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ORIGINAL ARTICLE

PD-1 blockade therapy augments the antitumor effects of lymphodepletion and adoptive T cell transfer

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Abstract

Lymphodepleting cytotoxic regimens enhance the antitumor effects of adoptively transferred effector and naïve T cells. Although the mechanisms of antitumor immunity augmentation by lymphodepletion have been intensively investigated, the effects of lymphodepletion followed by T cell transfer on immune checkpoints in the tumor microenvironment remain unclear. The current study demonstrated that the expression of immune checkpoint molecules on transferred donor $CD4^+$ and $CD8^+$ T cells was significantly decreased in lymphodepleted tumor-bearing mice. In contrast, lymphodepletion did not reduce immune checkpoint molecule levels on recipient $CD4^+$ and $CD8^+$ T cells. Administration of anti-PD-1 antibodies after lymphodepletion and adoptive transfer of T cells significantly inhibited tumor progression. Further analysis revealed that transfer of both donor $CD4^+$ and $CD8^+$ T cells was responsible for the antitumor effects of a combination therapy consisting of lymphodepletion, T cell transfer and anti-PD-1 treatment. Our findings indicate that a possible mechanism underlying the antitumor effects of lymphodepletion followed by T cell transfer is the prevention of donor T cell exhaustion and dysfunction. PD-1 blockade may reinvigorate exhausted recipient T cells and augment the antitumor effects of lymphodepletion and adoptive T cell transfer.

Keywords Lymphodepletion · PD-1 · T cell · Immune checkpoint

Abbreviations

CAR-T	Chimeric antigen receptor T
DCs	Dendritic cells
Foxp3	Forkhead box P3
i.v	Intravenous
i.p	Intraperitoneally
ICIs	Immune checkpoint inhibitors
mAbs	Monoclonal antibodies
MDSCs	Myeloid-derived suppressor cells
PD-1	Programmed-cell death-1
PD-L1	PD-ligand 1
s.c	Subcutaneous
TDLNs	Tumor-draining lymph nodes
TILs	Tumor-infiltrating lymphocytes

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Introduction

Lymphodepleting cytotoxic regimens, such as chemotherapy and radiotherapy, have shown the ability to enhance antitumor immunity. Previous studies have demonstrated that the function and proliferation of tumor-specific effector T cells are augmented when these cells are transferred into lymphodepleted hosts [1–3]. In clinical studies, the combination of adoptive transfer of tumor-infiltrating lymphocytes (TILs) and administration of a lymphodepleting therapy, such as high-dose chemotherapy or whole-body radiotherapy, has produced durable clinical responses [4, 5]. Chimeric antigen receptor T (CAR-T) cell therapy, which has been approved for use in hematologic malignancies, is administered after lymphodepleting chemotherapy [6]. We and others have found that transfer of naïve T cells after lymphodepletion augments antitumor immunity and inhibits tumor progression. Transferred naïve T cells proliferate in lymphopenic hosts and differentiate into tumor-specific effector T cells [7, 8]. Overall, lymphodepleting cytotoxic regimens enhance

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the antitumor effects of adoptively transferred effector and naïve T cells.

Several mechanisms have been proposed to underlie the augmentation of antitumor effects by lymphodepletion [9]. Previous studies have suggested that lymphodepletion enhances antitumor immunity through the depletion of suppressive immune cells [2, 3]. Recent studies have shown that lymphodepletion decreases host cell competition for activating cytokines, such as IL-7, IL-15, and IL-21, and increases the availability of these cytokines to transferred T cells [10, 11]. Although the mechanisms of antitumor effects augmentation in tumor-bearing hosts after lymphodepletion and T cell transfer have been intensively investigated, the influence of lymphodepletion followed by T cell transfer on immune checkpoint molecules expressed on tumor cells and immune cells remains to be elucidated.

Immune checkpoint inhibitors (ICIs), including anti-programmed-cell death-1 (PD-1) and PD-ligand 1 (PD-L1) antibodies, have demonstrated promising clinical benefits across a broad range of malignancies [12–18]. Although anti-PD-1/PD-L1 therapies produce durable responses in some cancer patients, not all patients with malignancies respond to these therapies. To enhance the antitumor effects and increase the number of patients who benefit from PD-1/PD-L1 blockade therapies, the combination of chemotherapy, radiotherapy, or molecular targeted therapy with ICIs has been intensively investigated [19–21]. Although some of these combinatorial immunotherapies have succeeded in improving survival and the overall response rate compared to standard therapies, it remains unclear whether the addition of cytotoxic therapy or molecular targeted therapy to ICIs produces synergistic effects. Anti-PD-1/PD-L1 antibodies reactivate exhausted T cells, which are capable of recognizing tumor-specific antigens, and augment antitumor immune responses against tumor cells [22]. It seems to be important to induce and increase tumor-specific T cells in cancer patients to enhance the antitumor effects of PD-1/PD-L1 blockade therapies. As mentioned above, lymphodepletion and transfer of naïve T cells increase tumor-specific T cells in tumor-bearing hosts; therefore, the combination of lymphodepletion, transfer of naïve T cells, and PD-1/PD-L1 blockade therapy may have synergistic antitumor effects.

In this report, we assessed the influence of lymphodepletion and naïve T cell transfer on immune checkpoints in the tumor microenvironments. We found that the expressions of immune checkpoint molecules were significantly reduced in transferred donor $CD4^+$ and $CD8^+$ T cells compared to recipient $CD4^+$ and $CD8^+$ T cells in lymphodepleted tumor-bearing mice. Administration of anti-PD-1 antibodies after lymphodepletion and adoptive transfer of T cells significantly inhibited tumor progression. Furthermore, transfer of both donor $CD4^+$ and $CD8^+$ T cells was responsible for this augmentation of antitumor effects. Our observations suggest that a possible mechanism of the antitumor effect augmentation mediated by lymphodepletion followed by T cell transfer is the prevention of donor T cell exhaustion and dysfunction. Blockade of PD-1 significantly enhanced the antitumor effects of lymphodepletion and T cell transfer.

Materials and methods

Animals

All animal studies were approved by the Niigata University Institutional Animal Care and Use Committee. Female C57BL/6 J (B6) mice were purchased from the CLEA Laboratory (Tokyo, Japan), and Ly5.1 congenic B6 mice were obtained from Sankyo Labo Service (Tokyo, Japan). All mice were housed in a specific pathogen-free environment and used between 8 and 12 weeks of age.

Tumors

The MCA205 cell line is a fibrosarcoma cell line of B6 origin that was initially induced through the intramuscular injection of 3-methylcholanthrene [23]. These tumor cells were routinely passaged in vivo and were used between the fifth and eighth passages. Single-cell suspensions were prepared from solid tumors by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO) for 3 h at room temperature. The cells were filtered through a 100-μm nylon mesh, washed, and suspended in HBSS for intravenous (i.v.) and subcutaneous (s.c.) inoculations. The B16F10 cell line is a melanoma cell line of B6 origin that was cultured in complete medium consisting of RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, antibiotics, and antifungal agents (Thermo Scientific, Waltham, MA) at 37 °C and 5% CO_2. Cells were harvested by brief treatment with 0.25% trypsin/0.02% EDTA (Thermo Scientific) and resuspended in HBSS for animal inoculations.

FACS analysis

FITC-conjugated monoclonal antibodies (mAbs) against CD11b (M1/70) and Ly5.1 (A20); PE-conjugated mAbs against CD4 (RM4-5), CD8 (53–6.7), CD95 (Jo2), Gr-1 (1A8), ICOS (7E.17Gg), LAG-3 (C9B7W), PD-1 (J43), PD-L1 (MIH5), TIGIT (1Gg) and TIM-3 (5D12);

Cy-chrome-conjugated mAbs against CD3 (145-2C11), CD4 (RM4-5), CD8 (53–6.7), CD11b (M1/70), CD11c (HL3), CD56 (PK136) and F4/80 (T45-2342); APC-conjugated mAbs against CD8 (53–6.7) and isotype-matched mAbs were purchased from BD Biosciences (San Jose, CA). A PE-anti-Forkhead box P3 (Foxp3) antibody (FJK-16 s) was purchased from eBioscience (San Diego, CA). Cell-surface phenotypes were determined by direct immunofluorescence staining with conjugated mAbs and analysis with a FACS-Calibur flow cytometer (BD Biosciences). Foxp3 staining was performed using a PE-Foxp3 staining set (eBioscience).

Adoptive transfer and PD-1 blockade therapy

B6 mice were lymphodepleted by sublethal irradiation with 500 cGy or were treated with cyclophosphamide (Sigma-Aldrich), etoposide (Sigma-Aldrich), paclitaxel (R & D Systems, Minneapolis, NE), or gemcitabine (R & D Systems). One day later, mice were reconstituted i.v. with 40×10^6 spleen cells from normal mice as a source of naïve T cells. The reconstituted mice were then inoculated s.c. with 1×10^5 MCA 205 tumor cells along the midline of the abdomen. An anti-PD-1 mAb (RMP1-14: Bio X cell, Lebanon, NH) was injected intraperitoneally (i.p.) into the mice at a dose of 250 μg per mouse on days 1, 7, and 13. The tumor size was measured in 2 perpendicular dimensions 2 to 3 times per week with digital calipers, and the tumor area (mm^2) was recorded.

Assessment of tumor-infiltrating immune cells

B6 mice were inoculated s.c. with 1×10^5 MCA205 or 1×10^5 B16F10 tumor cells along the midline of the abdomen. Seven days after tumor cell inoculation, these mice were sublethally irradiated and were then reconstituted with 40×10^6 spleen cells from Ly5.1 congenic mice or injected i.p. with an anti-PD-1 mAb (RMP1-14: Bio X cells) at a dose of 250 μg per mouse on days 7and 14. On day 21, tumor tissues were harvested, and single-cell suspensions were prepared by digestion using the three enzymes described above. These cell suspensions were labeled with fluorophore-conjugated antibodies for FACS analyses.

Cell separation

For the depletion of $CD4^+$ or $CD8^+$ cells from donor cells, naïve spleen cells were suspended at 3×10^8 cells/ml in 2.4 ml of magnetically activated cell sorting (MACS) buffer (0.5% bovine serum albumin in phosphate-buffered saline with 2 mM ethylenediaminetetraacetic acid) and then incubated at 4 °C with CD4 or CD8 MACS beads (Miltenyi Biotech, San Diego, CA) for 15 min. $CD4^-$ or $CD8^-$ cell populations were collected as flow-through cells from the MACS columns.

Antibody treatment in vivo

To deplete $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and regulatory T cells (Tregs), anti-CD4 mAb (L3T4), anti-CD8 mAb (Lyt-2), anti-NK1.1 mAb (PK136), and anti-CD25 mAb (PC61) were injected i.p. on the same day of tumor inoculation. The ability of depletion by antibody treatment was confirmed as ≥ 95% depletion using FACS analysis.

Statistical analysis

The significance of differences between groups was analyzed using the Wilcoxon rank-sum test or Student's t test. All statistical analyses were carried out using JMP 9 (SAS, Institute Inc., Cary, NC, USA). A 2-tailed p value < 0.05 was considered significant. All experiments were repeated at least twice.

Results

PD-L1 expression is reduced on transferred donor T cells in reconstituted irradiated hosts

Previous clinical studies have demonstrated that the expression of PD-L1 on tumor and immune cells is a predictive factor for the antitumor effects of PD-1 and PD-L1 inhibitors [24]. To examine whether PD-L1 expression is affected by lymphodepletion and T cell transfer, B6 mice were inoculated s.c. with MCA205 tumor cells (1×10^5) or B16F10 tumor cells (1×10^5) along the midline of the abdomen. On day 7, these mice were sublethally (500 cGy) irradiated. These irradiated lymphopenic mice were subsequently injected i.v. with spleen cells (40×10^6) from naïve Ly5.1 mice or injected i.p. with anti-PD-1 mAbs at a dose of 250 μg per mouse on days 7 and 14. On day 21, tumor tissues were harvested and single-cell suspensions were prepared for FACS analysis (Fig. 1A). As shown in Fig. 1, PD-L1 expression on MCA205, B16F10, $CD56^+$, $CD11b^+$, $CD11c^+$, and $F4/80^+$ cells, recipient $CD4^+$ T cells, and recipient $CD8^+$ T cells was not affected by irradiation followed by T cell transfer. In contrast, PD-L1 expression on donor $Ly5.1^+CD4^+$ and $Ly5.1^+CD8^+$ T cells was significantly reduced (Fig. 1C, D, J, K).

Expression of checkpoint receptors is suppressed on donor T cells

Previous studies have demonstrated that the checkpoint receptor axis is upregulated on T cells infiltrating tumors

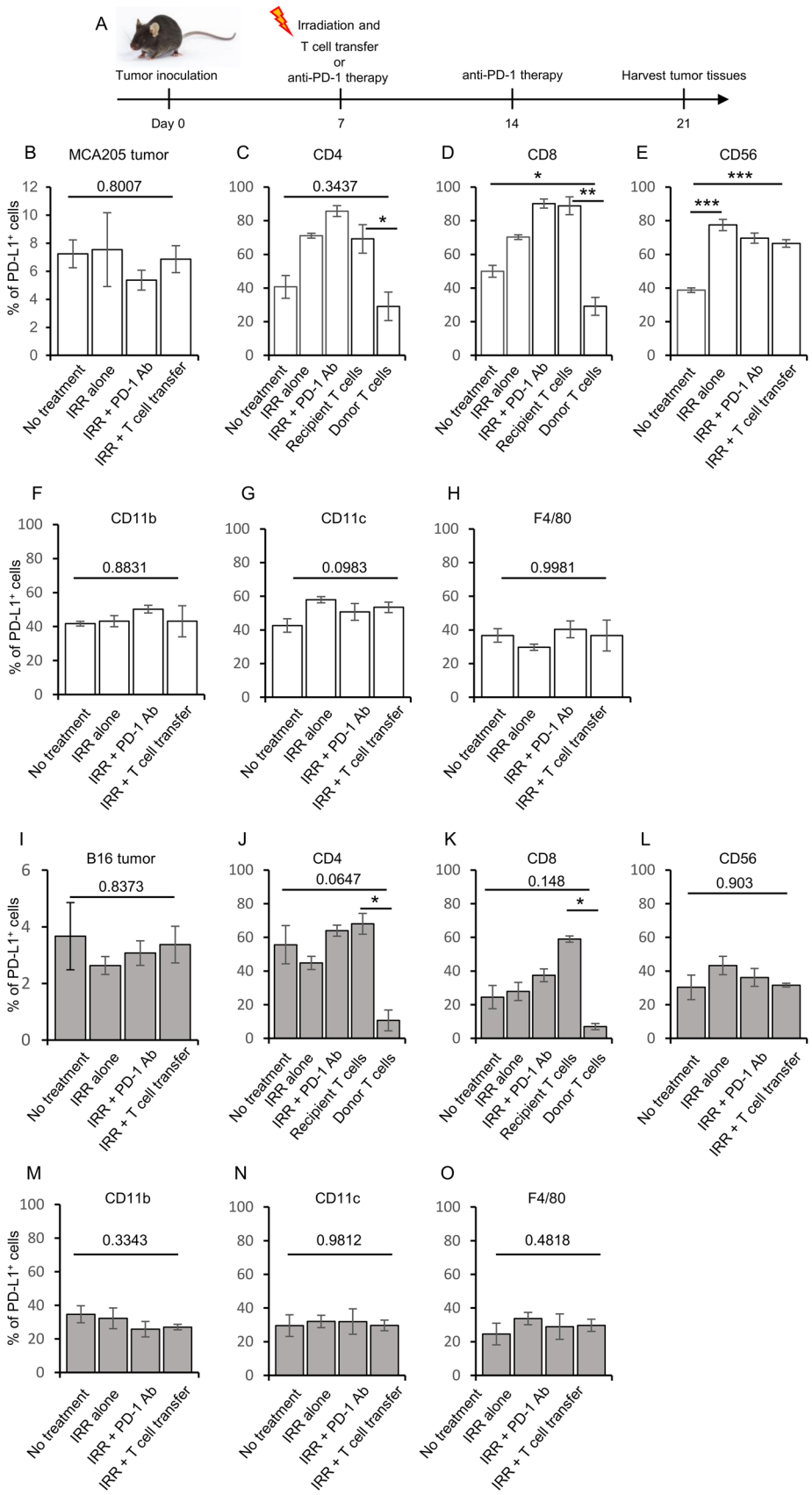


Fig. 1 PD-L1 expression on MCA205, B16F10, and immune cells in tumor tissues. **A** Experimental schema of the MCA205 and B16F10 tumor model. **B–O** B6 mice were inoculated s.c. with MCA 205 (**B–H**) or B16F10 (**I–O**) tumor cells along the midline of the abdomen. These mice were sublethally irradiated and were then reconstituted with spleen cells from Ly5.1 congenic mice 7 days after tumor cell injection, or injected i.p. with an anti-PD-1 mAbs (250 μg per mouse) on days 7 and 14. On day 21, tumor tissues were harvested, and single-cell suspensions were labeled with fluorophore-conjugated antibodies for FACS analyses. Data are shown as mean ± SE ($n = 3$/group). P values were estimated with the Student's t test and are shown as $^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$

[25]. Tumor cells mediate immunosuppression using immune checkpoints such as the PD-L1/PD-1 signaling pathway, and T cells in the tumor microenvironment are exhausted and poorly respond to tumor-antigens [26]. To assess immune checkpoint receptors on T cells after lymphodepletion and T cell transfer, we harvested MCA205 tumor tissues from irradiated mice subsequently reconstituted with spleen cells from normal Ly5.1 mice or treated with anti-PD-1 mAbs. As shown in Fig. 2A–F, expression of TIGIT, TIM-3, and LAG-3 on donor Ly5.1$^+$CD4$^+$ T cells were significantly reduced. Significant decreases in the expression of PD-1, ICOS, TIM-3, and LAG-3 on donor cells were also observed in the Ly5.1$^+$CD8$^+$ T cell population (Fig. 2G–I). In contrast, administration of anti-PD-1 mAbs following irradiation did not decrease the expression of immune checkpoint receptors on recipient CD4$^+$ and CD8$^+$ T cells (Fig. 2). We next evaluated checkpoint receptors on donor cells infiltrating B16 tumor tissues (Supplementary Fig. S1). Similar to the results for the MCA205 tumor models, most of checkpoint receptors were significantly less expressed on donor Ly5.1$^+$CD4$^+$ and Ly5.1$^+$CD8$^+$ T cells than those on recipient CD4$^+$ and CD8$^+$ T cells. In addition, we evaluated the effects of lymphodepletion on donor mice in this model system. Irradiated tumor-bearing mice were reconstituted with spleen cells from Ly5.1 mice that had been irradiated 14 days earlier. MCA205 tumor tissues were harvested and the expression of immune checkpoint receptors on T cells was assessed. Regardless of whether the donor mice had been irradiated or not, the expression of checkpoint receptors on donor T cells was significantly suppressed (Supplementary Fig. S2).

Next, we asked whether the expression of checkpoint receptors on donor T cells in tumor-draining lymph nodes (TDLNs) was also reduced after lymphodepletion and T cell transfer. TDLNs were harvested from irradiated mice subsequently reconstituted with spleen cells from normal ly5.1 mice and treated with anti-PD-1 mAbs. Unlike the immune checkpoint receptors on donor T cells in tumor tissues, the expression of checkpoint receptors on donor T cells in TDLNs was not reduced (Supplementary Fig. S3).

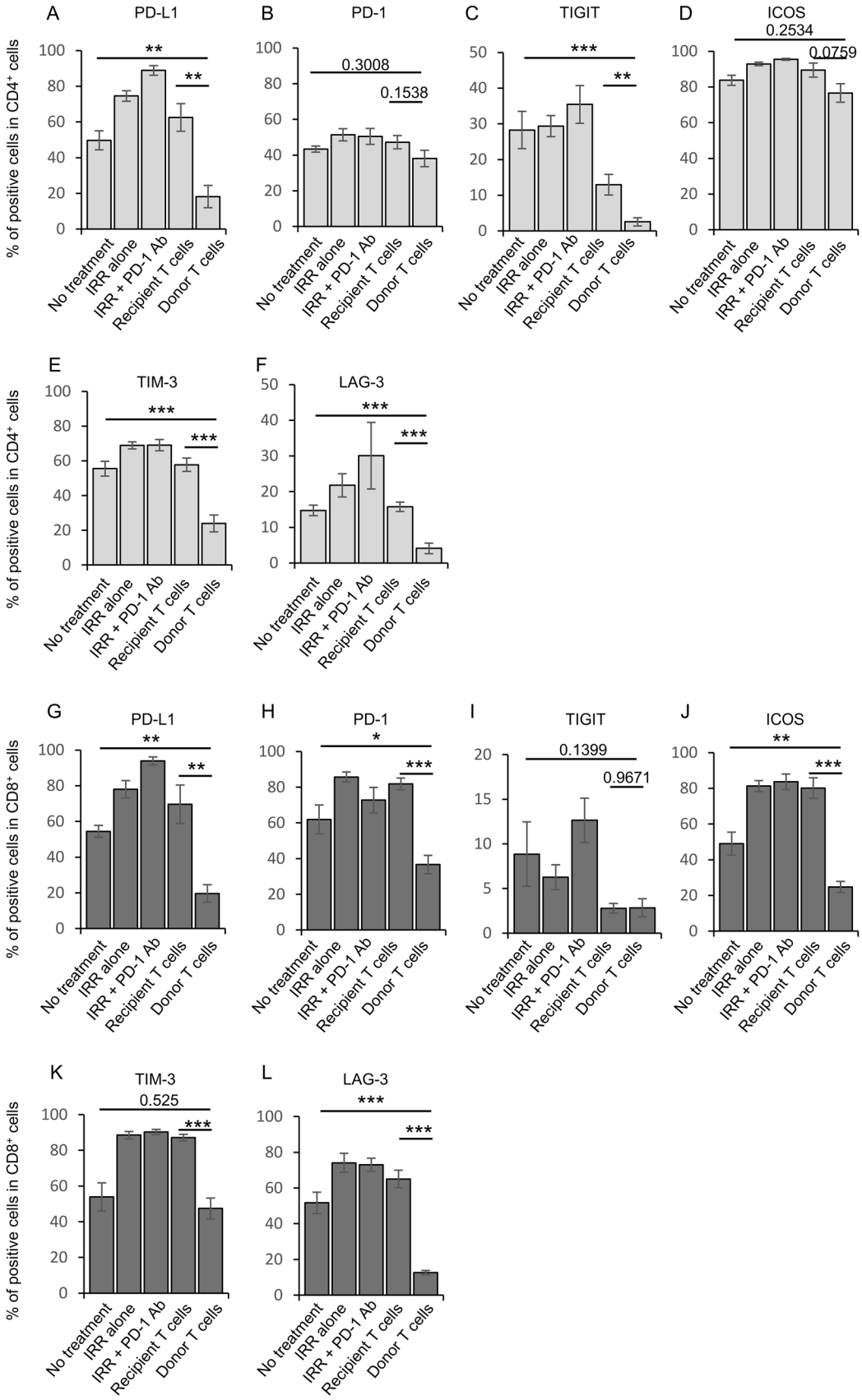
Anti-PD-1 mAbs enhance the antitumor effects of lymphodepletion followed by naïve T cell transfer

Recent studies have indicated that PD-1/PD-L1 blockade therapies restore the function of effector T cells and produce antitumor effects [27]. The presence of effector T cells appears to be required for the antitumor effects of PD-1/PD-L1 inhibitors. We previously demonstrated that tumor-specific effector T cells were induced and that antitumor immunity was augmented in tumor-bearing hosts that received lymphodepleting cytotoxic therapies followed by naïve T cell transfer [28]. To determine whether anti-PD-1 mAbs enhance the antitumor effects of lymphodepletion and naïve T cell transfer, sublethally irradiated mice were injected i.v. with spleen cells from normal mice and then inoculated s.c. with MCA205 or B16F10 tumor cells along the midline of the abdomen. Anti-PD-1 mAbs were injected i.p. into the mice at a dose of 250 μg per mouse on days 0, 6, and 12. As shown in Fig. 3A, B, the combination of irradiation, T cell transfer, and anti-PD-1 mAb treatment significantly inhibited skin tumor growth.

We next assessed the antitumor effects of the combination of other cytotoxic therapies with T cell transfer and anti-PD-1 mAb treatment. Mice were treated with various cytotoxic agents at half the median lethal dose (LD50; 300 mg/kg cyclophosphamide, 140 mg/kg etoposide, 12 mg/kg paclitaxel and 1000 mg/kg gemcitabine). One day later, these mice were reconstituted i.v. with spleen cells from normal mice and were then inoculated s.c. with MCA205 tumor cells. Anti-PD-1 mAbs were injected i.p. into the mice on days 1, 7, and 13. As shown in Fig. 3C, E, F, the retardation of skin tumor growth was observed in the cyclophosphamide-, paclitaxel- and gemcitabine-treated mice that were also injected with spleen cells and anti-PD-1 mAbs compared with untreated mice. Although the combination of cyclophosphamide or gemcitabine, T cell transfer, and anti-PD-1 mAb treatment did not show significant retardation of skin tumor growth when compared with the combination of cyclophosphamide or gemcitabine and T cell transfer (Fig. 3C, D, F), the combination of paclitaxel, T cell transfer, and anti-PD-1 mAb treatment significantly delayed tumor progression (Fig. 3E).

Donor CD4$^+$and CD8$^+$ T cells are responsible for the antitumor effects of the combination of irradiation, T cell transfer and anti-PD-1 mAb treatment.

Previous studies have demonstrated the key role of CD8$^+$ T cells in the antitumor effects of PD-1 blockade therapy [29, 30]. Other researchers reported that depletion of CD4$^+$ T cells augmented the antitumor effects of anti-PD-1/PD-L1 mAbs [31]. To evaluate the roles of CD4$^+$ and CD8$^+$ T cells in the antitumor efficacy of the combination therapy comprising irradiation, T cell transfer, and anti-PD-1 mAbs, we depleted CD4$^+$ or CD8$^+$ T cells from mice treated with anti-PD-1 mAbs after irradiation and reconstitution. The depletion of CD4$^+$ T cells or CD8$^+$ T cells abrogated the



◂**Fig. 2** Expression of checkpoint receptors after lymphodepletion and T cell transfer. B6 mice were injected s.c. with MCA205 tumor cells. These mice were irradiated and reconstituted with spleen cells from Ly5.1 mice 7 days after tumor injection or injected i.p. with anti-PD-1 mAbs (250 μg per mouse) on days 7 and 14. Tumor tissues were harvested and prepared for FACS analysis on day 21. **A–I** The percentage of PD-L1$^+$, PD-1$^+$, TIGIT$^+$, ICOS$^+$, TIM-3$^+$, LAG-3$^+$ cells among CD4$^+$ T cells are shown. B–l The percentage of PD-L1$^+$, PD-1$^+$, TIGIT$^+$, ICOS$^+$, TIM-3$^+$, LAG-3$^+$ cells among CD8$^+$ T cells are presented. Data are shown as mean ± SE ($n = 3$/group). P values were estimated with the Student's t test and are shown as $^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$

antitumor effects of this combination therapy (Fig. 4A, B). We next asked whether the transfer of CD4$^+$ and CD8$^+$ T cells was responsible for the antitumor efficacy of the combination of irradiation, T cell transfer, and anti-PD-1 treatment. CD4$^+$ and CD8$^+$ T cells were depleted from spleen cells before transfer using magnetic beads. As shown in Fig. 4C, the depletion of donor CD4$^+$ and CD8$^+$ T cells completely abrogated the antitumor effects of the combination therapy. Because a recent study showed that PD-1/PD-L1 blockade therapy activates the function of NK cells, we determined whether NK cells are responsible for the antitumor efficacy of this combination therapy [32]. Depletion of NK cells did not reduce the antitumor effects (Fig. 4D).

Effect of the combination therapy on regulatory T cells and myeloid-derived suppressor cells

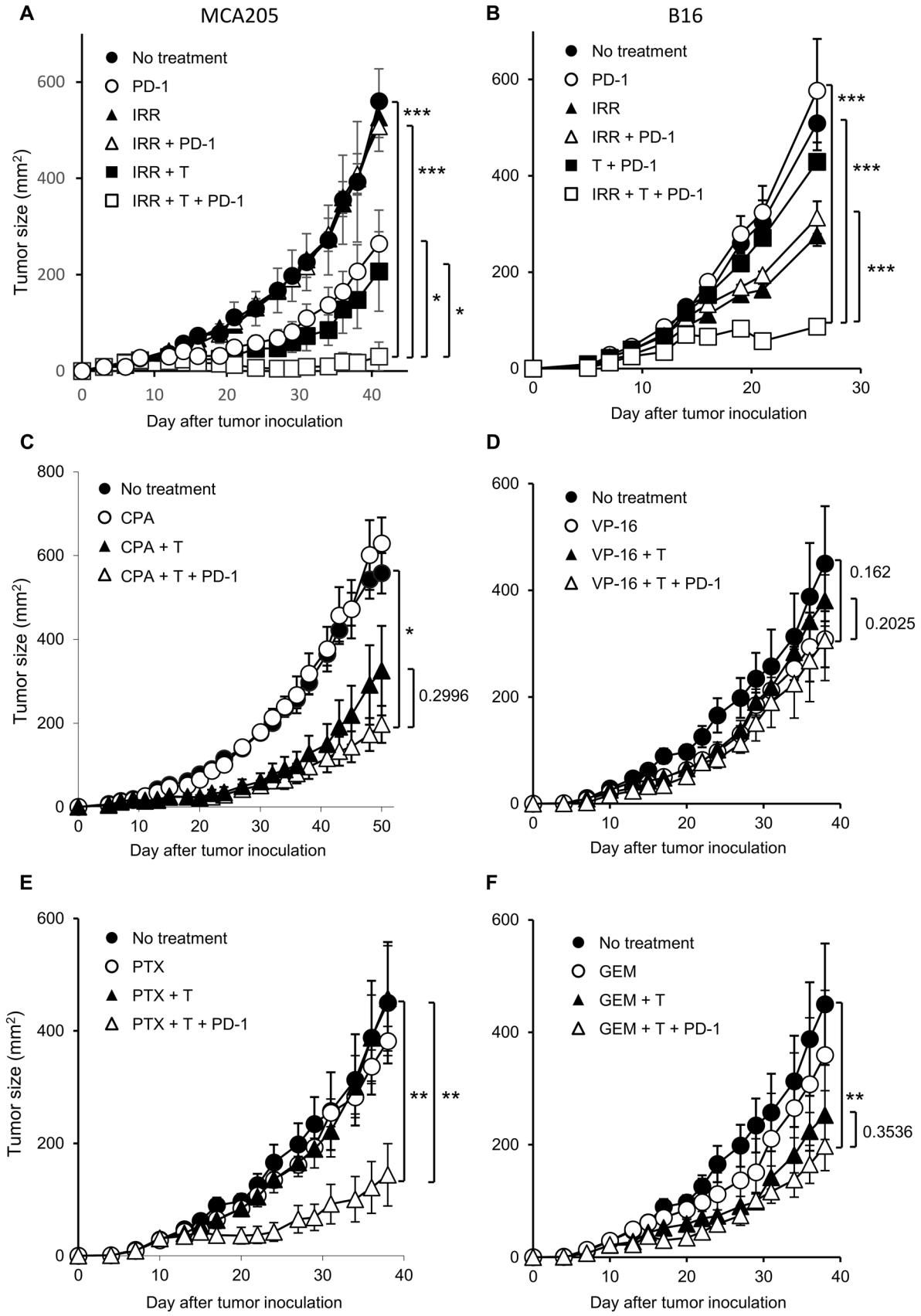
In previous studies, we demonstrated that the percentage of Tregs was increased after lymphodepleting regimens, including irradiation and cyclophosphamide administration [7, 28]. Tregs surviving lymphodepletion suppressed the development of antitumor immunity during recovery from lymphopenia. Myeloid-derived suppressor cells (MDSCs) are precursors of myeloid cells, dendritic cells (DCs), macrophages, and granulocytes [33]. MDSCs have the ability to suppress T cell immune responses [33]. We and others have previously demonstrated that MDSCs suppress the induction of tumor-specific T cells and that the accumulation of MDSCs is associated with poor outcomes [34, 35]. To elucidate the effect of the combination therapy on suppressive immune cells infiltrating tumors, mice were inoculated with MCA205 tumor cells. These mice were irradiated and adoptively transferred naïve T cells on day 14. Anti-PD-1 mAb was injected into the mice on days 14, 21, and 28. On day 35, MCA205 tumor tissues were harvested and single-cell suspensions were prepared for FACS analysis. As shown in Fig. 5A, C, the percentage of CD4$^+$Foxp3$^+$ Tregs was increased after irradiation, which was consistent with our previous study [7]. Although anti-PD-1 treatment and/or T cell transfer following irradiation reduced the percentage of Tregs, the frequency of Tregs was still higher than that in nonirradiated tumor-bearing mice. In contrast to Tregs, CD11b$^+$Gr-1$^+$ MDSCs in tumor tissues were not affected by irradiation, T cell transfer, or anti-PD-1 mAbs (Fig. 5B, D).

Depletion of the Tregs that survive irradiation enhances the antitumor effects of the combination therapy

Our previous studies showed that depletion of the Tregs that survive lymphodepletion increases tumor-specific effector T cells and augments antitumor immunity [7, 28]. To explore whether depletion of Tregs also enhances the antitumor effects of the combination of irradiation, T cell transfer, and anti-PD-1 treatment, MCA205 tumor-bearing mice were irradiated and reconstituted with spleen cells from normal mice 3 days after tumor cell injection. The mice were then injected i.p. with anti-CD25 mAbs (PC61). As shown in Fig. 6A, CD4$^+$Foxp3$^+$ Tregs were successfully depleted by the anti-CD25 mAbs. The mice were also treated with anti-PD-1 mAbs on days 3, 9, and 15. The depletion of Tregs augmented the antitumor effects of the combination therapy consisting of irradiation, T cell transfer, and anti-PD-1 mAbs and significantly inhibited skin tumor growth (Fig. 6B). To further evaluate the antitumor efficacy of the combination of lymphodepletion, mice with 20-day established skin tumor were sublethally irradiated and transferred i.v. with naïve T cells. The mice were then injected i.p. with anti-CD25 mAbs and treated with anti-PD-1 mAbs on days 20, 26, and 32. As shown in Supplementary Fig. S4, the combination therapy consisting of lymphodepletion, T cell transfer, anti-PD-1 treatment, and Treg depletion failed to inhibit the progression of advanced skin tumors.

Discussion

The enhancement of antitumor immunity by lymphodepletion has been well established [9]. In addition to the induction of tumor-specific effector T cells from naïve T cells, the function of effector memory T cells is augmented by lymphodepletion [8, 36]. Previous studies have suggested the mechanisms underlying the effectiveness of lymphodepletion in antitumor immunity to be as follows: depletion of suppressive immune cells, direct stimulation of T cells, upregulation of MHC-class I molecules on cancer cells, increase in the permeability of cancer cells to granzyme B, improvement in the availability of homeostatic cytokines,



◂**Fig. 3** Anti-PD-1 mAbs augment the antitumor effects of the combination of lymphodepletion and T cell transfer. **A** and **B** B6 mice were irradiated and reconstituted with spleen cells from normal mice. These mice were then inoculated s.c. with MCA205 (**A**) or B16F10 (**B**) tumor cells along the midline of the abdomen (5 mice per group). Anti-PD-1 mAbs (250 μg per mouse) were injected i.p. into the mice on days 0, 6, 12. c–f B6 mice were treated with cyclophosphamide (CPA, 300 mg/kg), etoposide (VP-16, 140 mg/kg), paclitaxel (PTX, 12 mg/kg) or gemcitabine (GEM, 1000 mg/kg). One day later, these mice were reconstituted with spleen cells from normal mice and were then injected s.c. with MCA205 tumor cells (5 mice per group). Anti-PD-1 treatment was administered i.p. on days 1, 7, and 13. Significance was assessed using the Student's t test and are shown as $^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$

enhancement of antigen presentation by DCs and induction of immunogenic cell death; however, precise mechanisms remain to be elucidated [9, 37, 38]. Recent evidence has shown that T cells are suppressed and exhausted in tumor-bearing hosts [39]. Exhausted T cells express different immune checkpoint receptors at various stages of exhaustion and increase the expression of these inhibitory receptors with progressive dysfunction. The PD-1/PD-L1 axis seems to be one of the most important immune checkpoints in T cell exhaustion because blockade therapy targeting PD-1/PD-L1 succeeds in reinvigorating T cell function and has demonstrated clinically meaningful antitumor effects on multiple cancer types [12–18]. In the current study, we first investigated whether PD-L1 expression in the tumor microenvironment is affected by lymphodepletion and adoptive transfer of T cells. MCA205 tumor cells, B16F10 tumor cells, macrophages, DCs, and NK cells did not show decreased expression of PD-L1 (Fig. 1). Next, we assessed whether lymphodepletion and adoptive transfer of T cells influence the expression of immune checkpoint receptors on T cells in tumor-bearing mice. Although recipient T cells expressed various inhibitory immune checkpoint receptors, the expression of immune checkpoint receptors was significantly reduced on transferred donor T cells that infiltrated tumors (Fig. 2 and Supplementary Fig. S1). The decrease of the expression of immune checkpoint receptors on donor T cells was not observed in TDLNs (Supplementary Fig. S3). The number of TDLN cells did not increase after the combination therapy consisting of lymphodepletion, T cell transfer, and anti-PD-1 mAbs, suggesting that this treatment did not promote the induction of antitumor effector T cells (data not shown). Our study indicates that prevention of donor T cell exhaustion in the tumor microenvironments may be one of the mechanisms underlying the effectiveness of lymphodepletion in antitumor immunity. Unlike donor T cells, recipient T cells did not show prevention of exhaustion by lymphodepletion and T cell transfer. The addition of anti-PD-1 antibodies to lymphodepletion and T cell transfer succeeded in augmenting antitumor effects (Fig. 3A, B). This augmentation might occur through the reactivation of exhausted recipient T cells.

In clinical studies and current clinical practice, CAR T cell therapy and adoptive transfer of TILs have been administered after lymphodepletion. Although these therapies have demonstrated durable antitumor effects, both T cell therapies require in vitro manipulation including stimulation with various cytokines [4–6]. In this study, naïve T cells were used as the source of antitumor effector T cells. Adoptive transfer of naïve T cells after lymphodepletion is different from the current clinical application of CAR T cells or TILs, but this method does not require in vitro stimulation.

Our previous study demonstrated that Tregs surviving lymphodepletion proliferate rapidly and suppress the antitumor immunity during recovery from lymphopenia [7, 28]. Further depletion of Tregs after lymphodepletion significantly enhanced antitumor immunity. Consistent with these previous findings, the percentage of Tregs was increased after irradiation (Fig. 5a, c). Recent evidence has shown that the Tregs are suppressed by signaling through PD-1 and that blockade of PD-1 significantly augments the suppressive ability of Tregs [40]. In the present study, anti-PD-1 treatment decreased the percentage of Tregs infiltrating tumors; however, the percentage of Tregs in mice treated with anti-PD-1 treatment following irradiation was still higher than that in untreated tumor-bearing mice (Fig. 5A, C). Depletion of Tregs following the combination of irradiation, T cell transfer, and anti-PD-1 treatment significantly reduced skin tumor progression (Fig. 6B). Our results indicate that anti-PD-1 treatment is insufficient to inhibit the suppressive function of Tregs after lymphodepletion and T cell transfer.

In previous clinical studies, whole-body irradiation, cyclophosphamide, and/or fludarabine were administered to deplete lymphocytes and enhance the antitumor effects of adoptive cell therapy [9]. Recent studies have demonstrated that other cytotoxic agents also augment antitumor immunity [37, 41, 42]. Furthermore, some of these cytotoxic drugs have been shown to enhance the antitumor effects of anti-PD-1/PD-L1 treatments [43, 44]. In the current study, we investigated whether the administration of

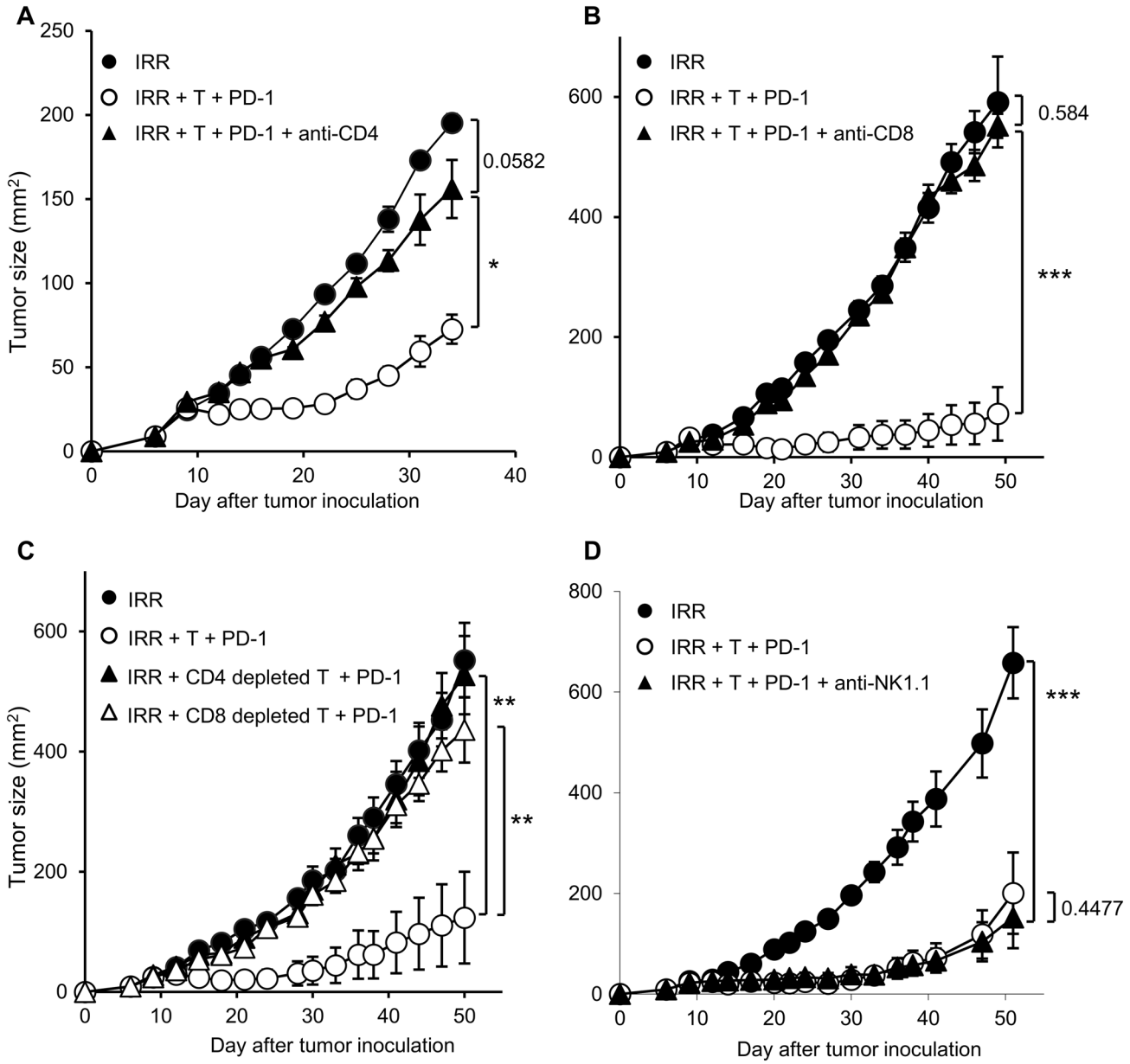


Fig. 4 Both donor CD4$^+$ and CD8$^+$ T cells but not NK cells are essential to augment the antitumor effects of the combination of irradiation, T cell transfer and anti-PD-1 treatment. **A** and **B** Irradiated lymphopenic mice were reconstituted with spleen cells from normal mice and inoculated s.c. with MCA205 tumor cells. These mice were then injected i.p. with anti-PD-1 mAbs. To deplete CD4$^+$ or CD8$^+$ T cells, the mice were further injected i.p. with anti-CD4 mAbs (**A**) or anti-CD8 mAbs (**B**). **C** Irradiated mice were reconstituted with either CD4- or CD8-depleted cell population. These mice were inoculated s.c. with MCA205 tumor cells (5 mice per group) and then treated with anti-PD-1 mAbs. **D** Reconstituted irradiated mice were inoculated s.c. with MCA205 tumor cells. These mice were injected i.p. with anti-PD-1 mAbs. For depletion of NK cells in vivo, anti-NK1.1 mAb was injected i.p. into the mice. *P* values were estimated with the Student's t test and are shown as $*p<0.05$, $**p<0.01$ or $***p<0.001$

cyclophosphamide, etoposide, paclitaxel, or gemcitabine would augment the antitumor efficacy of the combination therapy consisting of anti-PD-1 treatment and adoptive transfer of T cells (Fig. 3c–f). To increase the lymphodepleting effect of cytotoxic drugs, these drugs were used at a half the LD50. None of the examined drugs, except for paclitaxel, were capable of enhancing the antitumor efficacy of the combination therapy of anti-PD-1 therapy and adoptive transfer of T cells when compared with the combination of cytotoxic drugs and T cell transfer. Paclitaxel was reported to be able to increase the antigenicity of cancer cells by enhancing MHC-class I expression on tumor cells and improving antigen presentation by DCs through DC maturation [42, 45]. Considering

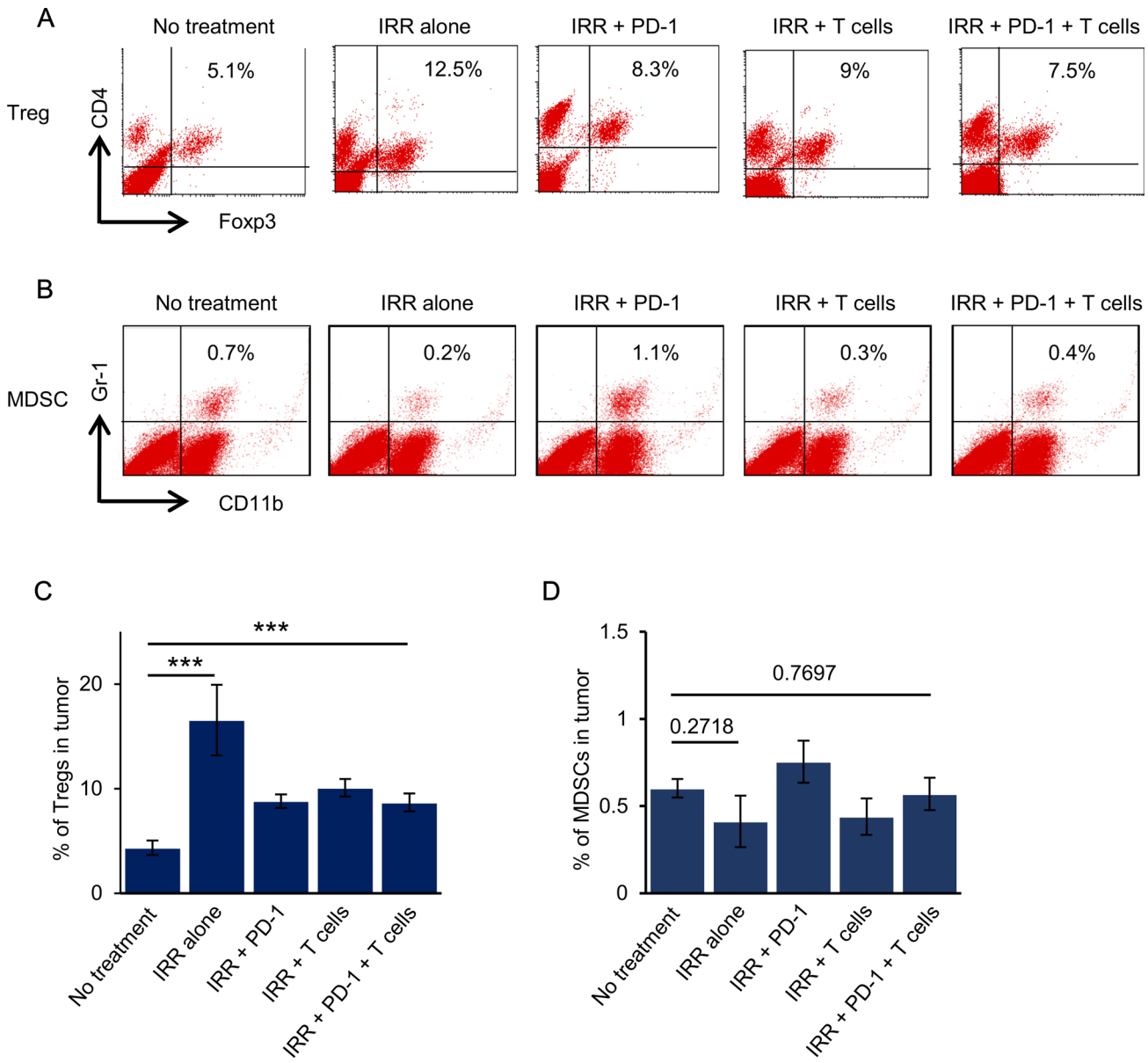


Fig. 5 The changes in the percentages of Tregs and MDSCs after the combination of irradiation, T cell transfer and anti-PD-1 mAbs in the tumor microenvironment. **A**, **B**, **C**, **D** MCA205 tumor-bearing mice were irradiated and reconstituted with naïve T cells 14 days after MCA205 tumor inoculation. Anti-PD-1 mAbs were injected i.p. into the mice on days 14, 21 and 28. MCA205 tumor tissues were harvested on day 35 for FACS analysis. Representative data are shown from 3 independent experiments. *P* values were estimated with the Student's *t* test and are shown as ***$p < 0.001$

clinical practice, administration of cytotoxic drugs seems to be more feasible than whole-body irradiation in cancer patients. Further investigation is required to determine which cytotoxic drugs are the best for lymphodepletion to augment the antitumor effects of anti-PD-1 therapy and adoptive transfer of T cells.

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Author Contributions SW, QZ, BH and LD designed experiments. MT, RS, MA, MS, YS, YA, FS, and SH performed experiments. MT, SW, and KS acquired data. SW and YS analyzed data and generated figures. MT and SW wrote the manuscript. AO, SS, and KN supervised the analysis. KI, RK, NA, YO, MH, TK, and TK discussed the results and conclusions and commented on the manuscript at all stages. All authors read and approved the final manuscript.

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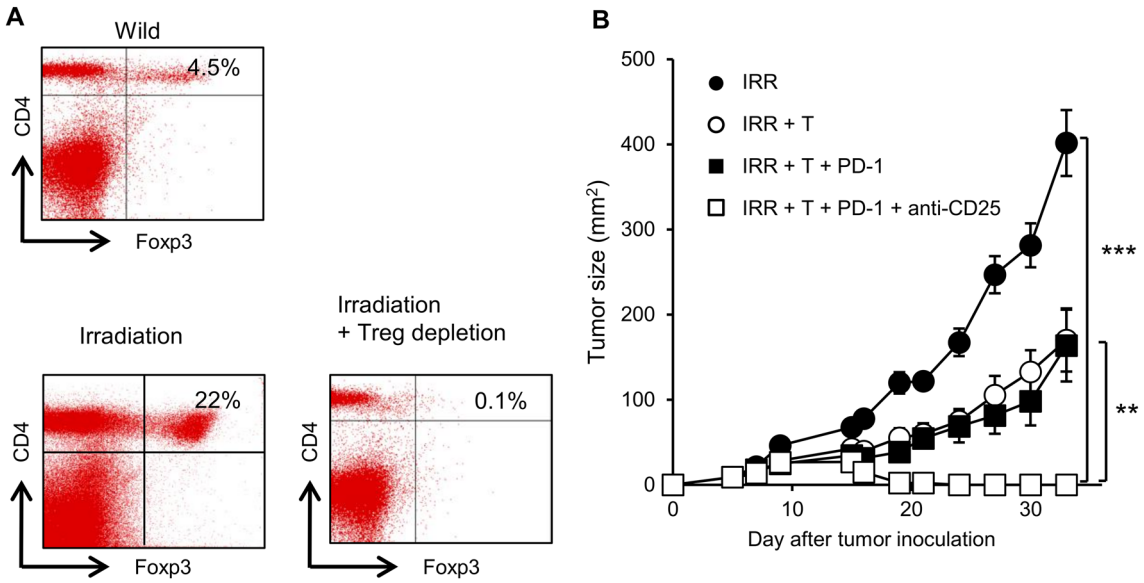

Fig. 6 Further depletion of Tregs augments the antitumor effects of the combination therapy. **A** Mice were irradiated and injected i.p. with anti-CD25 mAbs to deplete Tregs. Ten days after irradiation, spleen cells were harvested to assess the percentage of Tregs using flow cytometry. **B** MCA205 tumor-bearing mice were irradiated and adoptively transferred naïve T cells 3 days after tumor inoculation. The mice were treated with anti-CD25 mAbs to deplete Tregs on day 3 and received anti-PD-1 treatment on days 3, 9 and 15

Availability of data and material All the data are available under reasonable request. Material requests should be addressed to satoshi7@med.niigata-u.ac.jp.

Declarations

Conflict of interest The authors have no financial conflicts of interest.

Ethical approval All animal studies were approved by the Niigata University Institutional Animal Care and Use Committee.

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