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ORIGINAL ARTICLE

Vaccination with CD133$^+$ melanoma induces specific Th17 and Th1 cell–mediated antitumor reactivity against parental tumor

Takao Miyabayashi · Hiroshi Kagamu · Jun Koshio · Kosuke Ichikawa · Junko Baba · Satoshi Watanabe · Hiroshi Tanaka · Junta Tanaka · Hirohisa Yoshizawa · Koh Nakata · Ichiei Narita

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Abstract Accumulating evidence suggests that cancer cells possess a small subpopulation that survives during potentially lethal stresses, including chemotherapy, radiation treatment, and molecular-targeting therapy. CD133 is a putative marker that distinguishes a minor subpopulation from normal differentiated tumor cells in many cancers. Although it is necessary to eradicate all cancer cells to obtain a cure, effective treatment to eliminate the CD133$^+$ treatment–tolerant cells has not been elucidated. In this study, we demonstrated that a CD133$^+$ subpopulation in murine melanoma is immunogenic and that effector T cells specific for the CD133$^+$ melanoma cells mediated potent antitumor reactivity, curing the mice of the parental melanoma. CD133$^+$ melanoma antigens preferentially induced type 17 T helper (Th17) cells and Th1 cells but not Th2 cells. CD133$^+$ melanoma cell–specific CD4$^+$ T-cell treatment eradicated not only CD133$^+$ tumor cells but also CD133$^-$ tumor cells while inducing long-lasting accumulation of lymphocytes and dendritic cells with upregulated MHC class II in tumor tissues. Further, the treatment prevented regulatory T-cell induction. These results indicate that T-cell immunotherapy is a promising treatment option to eradicate CD133$^+$ drug-tolerant cells to obtain a cure for cancer.

T. Miyabayashi · H. Kagamu (✉) · J. Koshio · K. Ichikawa · J. Baba · S. Watanabe · I. Narita
Division of Respiratory Medicine,
Department of Homeostatic Regulation and Development,
Course for Biological Functions and Medical Control,
Graduate School of Medical and Dental Sciences,
Niigata University, Niigata, Japan
e-mail: hkagamu@med.niigata-u.ac.jp

H. Tanaka · J. Tanaka · H. Yoshizawa · K. Nakata
Bioscience Medical Research Center, Niigata University
Medical and Dental Hospital, Niigata, Japan

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Introduction

The cancer stem cells (CSCs) theory states that a minor subpopulation can initiate differentiated cancer cells and tumor tissues via self-renewal and asymmetrical cell division and that this plays a critical role in metastasis and recurrence [1–7]. It is controversial whether the classical CSC theory is applicable for all solid tumors. However, accumulating evidence suggests that a small subpopulation with unique features plays an important role in cancer recurrence after classical anticancer treatment and molecular-targeting therapy [8–12]. An excess of multidrug efflux transporters, antiapoptotic factors, DNA repair gene products, stem cell–specific growth signaling, and relative dormancy contribute to the ability of these cells to resist treatment. CD133 is a stem cell marker and putative CSC marker [13, 14]. It was demonstrated that all of examined cancer cells surviving after potentially lethal drug treatments uniformly express CD133 [15]. These drug-tolerant cancer cell populations use an altered chromatin state to induce a reversible drug-tolerant state and give rise to a permanent drug-tolerant cell population with genetic mutations. Unless these CD133$^+$ cancer cells are eradicated, it is impossible to achieve a lasting cure.

T-cell-mediated immunotherapy can mediate antitumor reactivity. We previously reported that effector T cells primed in tumor-draining lymph nodes (LNs) possessed antitumor therapeutic efficacy in brain, pulmonary, and skin metastasis models [16–18]. In this study, we found that LN T cells primed with the CD133$^+$ tumor vaccine

mediated potent antitumor therapeutic efficacy by eradicating $CD133^+$ tumor cells in tumors, thereby curing parental melanomas that comprised <1% $CD133^+$ tumor cells. Interestingly, $CD133^+$ melanoma antigens tended to prime type 17 helper T (Th17) cells and Th1 cells but not Th2 cells. These results indicate that T-cell immunotherapy may be a promising strategy to eradicate treatment-tolerant $CD133^+$ cancer cells.

Materials and methods

Mice

Female C57BL/6J (B6) mice were purchased from the CLEA Laboratory (Tokyo, Japan). They were maintained in a specific pathogen-free environment and used for experiments at the age of 8–10 weeks. All animal experiments were conducted with the permission of the Niigata University ethics committee for animal experiments.

Tumor cells

B16F10 melanomas, which originate from B6 mice, were maintained in vitro. Parental tumor cells were labeled with phycoerythrin (PE)-conjugated anti-CD133 monoclonal antibody (mAb; 13A4) and anti-PE microbeads (Miltenyi Biotec, Auburn, CA). $CD133^+$ and $CD133^-$ tumor cells were isolated with autoMACS™ (Miltenyi Biotec) according to the manufacturer's instructions. Cell purity was >90%.

mAbs and flow cytometry

Hybridomas producing mAbs against murine CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), CD3 (2C11), and CD62L (MEL14) were obtained from the American Type Culture Collection (Rocksville, MD). Anti-CD4, anti-CD8, and anti-CD62L mAbs were obtained from ascitic fluid of sublethally irradiated (500 cGy) DBA/2 mice. PE-conjugated anti-CD80 (16-10A), anti-CD86 (GL1), anti-CD62L (MEL14), anti-CD8 (2.43), and anti-CD25 (PC61) mAbs; fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (30-H12); and anti-CD4 (GK1.5) mAbs were purchased from BD PharMingen (San Diego, CA). Analyses of cell surface phenotypes were carried out by direct immunofluorescence staining of $0.5–1 \times 10^6$ cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan™ flow microfluorometer (Becton–Dickinson, Sunnyvale, CA). PE-conjugated subclass-matched antibodies used as isotype controls were also purchased from BD PharMingen. Samples were analyzed using the CellQuest™ software (BD PharMingen).

Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Wako Pure Chemical Industries, Osaka, Japan). To yield highly purified (>90%) cells with downregulated CD62L expression ($CD62L^{low}$), LN T cells were further isolated by a panning technique using T-25 flasks pre-coated with goat anti-rat immunoglobulin antibody (Ig Ab) (Jackson ImmunoResearch Laboratories, West Grove, PA)/anti-CD62L mAb (MEL14) and sheep anti-rat-Ig Ab/anti-CD62L mAb-coated DynaBeads M-450 (Dynal, Oslo, Norway). In some experiments, cells were further separated into $CD4^+$ and $CD8^+$ cells by depletion using magnetic beads, as described previously [18]. For in vitro experiments, highly purified $CD4^+$ cells were obtained using anti-CD4 mAb-coated Dynabeads and Detachabeads (Invitrogen) according to the manufacturer's instructions.

Bone marrow–derived dendritic cells

Dendritic cells (DCs) were generated from bone marrow cells (BMs), as described previously. In brief, BMs obtained from femurs and tibias of treatment-naïve mice were placed in T-75 flasks for 2 h at 37°C in complete medium (CM) containing 10 ng/ml of recombinant murine granulocyte–macrophage colony-stimulating factor (rmGM-CSF; a gift from KIRIN, Tokyo, Japan). Non-adherent cells were collected by aspirating the medium and transferred into fresh flasks. On day 6, non-adherent cells were harvested by gentle pipetting. CM consisted of RPMI 1640 medium supplemented with 10% heat-inactivated lipopolysaccharide (LPS)-qualified fetal calf serum (FCS), 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate (all from Life Technologies Inc.), and 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO).

DC/tumor-draining LN cells

BMs and DCs were co-cultured with the same number of irradiated tumor cells (5,000 cGy) in CM overnight. B6 mice were inoculated subcutaneously (s.c.) with 10×10^6 BM–DC and tumor cells in both flanks. Inguinal LNs draining BM–DC and tumor cells were harvested. Single-cell suspensions were prepared mechanically as described previously [19].

Adoptive immunotherapy

B6 mice were injected s.c. with parental B16-F10 tumor cells in 100 μl of Hank's balanced salt solution (HBSS) to establish subcutaneous tumors. Two or three days after the

inoculation, mice were sublethally irradiated (500 cGy) and then infused intravenously (i.v.) with T cells isolated from tumor-draining LNs. LN cells were stimulated with anti-CD3 mAb (2C11) and cultured in CM containing 40 U/ml of interleukin (IL)-2 for 3 days to obtain a sufficient number of T cells for in vivo experiments, as described previously [17]. The perpendicular diameter of subcutaneous tumors was measured with calipers.

Cytokine ELISAs

T cells were stimulated with immobilized anti-CD3 mAb or tumor antigen–pulsed BM–DCs in CM. Supernatants were harvested and assayed for IFN-γ, IL-4, and IL-17 content by a quantitative "sandwich" enzyme immunoassay using a murine IFN-γ, IL-4, and IL-17 ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer's instructions.

In vitro proliferation assay

Melanoma cells were labeled with 5 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes Inc., Eugene, OR) in HBSS at 37°C for 15 min and washed twice before CD3 stimulation. The ratio of CFSE-labeled tumor cells to unlabeled tumor cells was 1:10. Tumor cells were cultured in CM at 1×10^5/ml. Tumor cells were counted every day and were analyzed using a microfluorometer to determine the number of CFSE-labeled tumor cells. Three wells were analyzed for each condition.

Statistical analysis

Comparison between groups was made by Student's *t*-test. The dynamic tumor growth data was analyzed by multivariate general linear model. Differences were considered significant for $P < 0.05$. Statistical analysis was performed with SPSS statistical software (SPSS, Chicago, IL) or GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA).

Results

$CD133^+$ melanoma cells possessed distinct characteristics

For this study, we obtained purified $CD133^+$ tumor cells from murine B16-F10 melanomas (Fig. 1a) and then tested the properties of these cells. Skin tissues were not produced by 1×10^5 subcutaneously inoculated parental B16 melanoma cells, but 5×10^3 $CD133^+$ melanoma cells were sufficient to establish tumor tissues in vivo (Fig. 1b). In vitro proliferation assays showed that $CD133^-$ tumor cells proliferated more aggressively than $CD133^+$ tumor cells before they became confluent, but their proliferation was impeded by cell–cell contact inhibition. In contrast, the proliferation of $CD133^+$ tumor cells did not stop by contact inhibition, and cells piled together, developing into floating aggregates (Fig. 1c). We also tested whether $CD133^+$ melanoma cells could grow in an anchorage-independent manner. Although $CD133^-$ cells eventually died without anchorage, all $CD133^+$ tumor cells proliferated to become tumor spheres (Fig. 1d, e). $CD133^+$, but not $CD133^-$, tumor cells exhibited colony formation on soft agar (Fig. 1f).

Vaccination with $CD133^+$ tumor cells induced protective immunity against the parental melanoma

To examine whether the immune system can recognize $CD133^+$ melanoma cells, we immunized mice by subcutaneously inoculating them with 5,000 cGy-irradiated 1×10^7 parental, $CD133^-$, or $CD133^+$ melanoma cells mixed with 1×10^7 DCs. Fourteen days after immunization, 3×10^5 parental melanoma cells were subcutaneously injected. The tumor growth curves of mice that were immunized with parental tumor cells or $CD133^-$ tumor cells were identical to those of mice that had not received immunization (Fig. 2a). In contrast, tumor growth was significantly retarded in mice immunized with $CD133^+$ tumor cells.

$CD133^+$ tumor antigen–specific T cells mediated potent therapeutic efficacy

We examined the antitumor efficacy of LN T cells draining irradiated parental, $CD133^-$, or $CD133^+$ melanoma cell vaccinations with DCs. $CD62L^{low}$ (cells with downregulated CD62L expression) T cells that were isolated as antigen-primed T cells from LNs were cultured by the anti-CD3/IL-2 method, as described previously [20]. LN T cells were intravenously infused into mice bearing a 2-day-established parental melanoma skin tumor after sublethal whole-body irradiation (500 cGy). The tumors of mice treated with LN T cells primed with parental or $CD133^-$ tumor cells grew in a pattern similar to those of the untreated mice (Fig. 2b). In contrast, the tumors of mice treated by LN T cells primed with $CD133^+$ tumor cells did not grow, even though the mice had palpable tumors. Interestingly, the antitumor reactivity mediated by the LN T cells primed with $CD133^+$ tumor cells persisted for more than 60 days and no mice died of tumor. In this system, regulatory T (Treg) cells were eliminated by whole-body irradiation before antitumor T-cell infusion; however, generally, host lymphocytes recover approximately 20 days after irradiation, and Treg cells that recover as host

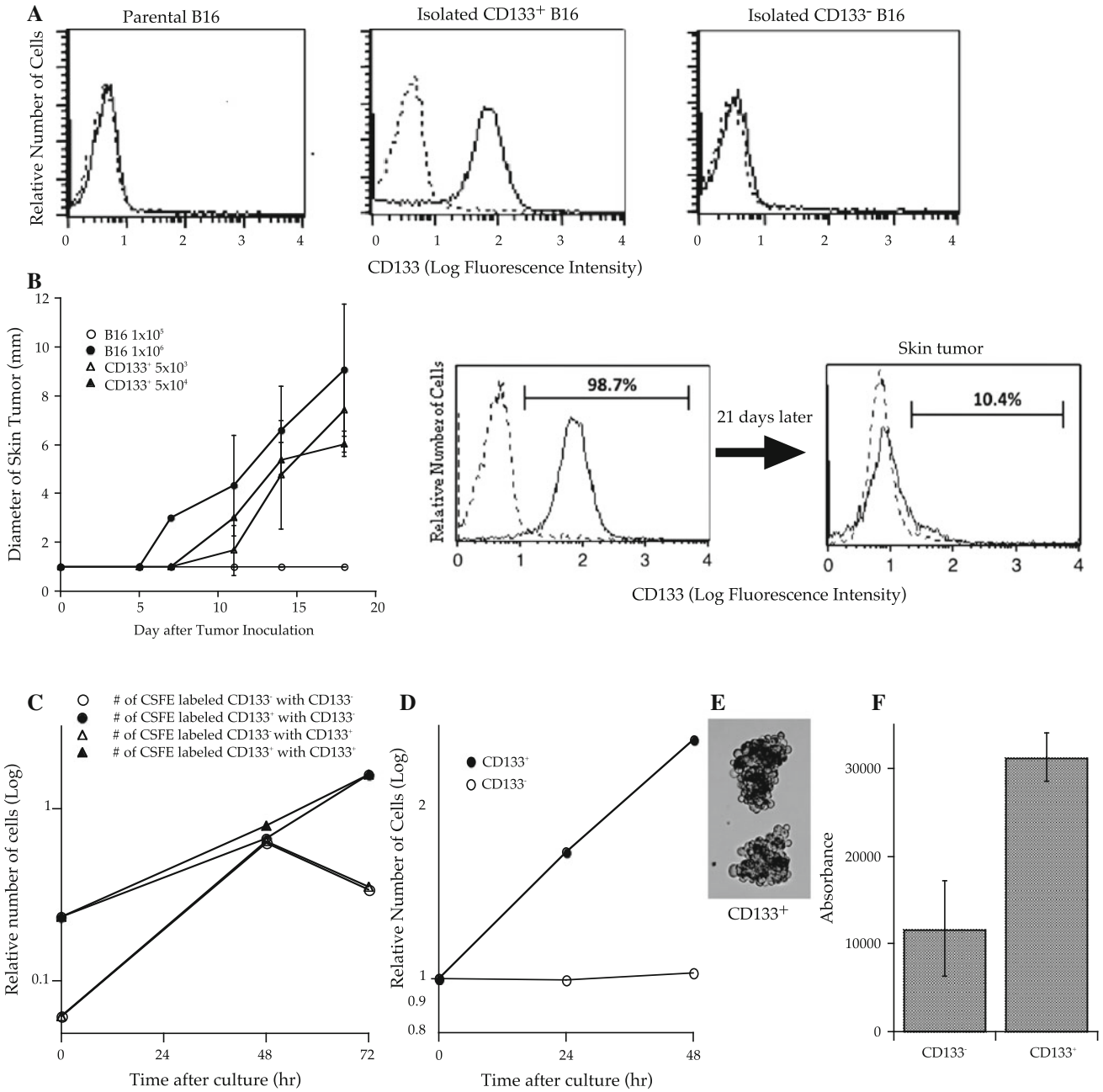


Fig. 1 CD133$^+$ B16 melanoma cells demonstrated high tumorigenicity in vivo (each group contained 5 mice) and proliferated in an anchorage- and cell–cell contact inhibition-independent manner in vitro. **a** One million B16 melanoma cells were stained with phycoerythrin (PE)-conjugated anti-CD133 or PE-conjugated isotype control monoclonal antibodies (mAbs). *Dotted lines* indicate the fluorescence intensity of tumor cells stained with PE-conjugated subclass-matched isotype control mAbs. Each frame consists of 10,000 cells. **b** B6 mice were subcutaneously inoculated along the midline of the abdomen with 5×10^3 or 5×10^4 CD133$^+$ cells, or 1×10^5 or 1×10^6 parental B16 cells. The diameter of the skin tumors was measured twice weekly with calipers, and the size was recorded as the average of 2 perpendicular diameters. Each group contained 6 mice. *Bars* indicate standard deviation. **c** CD133$^+$ or CD133$^-$ tumor cells (0.3×10^6) labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE) were mixed with non-labeled tumor cells (3×10^6) and cultured in complete medium (CM) at 1×10^5 cells/ml. Tumor cells were counted every day and analyzed using a microfluorometer to determine the number of CFSE-labeled tumor cells. Three wells were analyzed for each condition. **d**, **e** One million CD133$^+$ or CD133$^-$ tumor cells were cultured in 10 ml of CM in 50 ml conical tubes that were rotated to avoid cell attachment. Cell counts were performed every 24 h. After 72 h in culture, CD133$^+$ cells proliferated to build spheroid-like cell aggregates. **f** The soft agar colony assay was performed using CytoSelect^{TM} 96 Well Transformation Assay (Cell Biolabs Inc.) according to the manufacturer's instructions; 5×10^3 CD133$^+$ or CD133$^-$ B16 tumor cells were cultured in soft agar for 7 days, and colony formation was examined using a 96-well fluorometer

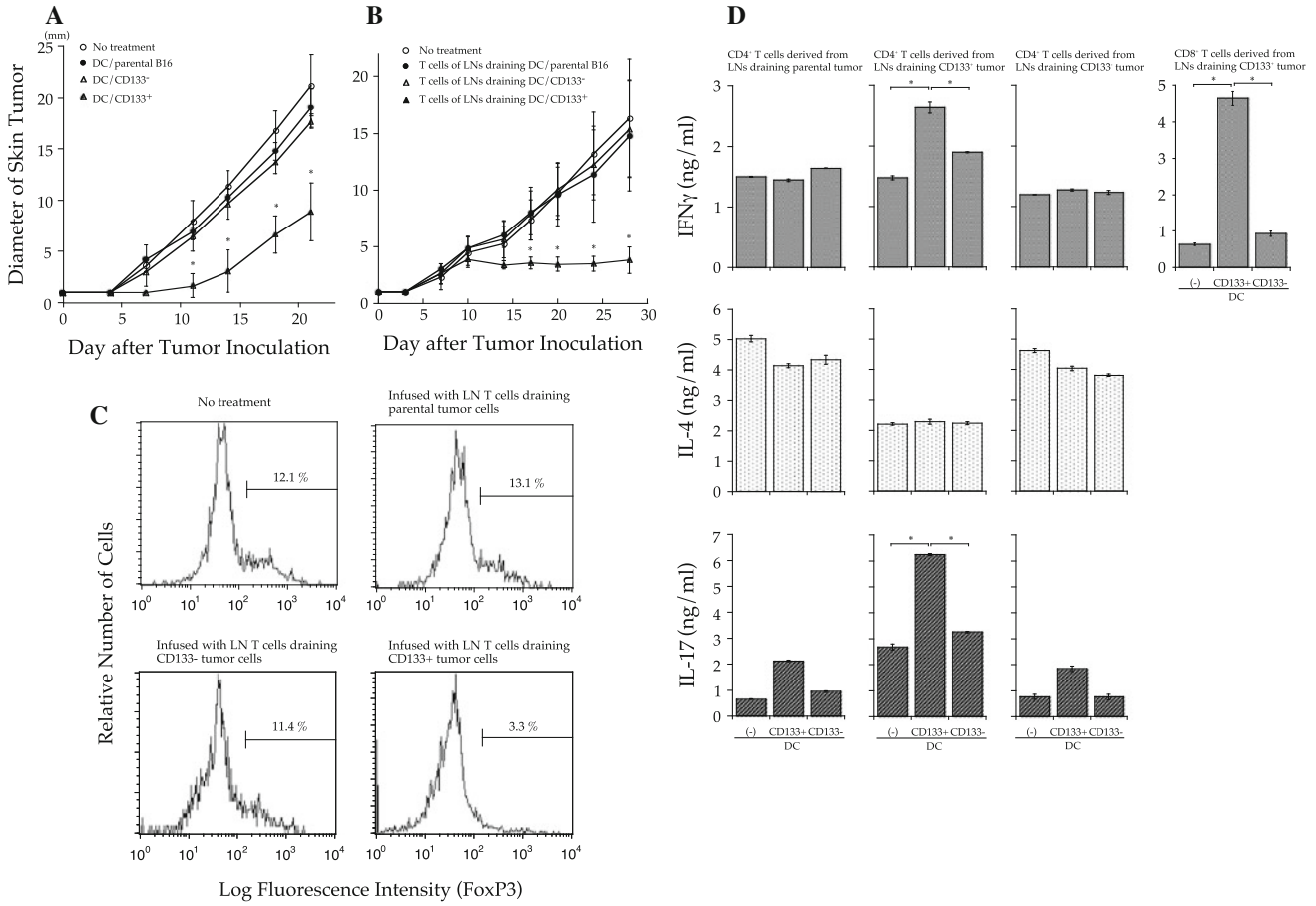


Fig. 2 Irradiated CD133$^+$ tumor cell/dendritic cell (DC) vaccine-primed CD133$^+$ tumor-specific CD8$^+$, and Th1 and Th17 CD4$^+$ T cells mediated therapeutic efficacy against parental B16 melanomas. **a** Bone marrow–derived DCs were co-cultured 1:1 overnight with irradiated (5,000 cGy) parental, CD133$^-$, or CD133$^+$ B16 cells in CM. Non-adherent cells were collected, and 1×10^6 cells were subcutaneously injected into mice. Two weeks later, mice were subcutaneously inoculated along the abdominal midline with 1×10^6 parental B16 cells. Data are from a representative experiment of 3, with 5 mice/group. *Asterisks* indicate $P < 0.01$. **b** Bone marrow–derived DCs co-cultured with irradiated parental, CD133$^-$, or CD133$^+$ tumor cells were subcutaneously injected into both flanks of mice. Inguinal lymph nodes (LN) were collected 8 days later. CD62L^{low} T cells were isolated as antigen-primed LN T cells and cultured by the anti-CD3/interleukin (IL-2) method. B6 mice were subcutaneously inoculated along the abdominal midline with 1×10^6 parental B16 cells in order to establish tumors. Two days later, mice were sublethally irradiated (500 cGy) and intravenously infused with 15×10^6 LN T cells. The diameter of skin tumors was measured twice weekly with calipers; size was recorded as the average of 2 perpendicular diameters. The data are from a representative experiment of 2, with 5 mice/group. *Asterisks* indicate $P < 0.01$. **c** Tumors were obtained 30 days after tumor inoculation and were digested with collagenase, hyaluronidase, and DNase. Cells in tumor tissues were stained using FITC-conjugated anti-CD4 mAb and PE-conjugated anti-Foxp3 mAb (e-Bioscience) with the staining kit according to the manufacturer's instructions. Cells in the lymphocyte region were gated for analyses. **d** IFN-γ, IL-4, and IL-17A secretion. In a 96-well plate, 1×10^5 CD62L^{low} CD4$^+$ T cells isolated from LNs draining irradiated tumor cells/DCs were stimulated with 2×10^4 DCs pulsed with tumor antigens in 200 μl CM for 48 h. DCs for stimulation were purified with CD11c microbeads after overnight co-culture with irradiated tumor cells. *Asterisks* indicate $P < 0.01$

immunity abrogate antitumor reactivity [18]. To elucidate whether T cells primed with CD133$^+$ tumor cells affected Treg induction, we examined Foxp3$^+$ regulatory T cells in tumor tissues 30 days after treatment. As shown in Fig. 2c, very few Foxp3$^+$ CD4$^+$ T cells were detected in tumor tissues of mice treated with LN T cells draining CD133$^+$ tumors. In contrast, there was almost the same number of Foxp3$^+$ CD4$^+$ T cells in tumor tissues of mice that were infused with LN T cells draining parental or CD133$^-$ tumors as in the untreated mice.

T cells primed in LNs draining CD133$^+$ melanoma cells/DCs were specific to CD133$^+$ tumor antigens and exhibited specific IFN-γ and IL-17 production

To examine cytokine release by T cells primed in LNs draining irradiated tumor cell/DC vaccine, CD62L^{low} CD4$^+$ LN T cells or CD62L^{low} CD8$^+$ LN T cells were stimulated with irradiated tumor cells in the presence of DCs for 48 h. Th1 cells have been considered the most important CD4$^+$ T cells for antitumor immunity.

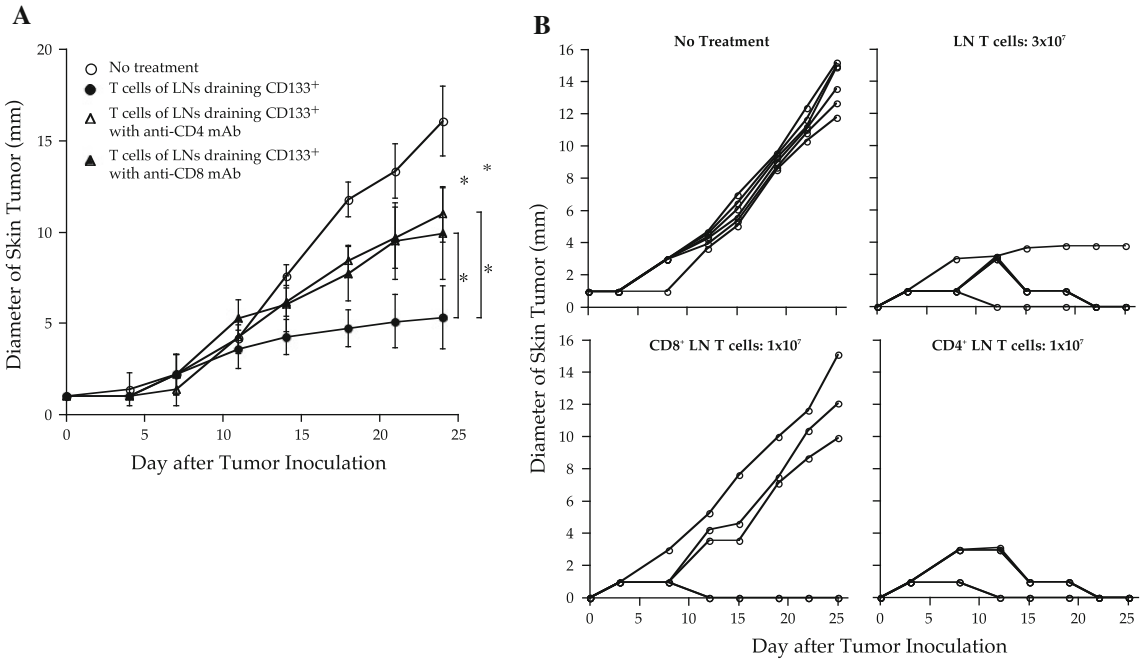


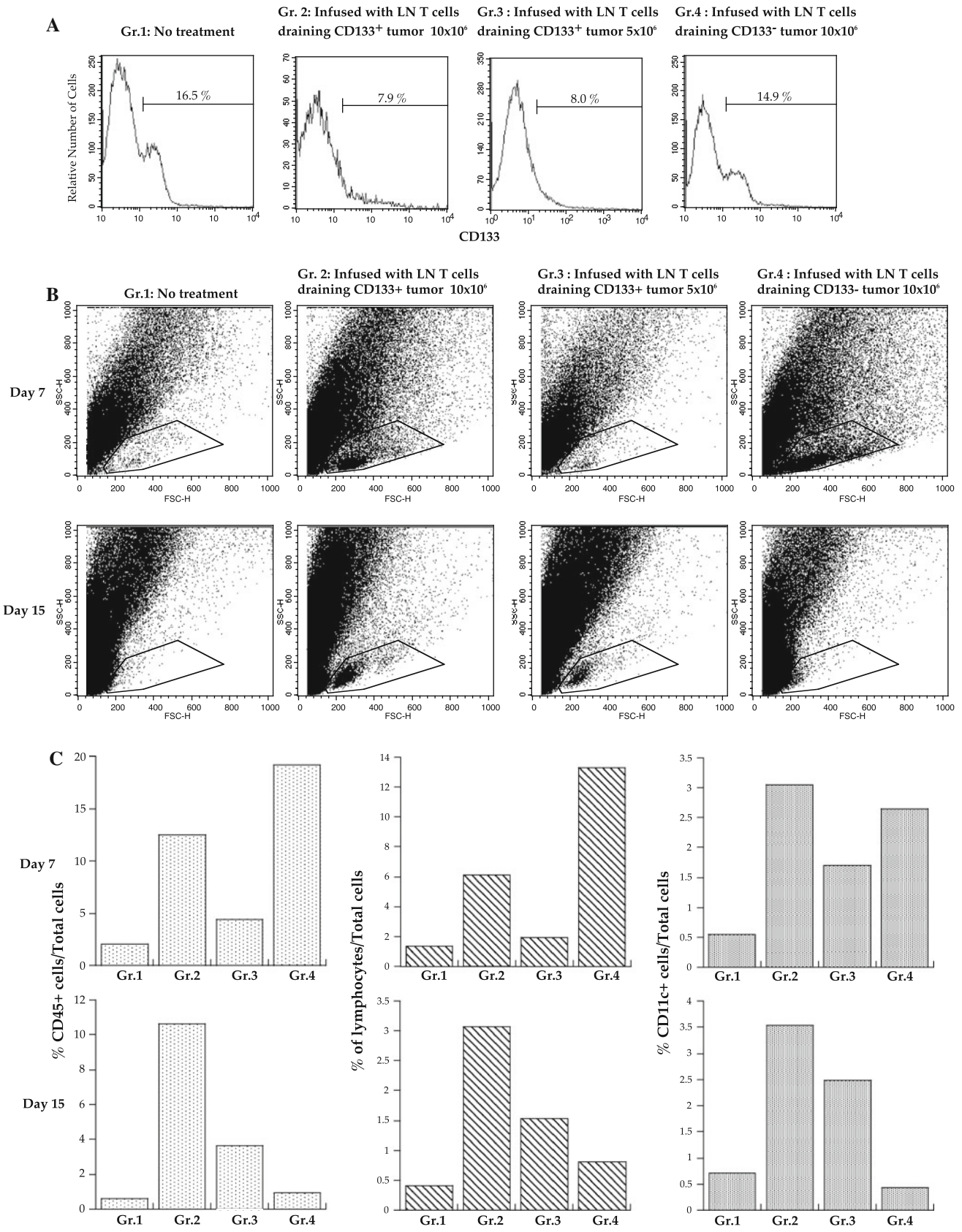
Fig. 3 $CD4^+$ T cells primed with irradiated $CD133^+$ tumor cell/dendritic cell (DC) vaccine mediated potent and long-lasting antitumor reactivity. Both $CD4^+$ and $CD8^+$ T cells were required for optimal antitumor efficacy. **a** Approximately 15×10^6 $CD62L^{low}$ T cells isolated from lymph nodes (LNs) draining irradiated $CD133^+$ melanoma cells/DCs for 8 days were cultured by the anti-CD3/interleukin (IL)-2 method and were infused intravenously into mice bearing 2-day-established skin tumors of parental melanoma. Mice were intraperitoneally injected with either anti-CD4 or anti-CD8 monoclonal antibody (mAb). The diameter of the skin tumors was measured twice weekly with calipers; size was recorded as the average of 2 perpendicular diameters. Each group contained 5 mice. *Asterisks* indicate $P < 0.01$. **b** $CD4^+$ and $CD8^+$ T cells were purified from $CD62L^{low}$ LN T cells draining irradiated $CD133^+$ melanoma cells/DCs with immuno-magnetic beads. Approximately 3×10^7 $CD62L^{low}$ T cells, 1×10^7 $CD62L^{low}$ $CD8^+$ T cells, or 1×10^7 $CD62L^{low}$ $CD4^+$ T cells were isolated from 8-day B16 $CD133^+$ tumor cell/DC-draining LN cells. $CD62L^{low}$ T cells were activated by the anti-CD3/IL-2 method and separated into $CD4^+$ and $CD8^+$ cells with magnetic beads. The diameters of the skin tumors were measured twice weekly with calipers; size was recorded as the average of 2 perpendicular diameters. Each group contained 5 mice. *Asterisks* indicate $P < 0.01$

Recently, it was reported that Th17 $CD4^+$ T cells that preferentially produce IL-17 and IL-6 play a critical role in antitumor immune responses [21–25]. The supernatants were analyzed for interferon (IFN)-γ, IL-4, and IL-17A using cytokine-specific enzyme-linked immunosorbent assays (ELISAs). $CD62L^{low}$ $CD4^+$ T cells derived from LNs draining $CD133^+$ melanoma cells exhibited specific and significantly greater IL-17A, but not IL-4, production upon $CD133^+$ tumor antigen stimulation (Fig. 2d). Conversely, T cells primed in LNs draining parental or $CD133^-$ tumor antigens did not show antigen-specific cytokine release. $CD62L^{low}$ $CD8^+$ T cells derived from $CD133^+$ tumor cell–draining LNs also produced IFN-γ upon stimulation with $CD133^+$ tumor antigens. These cytokine assays indicate that both $CD133^+$ tumor-specific $CD4^+$ and $CD8^+$ T cells were primed in LNs draining $CD133^+$ tumor antigens and that $CD133^+$ tumor antigens preferentially induced Th1 and Th17, but not Th2, cells.

$CD133^+$ melanoma-specific $CD4^+$ T cells mediated superior antitumor reactivity

To determine whether $CD4^+$ or $CD8^+$ T cells contributed to the antitumor efficacy mediated by $CD133^+$ tumor-specific LN T-cell treatment, $CD4^+$ and $CD8^+$ T-cell

Fig. 4 $CD133^+$ tumor-specific LN T cell treatment eradicated ▶ $CD133^+$ tumor cells and induced accumulation of leukocytes in tumor tissues. One million $CD133^+$ tumor cells mixed with 0.5×10^6 $CD133^-$ tumor cells were subcutaneously inoculated at both flanks. Zero (Gr. 1), 10×10^6 (Gr. 2), 5×10^6 $CD133^+$ tumor-specific LN T cells (Gr. 3), or 10×10^6 LN T cells draining $CD133^-$ tumor antigens (Gr. 4) were intravenously infused after sublethal whole-body irradiation. Each group contained 4 mice. Four tumors were collected and digested with collagenase, DNase, and hyaluronidase 7 and 15 days after T-cell infusion. The representative data of 3 independent experiments are presented. **a** CD133 expression of cells in the tumor region. **b** *Dot plots* of forward and side scatter. **c** The percentages of $CD45^+$ leukocytes based on the total number of cells (*left graphs*), of cells in the lymphocyte region (*middle graphs*), and of $CD11c^+$ cells (*right graphs*) in each group


A
Gr.1: No treatment
Gr. 2: Infused with LN T cells draining CD133+ tumor 10x10⁶
Gr.3 : Infused with LN T cells draining CD133+ tumor 5x10⁶
Gr.4 : Infused with LN T cells draining CD133- tumor 10x10⁶
Relative Number of Cells
16.5 %
7.9 %
8.0 %
14.9 %
CD133
B
Gr.1: No treatment
Gr. 2: Infused with LN T cells draining CD133+ tumor 10x10⁶
Gr.3 : Infused with LN T cells draining CD133+ tumor 5x10⁶
Gr.4 : Infused with LN T cells draining CD133- tumor 10x10⁶
Day 7
Day 15
SSC-H
FSC-H
C
Day 7
Day 15
% CD45+ cells/Total cells
% of lymphocytes/Total cells
% CD11c+ cells/Total cells
Gr.1
Gr.2
Gr.3
Gr.4

depletion studies were performed. In vivo depletion studies showed that both $CD4^+$ and $CD8^+$ T-cell depletion significantly diminished antitumor reactivity (Fig. 3a). However, these studies could not assess whether $CD4^+$ or $CD8^+$ LN T cells mediated superior antitumor reactivity, because host $CD4^+$ and $CD8^+$ T cells were also depleted. Therefore, we infused mice with 1×10^7 $CD4^+$ or $CD8^+$ cells purified from $CD133^+$ tumor-specific LN T cells. Two of 5 mice infused with $CD8^+$ LN T cells were cured (Fig. 3b). Interestingly, all mice infused with $CD4^+$ LN T cells were cured. Moreover, the duration of the antitumor effect of $CD133^+$ tumor-specific $CD4^+$ T cells was longer than that of $CD8^+$ T cells. In other words, skin tumors that were not cured within 10 days after tumor inoculation eventually grew in mice infused with 1×10^7 purified $CD8^+$ LN T cells. In contrast, mice infused with 1×10^7 purified $CD4^+$ LN T cells or 3×10^7 total LN T cells that contained approximately 6×10^6 $CD4^+$ LN T cells exhibited long-lasting antitumor reactivity and resulted in the complete remission of the parental melanoma.

$CD133^+$ tumor-specific T-cell treatment resulted in long-lasting accumulation of $CD4^+$ T cells and activated DCs in tumors

To understand the mechanism by which $CD133^+$ tumor-specific T cells mediated antitumor reactivity, we analyzed the cellular composition of skin tumors 7 and 15 days after treatment. One million $CD133^+$ tumor cells mixed with 0.5×10^6 $CD133^-$ tumor cells were inoculated in both flanks of mice. Although the percentage of $CD133^+$ cells did not differ among groups on the seventh day after tumor inoculation (data not shown), tumors of $CD133^+$ tumor-specific LN T-cell recipients lost their $CD133^+$ subpopulation. In contrast, tumors in mice that were infused with $CD133^-$ tumor-draining LN T cells contained approximately the same number of $CD133^+$ tumor cells as the control mice on the fifteenth day after treatment (Fig. 4a). Thus, it is likely that infused $CD133^+$ tumor-specific T cells indeed eradicated $CD133^+$ tumor cells prior to tumor regression.

On the seventh day, the number of $CD45^+$ leukocytes and lymphocytes in tumor tissues depended on the number of infused T cells, as more leukocytes were observed in tumor tissues of mice that were infused with 1×10^7 LN T cells draining $CD133^+$ or $CD133^-$ tumor cells. However, the tumor-infiltrating lymphocytes in mice that were infused with 1×10^7 LN T cells draining $CD133^-$ tumor antigen disappeared, leaving these mice with lymphocyte levels comparable to those in untreated mice by the fifteenth day (Fig. 4b). Conversely, mice treated with 10×10^6 $CD133^+$ tumor-specific LN T cells had 10 times more $CD45^+$ cells and 6 times more lymphocytes than the control animals did (Fig. 4b, c). $CD4^+$ T cells preferentially increased in the tumor tissues of $CD133^+$ tumor-specific LN T-cell recipients. Moreover, $CD133^+$ tumor-specific LN T-cell infusion resulted in a long-lasting increase in $CD11c^+$ DCs that had augmented the expression of MHC class II antigen in tumor tissues (Fig. 5a, b).

To determine whether $CD4^+$ or $CD8^+$ $CD133^+$ tumor-specific LN T cells induce the accumulation of lymphocytes and DCs, we analyzed tumors in mice infused with purified $CD4^+$ or $CD8^+$ $CD133^+$ tumor-specific LN T cells. Leukocyte accumulation was observed only when the mice were infused with $CD4^+$ $CD133^+$ tumor-specific T cells (Fig. 6a). Furthermore, 90 days after T-cell treatment, we examined splenocytes of mice cured with the $CD133^+$ tumor-specific T-cell infusion. Surprisingly, the mice cured with $CD4^+$ $CD133^+$ tumor-specific LN T cells had not only the $CD4^+$ T cells that produced IFN-γ and IL-17 upon $CD133^+$ tumor antigen stimulation but also the $CD4^+$ T cells that recognized and responded to $CD133^-$ tumor antigens, although the infused $CD4^+$ T cells were highly specific for $CD133^+$ tumor antigens (Figs. 2c, 6b).

Discussion

Our study demonstrates that $CD133^+$ melanoma-specific T cells are capable of mediating antitumor reactivity that results in the regression of established parental melanoma in mice. These results are surprising, as $CD133^+$ tumor cells comprised less than 1% of the parental melanoma. $CD133^+$ tumor cells may be so essential for the development of tumor tissues that eradication of $CD133^+$ tumor cells makes it difficult for melanoma cells to establish tumors in vivo, as $CD133^+$ tumor cells possess high tumorigenicity. However, this does not explain how $CD133^+$ tumor-specific T-cell therapy cured the mice with established skin tumors. Notably, $CD4^+$ T cells mediated superior long-lasting antitumor reactivity by inducing the accumulation of activated DCs and lymphocytes, but not Treg, in tumor tissues. Further, although $CD4^+$ T cells that were highly specific for $CD133^+$ melanoma antigens were infused, we detected T cells that secreted IFN-γ and IL-17 upon $CD133^-$ tumor antigen stimulation in cured mice. This observation is consistent with the previous report that Th17 cells expressing TCR for 1 tyrosinase-related protein-1 (TRP-1) epitope induced tumor antigen–specific T cells that were not specific for TRP-1 [21]. Thus, it is likely that the interaction between $CD4^+$ $CD133^+$ tumor-specific T cells and DCs that acquired $CD133^+$ tumor cells resulted in the induction of antimelanoma effector T cells with wide specificity, because $CD133^+$ and $CD133^-$ melanoma cells shared most antigens according to 2-D electrophoresis analyses (*data not shown*). Cumulatively, these results

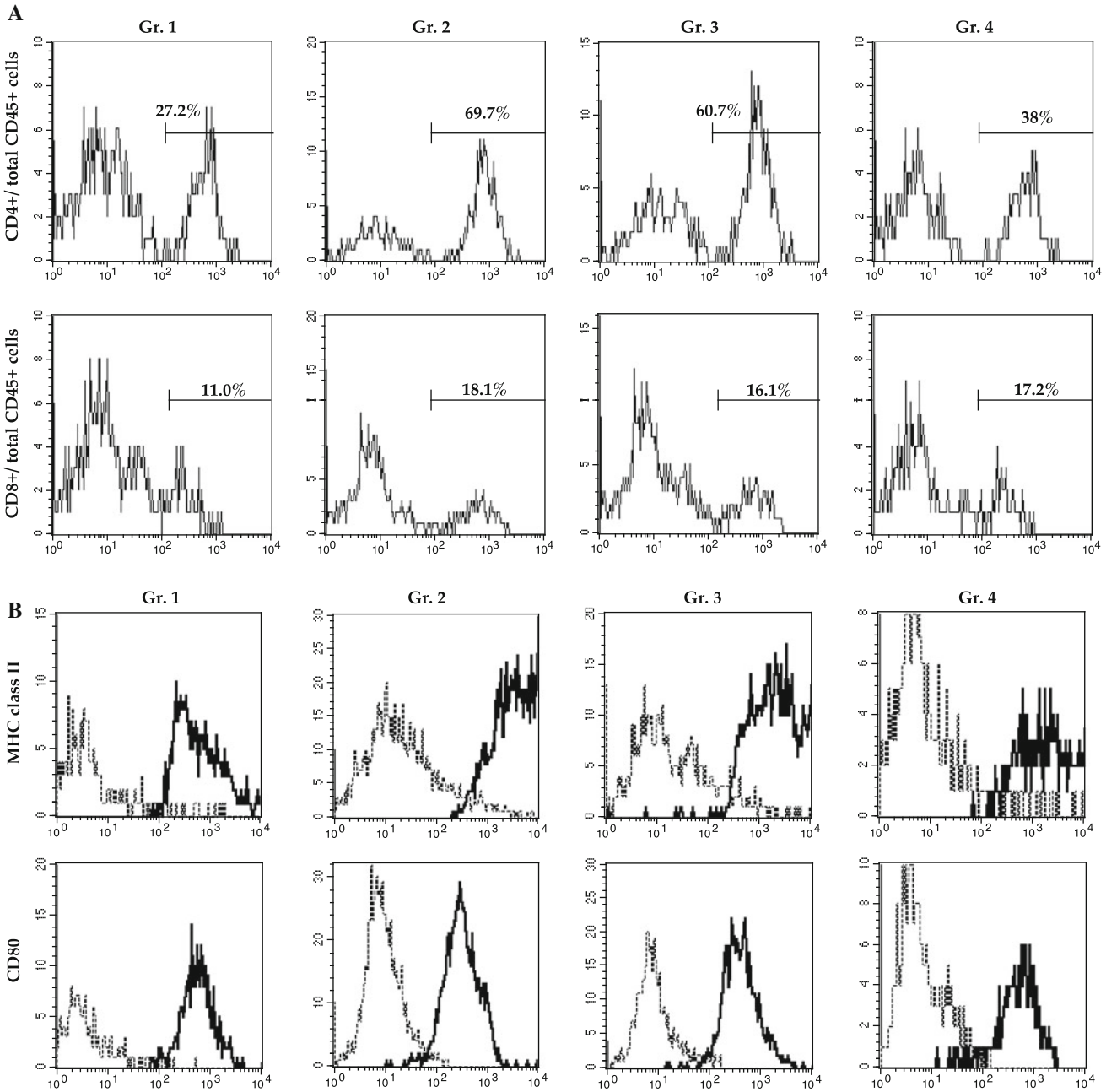


Fig. 5 $CD133^+$ tumor-specific LN T-cell treatment induced the accumulation of $CD4^+$ T cells and activated DCs. **a** CD4 (*upper graphs*) and CD8 (*lower graphs*) expression in gated $CD45^+$ cells. **b** $I\text{–}A^b$ (*upper graphs*) and CD80 (*lower graphs*) expression on gated $CD11c^+$ cells. All cells were derived from tumor tissues harvested 15 days after T-cell treatment

indicate that $CD133^+$ tumor-specific antigens are highly immunogenic and can induce tumor-specific $CD4^+$ and $CD8^+$ effector T cells to eradicate whole tumor cells.

Immunization with irradiated whole $CD133^+$ tumor cells induced $CD133^+$ tumor-specific T-cell priming. It is unclear why T cells were not primed by the majority of antigens but were instead primed by the minority of $CD133^+$ tumor-specific antigens. One possible explanation is that $CD133^+$ melanoma cells possessed molecules that stimulate DCs. However, co-culture of DCs with $CD133^+$ or $CD133^-$ melanoma cells showed no significant differences in the expression of MHC class I and II or co-stimulatory molecules or in the production of IL-4, IL-12, and IL-23 (data not shown). A second possibility is that the counterparts of effector T cells that abrogate effector T-cell expansion are not induced for $CD133^+$ melanoma epitopes. It is reported that Tregs are maintained with antigen presentation by DCs that acquire apoptotic cells without

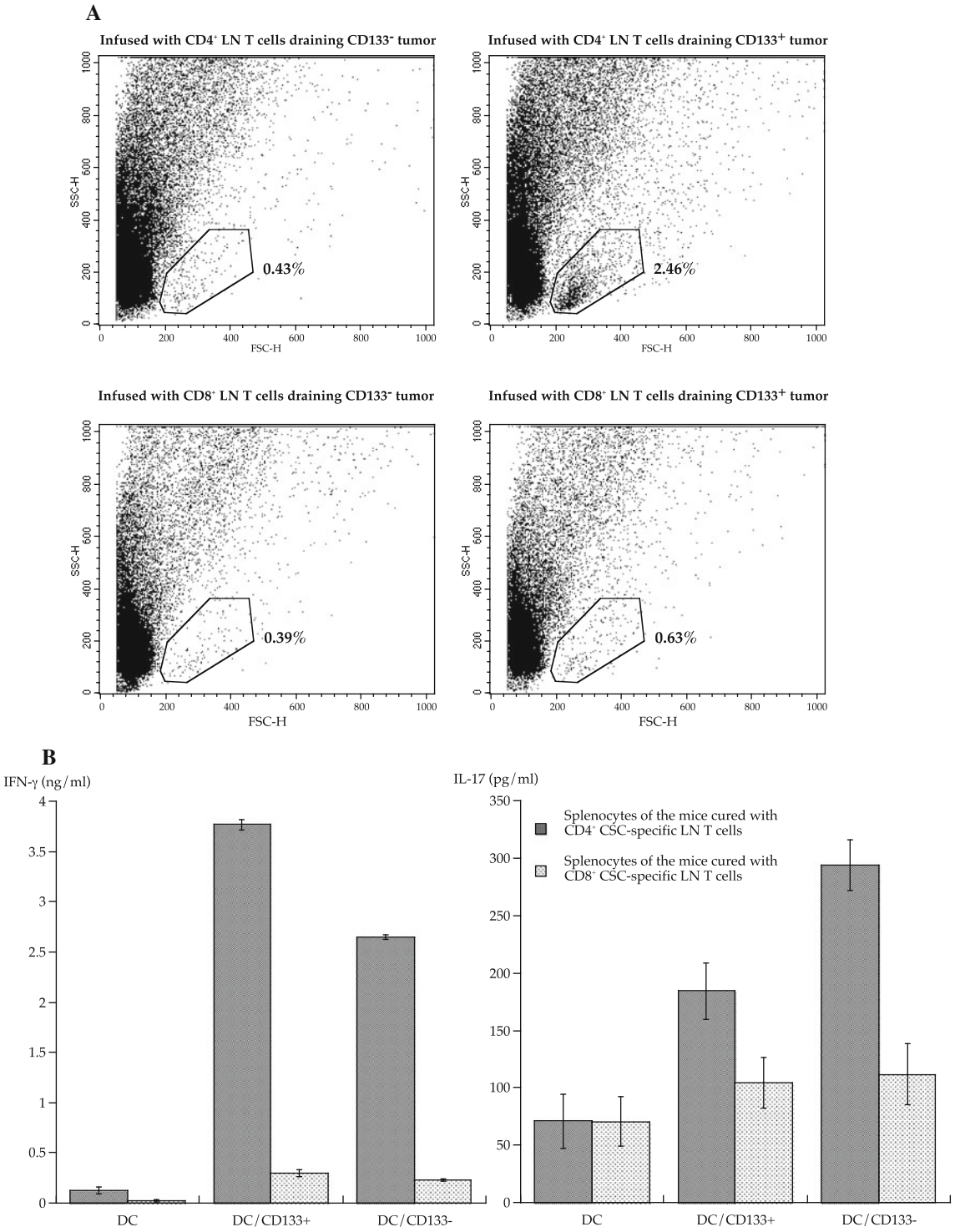


Fig. 6 Induction of $CD4^+$ T cells that recognized $CD133^-$ B16 melanoma antigens in mice cured with $CD133^+$ tumor-specific $CD4^+$ LN T-cell infusion. **a** One million parental B16 melanoma cells were subcutaneously inoculated. Isolated $CD4^+$ and $CD8^+$ T cells derived from LN T cells draining $CD133^+$ or $CD133^-$ tumor antigens were intravenously infused after sublethal whole-body irradiation. The figures present the microfluorometer analyses showing forward and side scatter plots of cells derived from digested tumor tissues 14 days after T-cell treatment. Each group contained 5 mice. **b** Spleens of mice cured with T-cell treatment were harvested 90 days after T-cell infusion. In a 96-well plate, 1×10^5 $CD62L^{low}$ $CD4^+$ T cells isolated from the spleens were stimulated with 2×10^4 DCs pulsed with tumor antigens in 200 μl CM for 48 h. DCs for stimulation were purified with CD11c microbeads after overnight co-culture with irradiated tumor cells. IFN-γ and IL-17A were measured with ELISA

danger signals [26, 27]. Because the $CD133^+$ tumor cell population is so small and immortal that sufficient antigens are not delivered for the DCs, it is possible that peripheral tolerance is not well established for $CD133^+$ tumor-specific antigens. It is difficult to quantify antigen-specific Tregs; however, because Th17 and Tregs have reciprocal developmental pathways and affect each other's generation [25], the absence of Tregs likely results in Th17 cell induction. This is consistent with our observation that $CD133^+$ tumor antigens tended to prime type Th17 $CD4^+$ T cells (Fig. 2c). Further, we observed that Treg induction was significantly suppressed in the tumor tissues and draining LNs of mice in the presence of $CD133^+$ tumor-specific $CD4^+$ T cells.

It is still unclear whether Th17 cells promote tumor growth or regulate antitumor responses [28]. It has been demonstrated that Th17 cells play critical roles in inflammation and autoimmune diseases since development of Th17 cells is reciprocally related to $Foxp3^+$ Treg cells, and Th17 cells can shift to Th1 lineage [25, 29]. Thus, the microenvironment that promotes Th17 differentiation likely facilitates antitumor immunity. It is demonstrated that Th17 cells promoted dendritic cell recruitment into the tumor tissues and increased tumor-specific $CD8^+$ T cells resulting in potent antitumor reactivity [21]. This is consistent with our data that treatment with $CD133^+$ tumor-specific $CD4^+$ T cells, which included Th17, resulted in long-lasting accumulation of T cells and activated DCs in tumors (Figs. 4, 5, and 6). On the other hand, Th17-associated cytokines, such as IL-17 and IL-6, may promote tumor growth through tumor neovascularization and carcinogenesis via STAT-3 signaling [30, 31]. In patients with prostate cancer, ovarian cancer, and small cell lung cancer, it is revealed that Th17 differentiation inversely correlated with tumor progression [23, 32, 33]. More recently, it was demonstrated that treatment with antibody against cytotoxic T lymphocyte antigen 4 induces Th17 cells in patients with melanoma and favors survival [34]. These data strongly suggest that Th17 cells play a protective role in human antitumor immunity [28].

$CD133^+$ melanoma-specific antigens preferentially induce Th17 and Th1, but not Treg or Th2, cells. Immunotherapy with $CD133^+$ tumor-specific T cells mediated potent therapeutic efficacy, effectively curing mice of the established parental tumors. These $CD133^+$ tumor-specific immune responses not only eradicated $CD133^+$ tumor cells but also promoted induction of T cells that recognized $CD133^-$ tumor antigens. It is still unclear why $CD133^+$ cells are superior to $CD133^-$ cells in inducing protective immunity. We examined whether $CD133^+$ cells possess molecules that could stimulate DCs; however, no differences were observed in the surface expression of CD80, CD86, CD40L, and OX40L (data not shown). Cytokines that affect DC differentiation, such as IL-4, IL-12, and IL-23, were also examined with ELISA. Either $CD133^+$ or $CD133^-$ tumor cells produced detectable cytokines. Further, we conducted proteome analyses and found 4 proteins that were preferentially expressed in $CD133^+$ tumor cells. Thus, it is possible that the $CD133^+$ tumor-specific proteins are immunogenic to induce antitumor protective immunity. Taken together, $CD133^+$ tumor-specific antigens are ideal immunogenic targets and have important implications in antitumor vaccination therapy.

Conflict of interest None.

References

1. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB (2004) Identification of human brain tumour initiating cells. Nature 432:396–401
2. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037
3. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, de Maria R (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
4. O'Brien CA, Pollett A, Gallinger S, Dick JE (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:106–110
5. Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C et al (2008) Identification of cells initiating human melanomas. Nature 451:345–349
6. Mimeault M, Batra SK (2007) Functions of tumorigenic and migrating cancer progenitor cells in cancer progression and metastasis and their therapeutic implications. Cancer Metastasis Rev 26:203–214
7. Diehn M, Clarke MF (2006) Cancer stem cells and radiotherapy: new insights into tumor radioresistance. J Natl Cancer Inst 98:1755–1757
8. Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, Sayegh MH, Sadee W, Frank MH (2005) ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. Cancer Res 65:4320–4333
9. Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C, Gritti A, Piccinini A, Porro D, Santinami M et al (2007) Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. Eur J Cancer 43:935–946
10. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:756–760
11. Malanchi I, Peinado H, Kassen D, Hussenet T, Metzger D, Chambon P, Huber M, Hohl D, Cano A, Birchmeier W et al (2008) Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. Nature 452:650–653
12. Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, Barow M, Mountford JC, Holyoake TL (2006) Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood 107:4532–4539
13. Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, Kornblum HI (2003)

Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA 100:15178–15183
14. Rappa G, Fodstad O, Lorico A (2008) The stem cell-associated antigen CD133 (Prominin-1) is a molecular therapeutic target for metastatic melanoma. Stem Cells 26:3008–3017
15. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA et al (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 141:69–80
16. Kagamu H, Shu S (1998) Purification of L-selectin (low) cells promotes the generation of highly potent CD4 antitumor effector T lymphocytes. J Immunol 160:3444–3452
17. Fujita N, Kagamu H, Yoshizawa H, Itoh K, Kuriyama H, Matsumoto N, Ishiguro T, Tanaka J, Suzuki E, Hamada H et al (2001) CD40 ligand promotes priming of fully potent antitumor CD4(+) T cells in draining lymph nodes in the presence of apoptotic tumor cells. J Immunol 167:5678–5688
18. Hiura T, Kagamu H, Miura S, Ishida A, Tanaka H, Tanaka J, Gejyo F, Yoshizawa H (2005) Both regulatory T cells and antitumor effector T cells are primed in the same draining lymph nodes during tumor progression. J Immunol 175:5058–5066
19. Watanabe S, Kagamu H, Yoshizawa H, Fujita N, Tanaka H, Tanaka J, Gejyo F (2003) The duration of signaling through CD40 directs biological ability of dendritic cells to induce antitumor immunity. J Immunol 171:5828–5836
20. Kagamu H, Touhalisky JE, Plautz GE, Krauss JC, Shu S (1996) Isolation based on L-selectin expression of immune effector T cells derived from tumor-draining lymph nodes. Cancer Res 56:4338–4342
21. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW, Dong C (2009) T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 31:787–798
22. Kryczek I, Wei S, Szeliga W, Vatan L, Zou W (2009) Endogenous IL-17 contributes to reduced tumor growth and metastasis. Blood 114:357–359
23. Koyama K, Kagamu H, Miura S, Hiura T, Miyabayashi T, Itoh R, Kuriyama H, Tanaka H, Tanaka J, Yoshizawa H et al (2008) Reciprocal CD4+ T-cell balance of effector CD62Llow CD4+ and CD62LhighCD25+CD4+ regulatory T cells in small cell lung cancer reflects disease stage. Clin Cancer Res 14:6770–6779
24. Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J, Wang RF (2008) Generation and regulation of human CD4+IL-17-producing T cells in ovarian cancer. Proc Natl Acad Sci USA 105:15505–15510
25. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441:235–238
26. Steinman RM, Turley S, Mellman I, Inaba K (2000) The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med 191:411–416
27. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 194:769–779
28. Zou W, Restifo NP (2010) T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol 10:248–256
29. Shi G, Cox CA, Vistica BP, Tan C, Wawrousek EF, Gery I (2008) Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. J Immunol 181:7205–7213
30. Numasaki M, Fukushi J, Ono M, Narula SK, Zavodny PJ, Kudo T, Robbins PD, Tahara H, Lotze MT (2003) Interleukin-17 promotes angiogenesis and tumor growth. Blood 101:2620–2627
31. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H (2009) IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. J Exp Med 206:1457–1464
32. Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ, DeMarzo AM, Meeker AK, Isaacs WB, Drake CG (2008) Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. Clin Cancer Res 14:3254–3261
33. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH et al (2009) Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. Blood 114:1141–1149
34. von Euw E, Chodon T, Attar N, Jalil J, Koya RC, Comin-Anduix B, Ribas A (2009) CTLA4 blockade increases Th17 cells in patients with metastatic melanoma. J Transl Med 7:35