumors in the liver. B16 cells before injection or isolated from primary or secondary tumors 14 days after injection were stained with anti-PD-L1 (open histograms) or control IgG (filled histograms). PD-L1 expression on liver-resident cells. Hepatocytes, LSECs, CD11b⁺ cells including Kupffer cells and macrophages, and intrahepatic lymphocytes were isolated from liver 7 days after tumor injection and stained with anti-PD-L1 (open histograms) or control IgG (filled histograms). Cryosections of liver at day 7 after tumor injection were stained with anti-PD-L1 (green) and anti-CD11b (red). Nuclei were counterstained with propidium iodide (blue). The asterisks represent tumor foci. PD-L1 expression on splenocytes. Splenocytes were isolated from spleen at day 0 or 5 after tumor injection and stained with anti-PD-L1 mAb (day 0, thin lines; day 5, thick lines) or control IgG (gray shadow). T cells, B cells, DCs and macrophages were gated as CD3⁺, CD19⁺, CD11c⁺ and CD11b⁺ cells, respectively.

macrophages in the spleen (in the inductive sites) but also on CD11b⁺ cells around tumors in the liver (in the effector sites).

The absence of PD-1 signal can augment homing of activated T cells to the effector sites

To examine the effect of PD-1/PD-L1 interaction in the inductive sites, T cell phenotype was analyzed by flow cytometry 5 days after tumor injection (Fig. 3). The percentage of CD4 and CD8 T cells that showed an activated phenotype (CD44^{high}) increased in the spleen of PD-1^{-/-} mice compared with that of wt mice (percentages of CD44^{high} population in CD4 T cells; wt 20%, PD-1^{-/-} 46%; percentages of CD44^{high} population in CD8 T cells; wt 22%, PD-1^{-/-} 34%). The number of CD4 and CD8 T cells with a phenotype of homing to peripheral tissues (CD62L^{low}) was also increased in PD-1^{-/-} mice. The percentages of CD62L^{low} population in CD4 T cells in wt and PD-1^{-/-} mice were 17 and 42%, respectively. The percentages of CD62L^{low} population in CD8 T cells in wt and PD-1^{-/-} mice

were 3 and 16%, respectively. To prove a direct role of PD-1 in the enhanced T-cell activation and homing into the periphery, we injected anti-PD-1 mAb or control IgG on days 0, 1 and 3 after tumor inoculation. Injection with anti-PD-1 mAb induced increase of CD44^{high} and CD62L^{low} population both in CD4 and CD8 T cells (percentages of CD44^{high} population: CD4 T cells, 28%; CD8 T cells, 30% and percentages of CD62L^{low} population: CD4 T cells, 24%; CD8 T cells, 6%). Injection with control IgG had no effect (data not shown). These results suggest that the absence of negative signal through PD-1 can augment activation levels and homing capacities of spleen T cells to the effector sites.

The absence of PD-1 signal induced accumulation of cytotoxic T cells in tumor sites

To examine whether activated T cells accumulated in the effector sites, we analyzed histopathologically liver sections of PD-1^{-/-} or wt mice which were injected with tumor cells

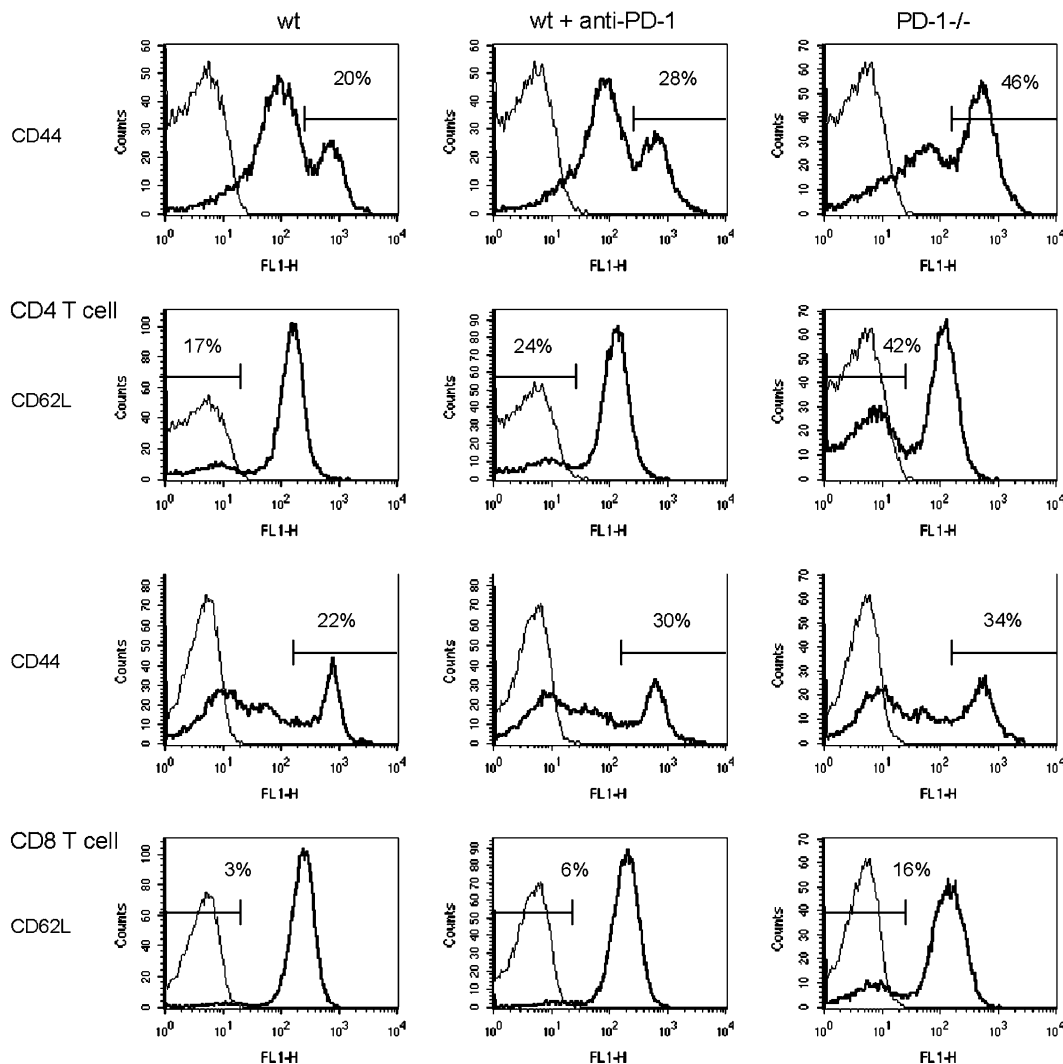


Fig. 3. Cell surface phenotype of splenic T cells. B16 cells (1×10^6) were injected into the spleen of syngenic B6 wt or B6-PD-1^{-/-} mice. B6 wt mice were intraperitoneally injected with anti-PD-1 mAb (100 μ g/dose) on days 0, 1 and 3. At day 5 after injection, T cells were isolated from the spleen and stained with anti-CD44 or anti-CD62L (thick lines) and control IgG (thin lines).

(Fig. 4A). At day 7, larger clusters of inflammatory cell infiltration was observed in the liver of PD-1^{-/-} mice compared with that in wt mice. Microscopic tumor foci were detectable in wt liver on day 7, while no obvious tumor foci were found in PD-1^{-/-} liver on day 7. At day 12, in wt mice, tumors were

surrounded by fibroblast-like cells and separated from normal tissues by the septum. There was no cellular infiltration within the tumors inside the septum. On the other hand, tumor foci were not observed in the liver of PD-1^{-/-} mice on day 12. These results suggest that, in PD-1^{-/-} mice, early and strong

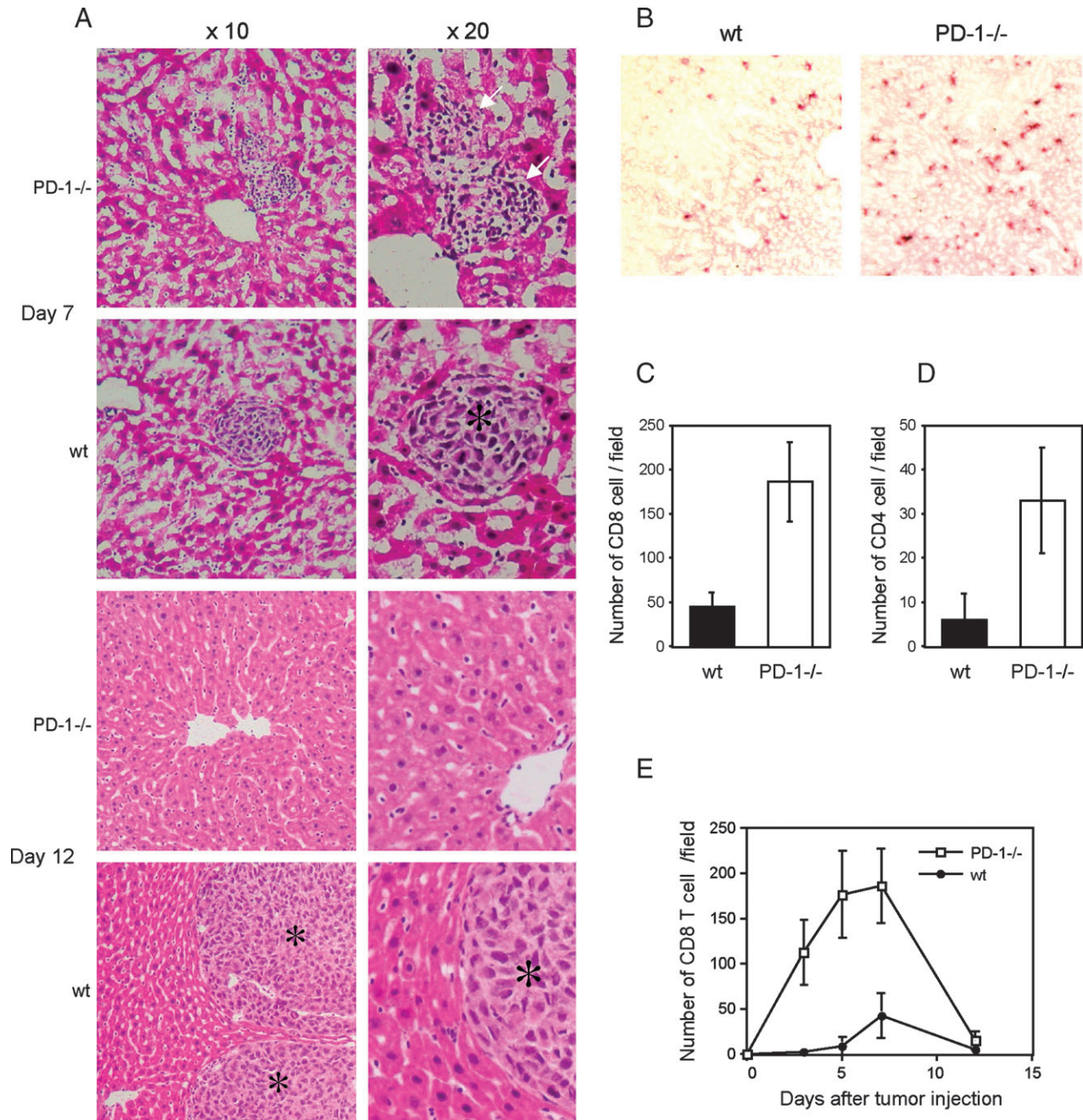


Fig. 4. CD8 T cells significantly infiltrated into the tumor sites in PD-1^{-/-} mice. B16 cells (1×10^6) were injected into the spleen of syngenic B6 wt or B6-PD-1^{-/-} mice to examine tumor formation in the liver. At day 7 and 12, liver sections of PD-1^{-/-} or wt mice were analyzed by hematoxylin and eosin staining. Original magnification; left panel, $\times 10$; right panel, $\times 20$. The asterisks represent tumor focus. The arrows represent cellular infiltration. At day 7, cryosections of the liver of PD-1^{-/-} or wt mice were stained with anti-CD8 (red). Original magnification, $\times 20$. The number of CD8 T cell in the liver. At day 7, cryosections of the liver of PD-1^{-/-} or wt mice were stained with anti-CD8. The number of CD8⁺ cells per field ($\times 20$ magnification) was counted. The number of CD4 T cell in the liver. At day 7, cryosections of the liver of PD-1^{-/-} or wt mice were stained with anti-CD4. The number of CD4⁺ cells per field ($\times 20$ magnification) was counted. Time course of the number of CD8 T cells in the liver. Liver sections at day 0, 3, 5, 7 and 12 after tumor injection were stained with anti-CD8. The number of CD8⁺ cells per field ($\times 20$ magnification) was counted. The data represent mean \pm SD of 10 fields randomly chosen.

cellular infiltration occurred and killed tumor cells before tumors were surrounded by the septum, while weak and slow cellular infiltration in wt mice allowed tumors to form the septum, resulting in the failure to attack tumors by the host immune system.

To examine which type of T cells infiltrated into tumors, liver sections were stained with anti-CD8 and anti-CD4 mAbs. At day 7 after tumor injection, CD8 T cells increased in the liver of PD-1^{-/-} mice compared with that of wt mice (Fig. 4B). The number of infiltrating CD8 T cells in PD-1^{-/-} liver was almost 4-fold of wt liver (PD-1^{-/-}, 186; wt, 45 per ×20 field) (Fig. 4C). At day 7, the number of CD4 T cells in PD-1^{-/-} liver was 5-fold that in wt liver (PD-1^{-/-}, 33; wt, six per ×20 field) (Fig. 4D). To examine the time course of infiltrating CD8 T cell numbers, liver sections at various time points were analyzed by immunohistochemistry using anti-CD8 mAb (Fig. 4E). In PD-1^{-/-} mice, the number of CD8 T cells in the liver began to increase at day 3, reached a peak at day 7 and declined to a wt level by 12 days. The increase of CD8 T cells lasted for almost 10 days. On the other hand, in wt mice, CD8 T cells could be detected at day 5, reached a small peak at day 7 and disappeared by day 12. These results suggest that the absence of PD-1 signal induced more rapid infiltration and augmented accumulation of effector CD8 and CD4 T cells into the tumor sites. The reason for such an early immune response at day 3 is probably because direct injection of tumor cells into the spleen saved time otherwise required for APCs to migrate from the periphery to lymphoid organs.

The absence of PD-1 signal enhances effector functions of T cells

To investigate the role of PD-1 in the induction of cytotoxic activity, B16 cells were directly injected into the spleen of PD-1^{-/-} or wt mice. At day 4 after injection, splenocytes were recovered and subjected to secondary *in vitro* stimulation with B16 cells. CTL activity was measured using a non-radioactive cytotoxic detection kit. PD-1^{-/-} splenocytes showed cytotoxic activity against B16 cells, but wt splenocytes did not (Fig. 5A). Neither PD-1^{-/-} nor wt splenocytes showed cytolytic activity against EL4 cells. Next we isolated T cells from spleen 4 days after tumor injection, and used the T cells as effector cells (Fig. 5B). While wt T cells showed slight cytolytic activity against B16 cells, PD-1^{-/-} T cells showed much stronger cytolytic activity. These data indicate that the absence of PD-1 could induce strong cytotoxic activity against weakly immunogenic tumor cells.

To examine whether strong cytolytic activity is due to the increase of the number of effector T cells in PD-1^{-/-} mice, we isolated polyclonal effector T cells using transmigration assay as shown in Fig. 5(D). When naïve T cells differentiate into effector T cells, they acquire the ability to migrate through endothelial cells into peripheral tissues (41,45–47). To isolate effector T cells, we purified T cells from the spleen 4 days after tumor injection and placed them on the monolayer of LSEC in the upper well. After 3 h of incubation, we harvested the migrated T cells from the lower well. PD-1^{-/-} T cells showed higher transmigration ability than wt T cells (Fig. 5E). Then the same number of wt and PD-1^{-/-} migrated T cells were subjected to secondary *in vitro* stimulation with B16 cells. The

cytotoxic activities of PD-1^{-/-} and wt migrated T cells were almost equivalent (Fig. 5C), suggesting that stronger cytolytic activity of PD-1^{-/-} T cells is due to the increase in the number of effector T cells in PD-1^{-/-} mice.

We next examined the function of PD-1 in T cell proliferation and cytokine production at the effector phase (Fig. 5F and G). We hypothesized two situations; (i) T cells interact with target tumor cells; and (ii) T cells interact with PD-L1-expressing CD11b⁺ cells in the liver. To mimic these two environments *ex vivo*, B16 cells were injected into the spleen of PD-1^{-/-} or wt mice, and then T cells isolated from the spleen 4 days after injection were co-cultured with (i) mitomycin C (MMC)-treated B16 cells or (ii) MMC-treated CD11b⁺ cells isolated from the liver. When T cells alone were cultured, PD-1^{-/-} T cells showed high proliferative response and secreted a significant amount of IFN- γ , whereas wt T cells neither proliferated nor secreted IFN- γ . These results suggest that PD-1^{-/-} T cells were more activated than wt T cells before they encountered tumors. When co-cultured with B16 cells, PD-1^{-/-} T cells showed stronger responses of both proliferation and cytokine production than wt T cells. When co-cultured with CD11b⁺ cells, wt T cells proliferated slightly and did not produce cytokines, while PD-1^{-/-} T cells showed strong proliferative response and cytokine production. These results show that PD-1^{-/-} T cells are more activated and sustained proliferation and cytokine production for longer periods than wt T cells even, in the absence of stimulation with tumor cells.

Taken together, hematogenous tumor spread was inhibited in PD-1^{-/-} mice by the combination of the following mechanisms; (i) enhanced induction of effector T cells in the spleen, (ii) augmented migration and accumulation of effector T cells into the tumor sites, and (iii) prolonged proliferation and cytokine production and high cytotoxic activity of effector T cells.

PD-1 blockade can inhibit hematogenous spread of B16 melanoma and CT26 colon cancer cells

To examine the possibility of PD-1 blockade as a therapy against hematogenous dissemination of tumor cells, anti-PD-1 mAb or control IgG was intraperitoneally injected into B6 syngenic mice every 2 days after intrasplenic injection of B16 melanoma cells. Injection with anti-PD-1 mAb inhibited tumor growth in the liver, while injection of control IgG had no therapeutic effect, resulting in progressive growth of disseminated tumors in the liver (Fig. 6A). At day 16, the weight of liver from mice treated with anti-PD-1 mAb was almost half that of control mice (Fig. 6B).

To examine whether the absence of PD-1 can inhibit hematogenous spread of other tumors into other organs on the different genetic background, BALB/c-PD-1^{-/-} or wt mice were intravenously injected with CT26 colon cancer cells to form tumors in the lung. Tumor formation of CT26 colon cancer in the lung was inhibited in PD-1^{-/-} mice (Fig. 6C). At day 18, the weight of PD-1^{-/-} lung was almost half that of the wt lung (Fig. 6D). Injection with anti-PD-1 mAb also resulted in the reduction of CT26 tumor formation in the lung (Fig. 6E and F). These results suggest that PD-1 blockade may be useful in the treatment for hematogenous spread of various tumor cells.

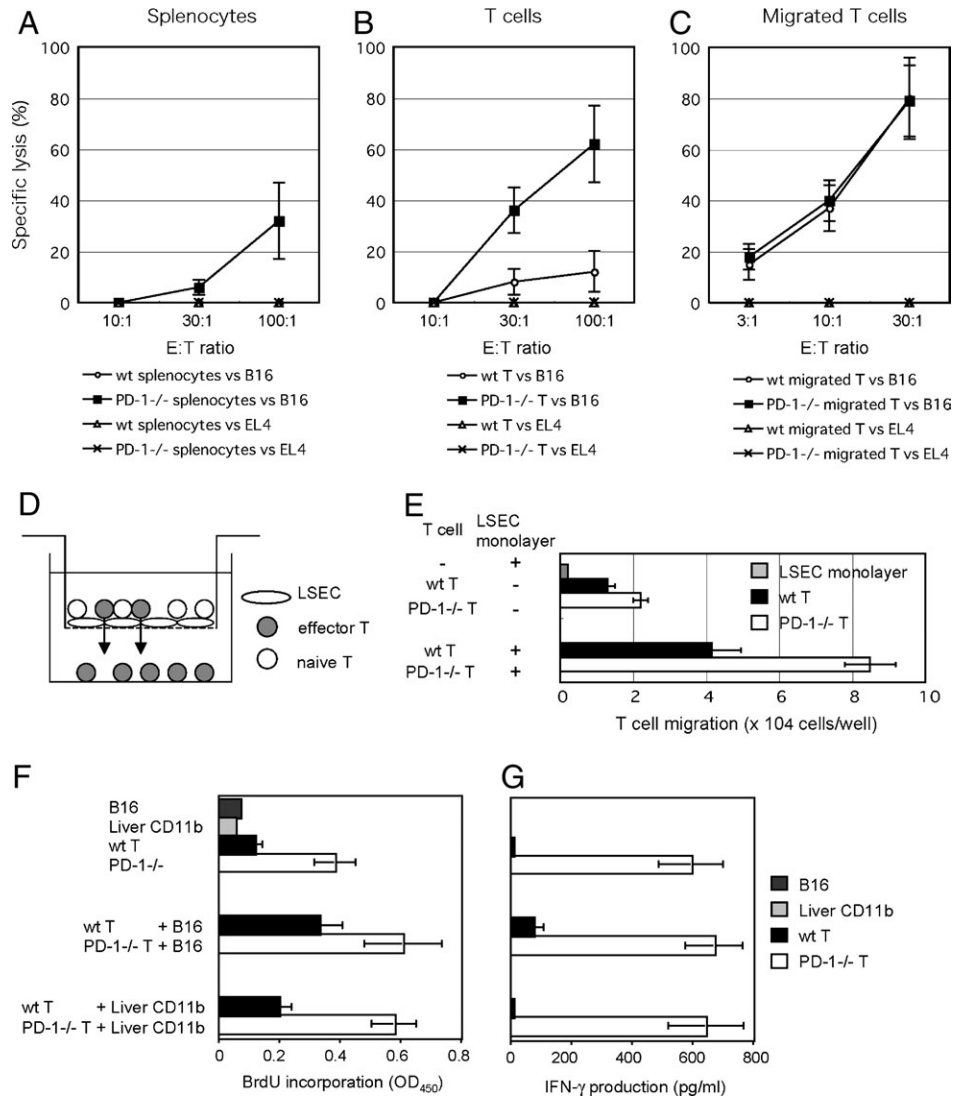


Fig. 5. The absence of PD-1 signal induced strong T cell response. B16 cells (1×10^6) were injected into the spleen of syngenic B6 wt or B6-PD-1^{-/-} mice. (A–C) The effect of PD-1 deficiency on cytotoxic activity. At day 4, spleens were harvested and T cells were purified from the splenocytes. Polyclonal effector T cells were collected from the lower well using transmigration assay as shown in (D). Whole splenocytes (A), T cells (B) or migrated T cells (C) were subjected to secondary *in vitro* stimulation with B16 cells or EL4 cells for 8 h, and CTL activity was measured using non-radioactive cytotoxic detection system. The data were expressed as mean \pm SD of triplicate samples. (D and E) The effect of PD-1 deficiency on transmigration ability of T cell. (D) After 4 days after tumor injection, T cells (2×10^5) were isolated from spleens and applied to the monolayer of LSECs (3×10^5 /insert) in the upper well. After 3 h incubation, the number of migrated cells in the lower wells was counted. The data were expressed as mean \pm SD of triplicate samples. (F and G) The effect of PD-1 deficiency on T cell proliferation and cytokine production. At day 4, T cells were isolated from the spleen and co-cultured with for 48 h with or without mitomycin C-treated B16 cells or CD11b⁺ cells isolated from liver. T cell proliferation was measured by BrdU incorporation (F). The culture supernatants were harvested and IFN- γ production was measured by ELISA (G). The data were expressed as mean \pm SD of triplicate samples.

Discussion

In this study we demonstrated that PD-1 blockade immunotherapy could inhibit hematogenous cancer spread, using a tumor formation model in the liver after intrasplenic injection of poorly immunogenic B16 melanoma on the B6 background. Although B16 is one of the most poorly immunogenic tumors (44), we demonstrated the effectiveness of PD-1 blockade against B16 melanoma by a single therapy. CTLA-4 blockade as a single agent does not appear to be universally effective: growth of tumors such as the B16 melanoma and the SM1 mammary carcinomas was unaffected by administration of

anti-CTLA-4 (11,12). Our results suggest that the PD-1 blockade may have a stronger therapeutic effect than the CTLA-4 blockade.

In our studies, the absence of PD-1 signal inhibited systemic B16 dissemination but not B16 subcutaneous tumors. The most striking difference between disseminated and subcutaneous tumors is that CTL can easily access disseminated sites via the vascular system, while primary subcutaneous tumors do not have physiological vessels to approach tumor cells. The vascular system plays two important roles in tumor dissemination. One is to provide a path for tumor migration into

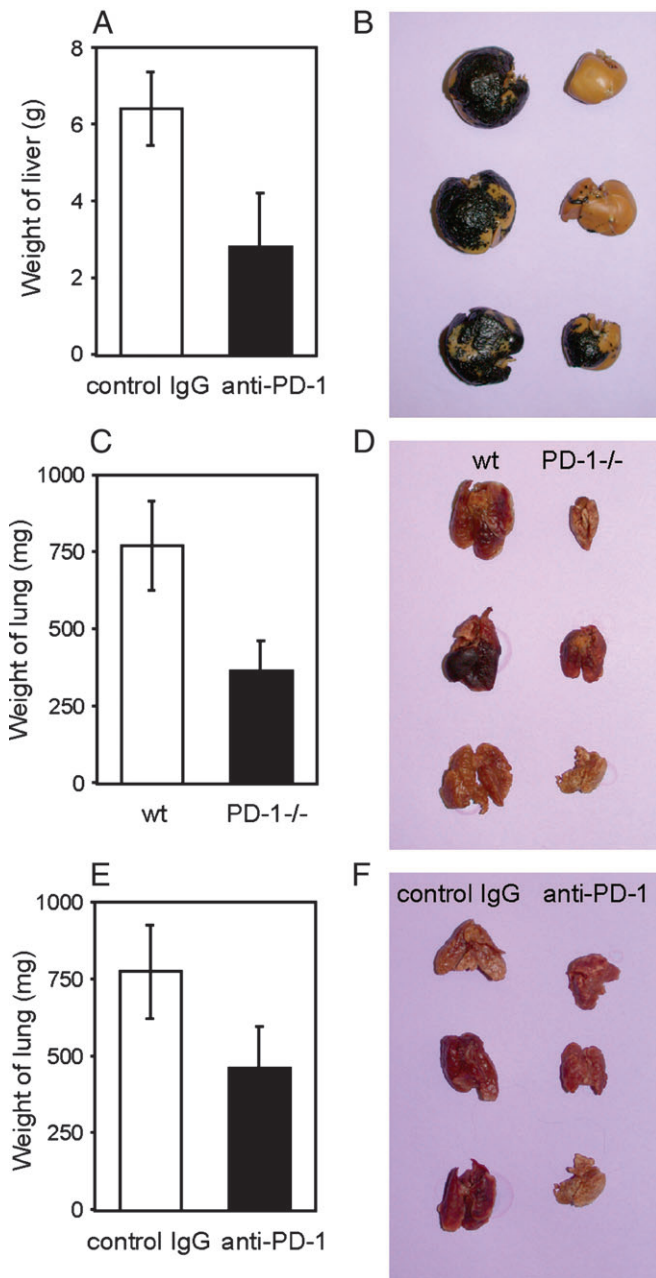


Fig. 6. PD-1 blockade inhibited disseminated tumors of B16 in the liver and CT26 in the lung. (A and B) B16 cells (1×10^6) were injected into the spleen of syngenic B6 wt or B6-PD-1^{-/-} mice and anti-PD-1 mAb (100 μ g/dose) were intraperitoneally injected every 2 days. At day 16, the liver was harvested (B) and the weight was measured (A). (C and D) CT26 cells (1×10^6) were intravenously injected into syngenic BALB/c wt or BALB/c-PD-1^{-/-} mice. At day 18, the lung was harvested (D) and the weight was measured (C). (E and F) CT26 cells (1×10^6) were intravenously injected into syngenic BALB/c wt or PD-1^{-/-} mice and anti-PD-1 mAb (100 μ g/dose) were intraperitoneally injected every 2 days. At day 18, the lung was harvested (F) and the weight was measured (E). The results were expressed as mean \pm SD of five mice.

distantly located organs. The other is to provide a path for CTL to approach tumors. Because of the more efficient access of CTL to disseminated tumors than to primary tumors, disseminated tumors may be more susceptible to immunotherapy

than primary tumors. Our results suggest that PD-1 blockade can be a powerful tool for the treatment of hematogenous spread of tumors. We also tested the effect of PD-L1 on tumor cells on hematogenous spread using PD-L1 transfected B16 cells. Although the number of B16/PD-L1 disseminated tumors in the liver was the same as that of B16 tumors, the size of B16/PD-L1 disseminated tumor was larger than that of parental B16 cells (data not shown). These data suggest that PD-L1 on tumor cells could affect the tumor growth capacity in the liver but not the spreading capacity to the liver.

The strong inhibitory mechanisms of hematogenous tumor dissemination by PD-1 blockade are likely due to a combination of the following: (i) an increase in the number of effector cells (enhanced T-cell priming in the spleen); (ii) prolonged T cell activation (prolonged proliferation and cytokine production without any stimulation); and (iii) enhanced recruitment of effector cells into tumor sites (increased homing to the periphery). These mechanisms allow effector T cells to accumulate and proliferate in tumor sites at an early stage and to reject tumors before tumors are surrounded by the septum. A large amount of IFN- γ secretion from effector T cells for long periods can induce inflammatory cellular infiltration such as macrophages, which contribute to enhance anti-tumor immunity.

Previously we reported the effectiveness of PD-L1 blockade against tumors which highly express PD-L1 (16). In the present study, we have shown that PD-1 blockade is also effective against tumors which hardly express PD-L1 on their surface. Compared with tumor cells, PD-L1 was strongly expressed on APCs (Fig. 2). In addition, PD-1^{-/-} T cells were more activated than wt T cells before they encountered tumors in the liver (Fig. 5F and G). These results suggest that PD-1 signal may regulate anti-tumor immune responses through PD-L1 on APCs in spleen (at the activation phase) rather than through PD-L1 on tumor cells in liver (at the effector phase).

Several studies suggested that PD-L1 might function as a positive regulator of the immune system through a different receptor from PD-1 (22,48). However, our previous results using anti-PD-L1 mAb strongly suggest that PD-L1 functions as a negative regulator in anti-tumor immune responses (16). We tried both anti-PD-1 and anti-PD-L1 mAbs for the treatment of hematogenous tumor spread. Although anti-PD-1 mAb showed the striking effect on tumor spread, anti-PD-L1 had no effect on the treatment (data not shown). One possible explanation is that the amount of the anti-PD-L1 mAb is not enough to block PD-1 signal. Because PD-L1 is expressed on so many cells in various organs (18,22,25), it is difficult to target the specific sites. In contrast, PD-1 expression is limited to activated T cells. Restricted expression of PD-1 on T cells only after activation with specific antigens, make it possible to target the T cells at the specific sites with smaller amounts of anti-PD-1 mAb and to minimize side effects. In our metastasis models, PD-L2 surface expression was not observed on any cells, including antigen-presenting cells such as DCs and macrophages, tumor cells such as B16 cells and CT 26 cells, T cells, B cells, NK cells and NKT cells (data not shown).

CTLA-4^{-/-} mice showed much more severe phenotypes with lethal lymphoproliferative disorder (49), while PD-1^{-/-} mice showed milder phenotypes of autoimmune diseases with slow onsets. Such mild phenotype of PD-1^{-/-} mice, combined with the effective data to inhibit B16 tumor dissemination,

suggest that PD-1 blockade may have more advantageous therapeutic effects with fewer side effects than CTLA-4 blockade.

In this study we demonstrated that the PD-1 blockade is effective against not only the disseminated tumors of B16 in the liver but also the disseminated tumors of CT26 in the lung. These results suggest that PD-1 blockade may be applicable to other metastatic tumors, in other organs, and on other genetic backgrounds. Costimulatory immunotherapy does not target tumor antigens and can be applied to various kinds of tumors. PD-1 blockade immunotherapy may be useful to prevent occult metastasis when used as a perioperative prophylaxis or post-operative adjuvant therapy.

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Abbreviations

APC	antigen-presenting cell
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
IHL	intrahepatic lymphocyte
LNPC	liver nonparenchymal cell
LSEC	liver sinusoidal endothelial cell
MMC	mitomycin C
Tg	transgenic
wt	wild type

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