Anti-CD137 Monoclonal Antibody Administration Augments the Antitumor Efficacy of Dendritic Cell-Based Vaccines

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ABSTRACT

In weakly and poorly immunogenic tumor models, we examined the effects of stimulating CD137 (4-1BB) *in vivo* by administering anti-CD137 monoclonal antibody after tumor lysate-pulsed dendritic cell (TP-DC) vaccination. TP-DC subcutaneous vaccination induced a transient up-regulation of CD137 on T cells and natural killer (NK) cells within vaccine-primed lymph nodes (VPLNs). In established pulmonary and subcutaneous tumor models, anti-CD137 synergistically enhanced tumor regression after TP-DC vaccination. In the subcutaneous tumor model, the combined therapy resulted in improved survival. Combined therapy also resulted in improved local control of subcutaneous tumor after surgical resection. Anti-CD137 polarized the cytokine release of VPLNs and spleen cells in response to tumor antigen toward a type 1 (interferon-γ) *versus* a type 2 (interleukin-4) profile. Cell depletion and the use of knockout animals identified that CD8^{+}, CD4^{+}, and NK cells were involved in the tumor rejection response and that CD8^{+} cells had the major effector role. Anti-CD137 administration resulted in increased proliferation of adoptively transferred OT-1 CD8^{+} T cells in the VPLNs of mice inoculated with B16-OVA TP-DCs. Polarization toward type 1 (interferon-γ) *versus* type 2 (interleukin-4) was also observed with the OT-1 cells from VPLNs and spleen cells after anti-CD137 injections. This polarization effect was abrogated by the *in vivo* depletion of NK cells. These findings indicate that the adjuvant effect of anti-CD137 given in conjunction with TP-DC vaccination is associated with the polarization of T effector cells toward a type 1 response to tumor antigen and is mediated via NK cells.

INTRODUCTION

The use of dendritic cells (DCs) as vaccine reagents has become an area of intense focus in the field of cancer immunotherapy. DCs can be pulsed with tumor-associated antigen by a variety of methods that result in the ability of DCs to prime naïve T cells, and DCs can mediate regression of established tumor when given as a vaccine in animal models (1–5). Despite these promising preclinical observations, the initial clinical studies evaluating DC-based vaccines have demonstrated limited success (6–10). Several of these studies have found that immune responses to tumor antigen as measured by *in vitro* assays are up-regulated by vaccination but were insufficient in inducing meaningful clinical tumor regression (8–10). Methods to enhance these immune responses would potentially improve the therapeutic efficacy of this vaccine approach.

CD137 (4-1BB) is an important costimulating receptor that is expressed by activated natural killer (NK) cells, T cells, and DCs (11, 12). The natural ligand for CD137 is found on activated B cells, macrophages, and DCs. Ligation of CD137 results in cellular proliferation and protection of cells from activation-induced cell death (13,

14). CD137 ligation can be accomplished with agonistic monoclonal antibody (mAb) to CD137. Melero *et al.* (15) were the first to report that anti-CD137 mAb (anti-4-1BBmAb) can be administered *in vivo* to mediate antitumor responses against established weakly immunogenic tumors in animal models. Against poorly immunogenic or nonimmunogenic tumors, it was reported by the same group that tumor regression required active immunization with a peptide vaccine + anti-CD137 (16). It was apparent from these and other studies that anti-CD137 administration can provide potent *in vivo* costimulation of cellular immune responses and may be a useful reagent to examine with DC-based vaccines.

In this report, we examined the role of anti-CD137 administration in modulating the immune responses induced by tumor lysate-pulsed DC (TP-DC) vaccinations. Tumor lysates provide the ability to sensitize T cells to the entire spectrum of tumor-associated antigens specific for an individual tumor. This has formed the rationale for clinical trials we and others have conducted (6, 10). The studies in this report involve the treatment of both weakly and poorly immunogenic established tumors. We describe herein the effects of anti-CD137 on the immune function of vaccine-primed T cells and how NK cells modulate this function.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6) mice, 6–8 weeks of age, were purchased from Harlan Laboratories (Indianapolis, IN). Female B6 129-Il15[ratm1Ama], B6 129S2-Cd4[tm1Mak], and B6 129S2-Cd8[tm1Mak] knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Hemizygous OT-1 T-cell receptor transgenic mice maintained on a C57BL/6-CD45.1 background were a generous gift from Dr. Kevin McDonagh (University of Michigan, Ann Arbor, MI). All mice were maintained in specific pathogen-free conditions and used at 10 to 14 weeks of age. The University of Michigan Laboratory of Animal Medicine approved all animal protocols.

Tumors. MCA 205 and MCA 207 murine tumors are 3-methylcholanthrene–induced fibrosarcomas, syngeneic to B6 mice. These tumors have been characterized previously as weakly immunogenic with distinct tumor-specific transplantation/rejection antigens (17). These tumors have been maintained *in vivo* by serial subcutaneous transplantation in B6 mice and were used within the 8th passage. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 40 mL of Hanks' balanced salt solution (Life Technologies, Inc., Grand Island, NY) containing 40 mg of collagenase, 4 mg of DNase I, and 100 units of hyaluronidase (all enzymes were from Sigma, St. Louis, MO) for 2 to 3 hours at room temperature, as described previously (18). Tumor cells were washed three times in Hanks' balanced salt solution for administration to mice or resuspended in complete medium (CM) for *in vitro* assays. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L fresh ʟ-glutamine, 100 mg/mL streptomycin, 100 units/mL penicillin, 50 μg/mL gentamicin, 0.5 μg/mL Fungizone (all from Life Technologies, Inc.), and 0.05 mmol/L 2-mercaptoethanol (Sigma).

The B16-BL6 melanoma is a poorly immunogenic melanoma of spontaneous origin (19). B16-OVA is a tumor line transfected to express ovalbumin (OVA) that serves as a tumor-associated antigen. This line was obtained from Dr. Kenneth Rock (University of Massachusetts, Amherst, MA). EL-4 is a T-cell thymoma syngeneic to B6 mice. RMAS cells are TAP-deficient cells that are defective in transporters associated with antigen processing and

express functionally "empty" class I major histocompatibility complex molecules on the cell surface (20).

Antibody and Cytokines. Agonistic anti-CD137 mAb (1D8) was kindly provided by Bristol-Myers Squibb Co., Pharmaceutical Research Institute (Princeton, NJ). ID8 is a rat IgG2a class of antibody. Recombinant murine granulocyte macrophage colony-stimulating factor and recombinant murine interleukin (IL)-4 were purchased from PeproTech (Rocky Hill, NJ).

Generation of Bone Marrow-derived Dendritic Cells and Tumor Lysate Pulsing. Erythrocyte-depleted bone marrow cells were cultured in CM supplemented with 10 ng/mL granulocyte macrophage colony-stimulating factor and 10 ng/mL IL-4 at 1×10^6 cells per mL. On day 5, nonadherent cells were harvested by gentle pipetting. DCs were enriched by density centrifugation over 14.5% (w/v) metrizamide (Sigma), and the low-density interface was collected. DCs were washed twice, enumerated, and used for *in vitro* and *in vivo* functional studies. For preparation of tumor lysate, MCA 205, B16-BL6, B16-OVA, and EL-4 cells were suspended in PBS and subjected four times to rapid freeze-thaw exposures and then spun at $\sim 100 \times g$ for 5 minutes to remove cellular debris. The DCs were resuspended at 1×10^6 cells per mL in CM and incubated with tumor lysate at a tumor cell equivalent to DC ratio of 3:1 for 18 hours.

OT-1 Cell Purification and Labeling. Freshly isolated splenocytes and popliteal, inguinal, mesenteric, axillary, and brachial lymph node (LN) cells from OT-1 transgenic mice (Ly5.1) were prepared as single cell suspensions and pooled. Lymphoid cells were enriched by the Magnetic Activated Cell Sorting device (Miltenyi Biotec, Auburn, CA), using the CD8a$^+$ T-cell isola-

tion kit according to the manufacturer's protocol (Miltenyi Biotec). The fraction of tetramer-positive CD8$^+$ T cells averaged between 93% and 97% of the total population by flow cytometry. Cells were suspended at 10^7 cells per mL in PBS and incubated with 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE; C-1157; Molecular Probes, Inc., Eugene, OR) at concentrations of 1 μmol/L for 10 minutes at room temperature in the dark and washed twice with PBS. Based on flow cytometry, a total lymphoid cell suspension containing 4 to 5×10^6 tetramer-positive CD8$^+$ T cells was injected intravenously into C57BL/6 (Ly5.2) mice. To assess proliferation and CD62L expression of OT-1 cells in vaccine-primed lymph nodes (VPLNs), 0.5 to 1.0×10^6 events were collected and analyzed by flow cytometry.

Flow Cytometry. The following antibodies were used in this study: purified rat anti-CD16/32 (2.4G2), fluorescein isothiocyanate (FITC)-labeled anti-CD3ϵ (145-2C11), phycoerythrin (PE)-labeled anti-NK1.1 (PK136), FITC- and PE-labeled anti-CD4 (GK1.5: rat IgG2b), CyChrome anti-CD8a (53-6.7), PE-labeled anti-CD62L (Mel-14), PE- and biotin-labeled anti-CD45.1 (Ly5.1; A20), streptavidin CyChrome conjugate (all from BD PharMingen, San Diego, CA), and FITC-labeled anti-CD8a (5H10; rat IgG2b; Caltag, Burlingame, CA).

Before staining, cells were incubated with 2.4G2 mAb on ice for 15 minutes to prevent nonspecific binding of mAbs. All procedures were performed using cold PBS containing 5% fetal bovine serum. VPLN cell surface expression of CD137 was assessed by indirect immunofluorescence assays using purified anti-CD137 mAbs (1D8; rat IgG2a) followed by biotin-labeled antirat IgG2a (RG7/1.30), and purified rat IgG2a isotype standard (R35-95) was used as a control (both from BD PharMingen). To assess CD137 expression on NK cells
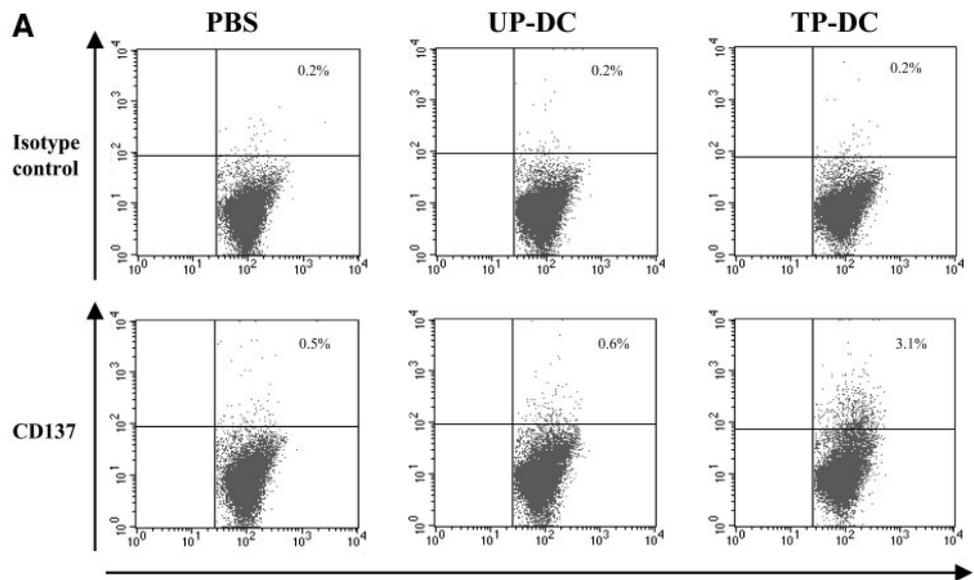

Fig. 1. CD137 up-regulation on CD3$^+$ cells. *A.* CD137 expression on CD3$^+$ cells. VPLN cells were stained for CD137 expression by indirect immunofluorescence assays 3 days after immunization with PBS, UP-DCs, or TP-DCs. Data are representative of three independent experiments. *B.* Kinetics of CD137 expression on CD3$^+$ cells. VPLN cells and splenocytes were stained for CD137 expression by indirect immunofluorescence assays on day 0, 3, and 7 after immunization with PBS, UP-DCs, or TP-DCs.

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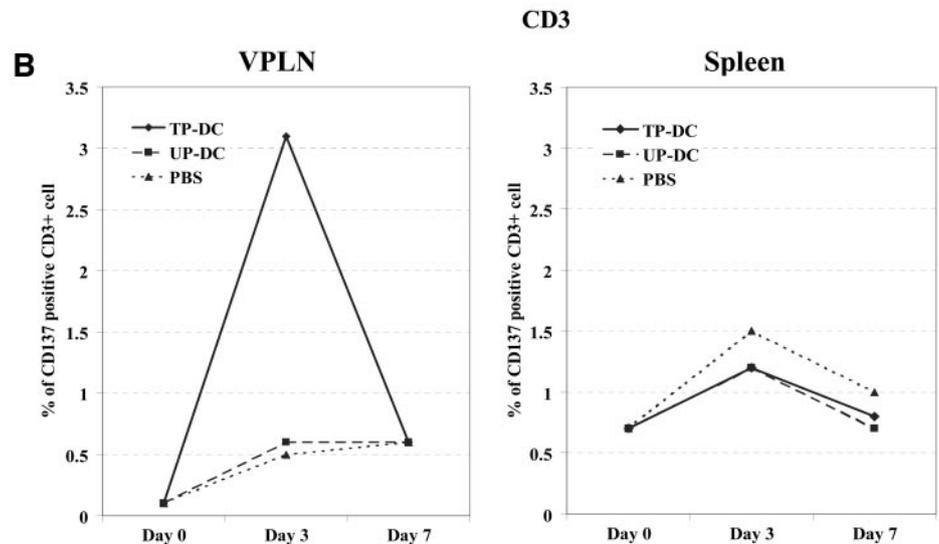
and CD4$^+$ and CD8$^+$ T cells in VPLNs, 3×10^6, 0.5×10^6, and 0.5×10^6 events were collected in a FACScan flow cytometer, respectively. FACSCalibur with CellQuest software was obtained from Becton Dickinson (San Diego, CA).

Assessment of Cytokine Release in Response to Tumor Cells. To measure cytokines released by VPLNs and spleen cells in response to tumor stimulation, 1.0×10^6 lymphoid cells were cocultured with 0.1×10^6 irradiated (6,000 rads) MCA 205 tumor cells or irradiated (10,000 rads) RMAS cells. RMAS cells were loaded with OVA–peptide$_{257-264}$ (SINFEKL) at 10 μmol/L per mL in 2-mL volumes per well in 24-well tissue culture plates for 24 hours at 37°C. The culture supernatants were then collected and analyzed for cytokine production using ELISAs. Irradiated MCA 207 and RMAS cells without peptide were used as specificity controls.

Treatment of Established Pulmonary Metastases. B6 mice received 2×10^5 MCA 205 or 1×10^5 B16-BL6 viable tumor cells intravenously on day 0. The mice were then immunized intradermally with 1.5 to 2.0×10^6 MCA 205, B16-BL6, or EL-4 TP-DCs on day 3 (treatment of 3-day–old lung metastases) or on days 10 and 14 (treatment of 10-day–old lung metastases). Anti-CD137 mAb (1D8) or control rat IgG (Sigma) was given intraperitoneally (100 μg) in 0.5 mL of PBS on days 4 and 7 (treatment of 3-day–old lung metastases) or on days 11, 14, and 17 (treatment of 10-day–old lung metastases). Mice were sacrificed on day 15 (for 3-day–old lung metastases) or 20 (for 10-day–old lung metastases) to enumerate lung metastases. Five mice were used in each experimental group. Intratracheal instillation with India ink was performed to stain the lung parenchyma (21). The MCA 205 metastases appeared as discrete white nodules on the black surface of lungs. For enumeration of B16-BL6 tumors, Fekete's solution was instilled in the lungs via the trachea.

Treatment of Established Subcutaneous Tumor. B6 mice were inoculated subcutaneously in the mid-right flank with 2×10^6 MCA 205 viable tumor cells on day 0. The mice were then immunized intradermally with 1.5 to 2.0×10^6 MCA 205 TP-DCs on days 7 and 11. Anti-CD137 mAb (1D8) or control rat IgG was given intraperitoneally (100 μg) in 0.5 mL of PBS on days 8, 11, and 14. The largest perpendicular diameters of tumors were measured in a blinded coded fashion using vernier calipers, and size was recorded as tumor area (in mm^2). Data are reported as the average tumor area $\pm$ SE of six or more mice per group.

In vivo Cell Depletion. Anti-NK1.1 hybridoma cells were used to produce the PK136 mouse IgG2a mAb (HB191; American Type Culture Collection, Manassas, VA). Hybridoma cells were inoculated into pristine-primed immunocompromised DBA/2 mice as described previously to generate ascites (22). MAb was purified from ascites fluid by ImmunoPure Immobilized Protein A/G gel (Pierce, Rockford, IL). The optimal amount of purified PK136 mAb for *in vivo* depletion was 400 μg intraperitoneally and was determined in preliminary functional assays of NK activity and flow cytometry (data not shown). The anti-CD4$^+$ mAb (GK1.5; rat IgG2b) and the anti-CD8$^+$ mAb (2.43; rat IgG2a) were purchased from Ligocyte (Bozeman, MT). The optimal amount of mAbs for *in vivo* depletion of CD4$^+$ or CD8$^+$ cells was determined in preliminary assays and found to be 800 and 400 μg intraperitoneally, respectively.

Statistical Analysis. For comparison of treatment groups, a one-way analysis of variance (followed by a Newman-Keuls *post hoc* test) was performed using tumor measurements taken on the last day recorded for comparison of treatment groups. Student's *t* test was used to analyze cell proliferation and cytokine release data. All statistical analysis was performed using GraphPad Prism software (San Diego, CA). Statistical significance was achieved when P was <0.05.

RESULTS

TP-DC Vaccination Modulates CD137 Expression. CD137 expression was assessed before and after DC vaccination in the VPLNs and spleen. As shown in Fig. 1A, CD137 expression on CD3$^+$ VPLN cells was negligible before vaccination. TP-DC vaccination resulted in 3.1% of CD3$^+$ cells in the VPLNs expressing CD137 on day 3 compared with $<1\%$ of CD3$^+$ cells in VPLN cells from animals inoculated subcutaneously with PBS or unpulsed DCs (UP-DCs). In the same experiment, CD3$^+$ splenocytes from vaccinated mice did not

have significantly altered CD137 expression at 3 or 7 days after vaccination (Fig. 1B).

Analysis of different lymphoid subsets (NK, CD4$^+$, and CD8$^+$) for CD137 expression was performed after TP-DC vaccination. Three days after TP-DC vaccination, the percentage of NK, CD4$^+$, and CD8$^+$ cells was 1.4%, 16.1%, and 57.1%, respectively (Fig. 2A). The expression of CD137 in these subpopulations is depicted in Fig. 2B. There was up-regulation of CD137 after TP-DC vaccination in all subpopulations compared with PBS-treated control mice. This was most notable in the CD3$^-$ NK1.1$^+$ cells population. No CD137 up-regulation was observed on CD3$^+$ NK1.1$^+$ cells (data not shown).

Anti-CD137 Monoclonal Antibody Administration Augments the Antitumor Efficacy of Dendritic Cell-Based Vaccines. We evaluated the antitumor activity of anti-CD137 administration in a 3-day pulmonary metastasis model. The first tumor evaluated was MCA 205 sarcoma, a weakly immunogenic tumor. As shown in Fig. 3A, mice treated with anti-CD137 alone or TP-DC vaccine had significantly reduced numbers of lung metastases compared with control animals. However, the combination of anti-CD137 + TP-DCs resulted in the greatest reduction of metastatic tumor, indicating an additive effect of the combined treatment. Using the nonimmunogenic B16-BL6 melanoma, we found that the combination therapy using anti-CD137 and TP-DCs was synergistic in mediating the regression of 3-day–old lung tumors (Fig. 3B). There was no antitumor effect with anti-CD137 alone or in combination with UP-DCs or EL-4 lysate-pulsed DCs. The latter represented a specificity control for B16-BL6. However, the combined therapy using anti-CD137 + B16-BL6 lysate-pulsed DCs resulted in significant tumor regression compared with all other groups.
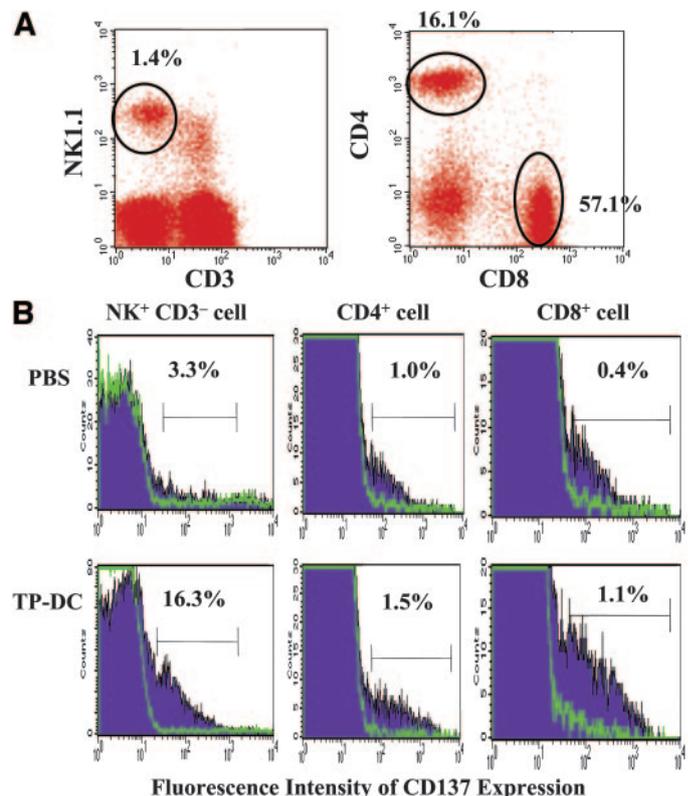

Fig. 2. CD137 up-regulation on VPLN cells. *A.* VPLN cells were stained for CD3, NK1.1, CD4, and CD8 markers. *B.* VPLN cells were stained for CD137 expression by indirect immunofluorescence 3 days after immunization with PBS or TP-DCs. Data are representative of four independent experiments. Control staining was performed with a purified rat IgG2a isotype standard. No CD137 up-regulation on CD3$^+$ NK1.1$^+$ cells was observed (data not shown).

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We next looked at the treatment of advanced (10-day–old) MCA 205 pulmonary metastases. As shown in Fig. 4, immunotherapy using anti-CD137 alone or in combination with UP-DCs had no antitumor activity against 10-day–old metastases. Similarly, UP-DCs or TP-DCs alone showed no therapeutic activity. However, the combination of TP-DC vaccination + anti-CD137 administration resulted in significant tumor regression. This demonstrated a synergism between
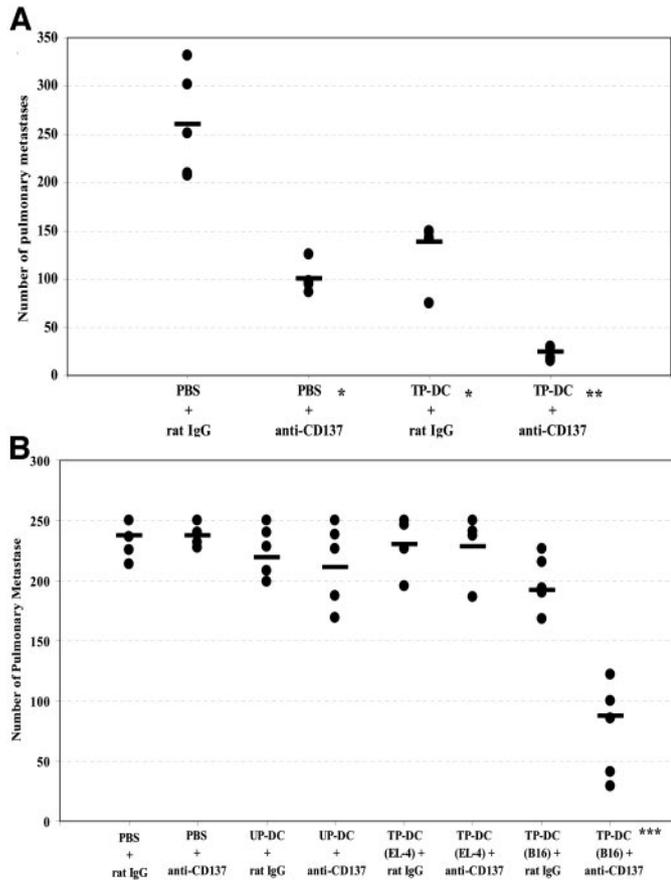

A

B

Fig. 3. Anti-CD137 mAb administration augmented antitumor reactivity of TP-DC immunization. Treatment of 3-day–old MCA 205 pulmonary metastases (*A*) and 3-day–old B16-BL6 pulmonary metastases (*B*). *A*: *, $P < 0.001$ *versus* PBS + rat IgG; **, $P < 0.001$ *versus* PBS + rat IgG and PBS + anti-CD137, $P < 0.01$ *versus* TP-DCs + rat IgG. *B*: ***, $P < 0.001$ *versus* any other groups. *A* and *B* are representative of three and two independent experiments, respectively.
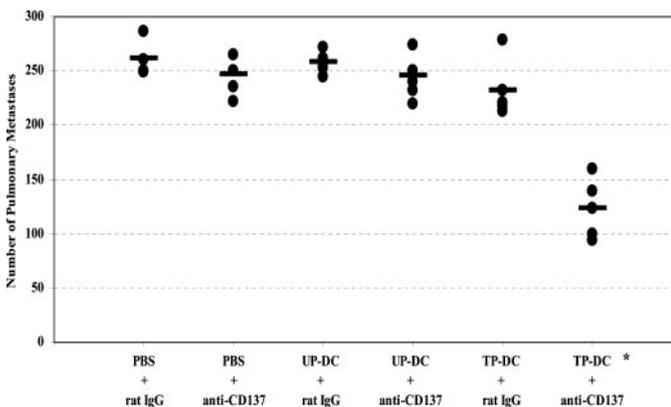

Fig. 4. Anti-CD137 mAb administration augmented antitumor reactivity of TP-DC immunization in the treatment of 10-day–old MCA 205 pulmonary metastases. *, $P < 0.001$ *versus* any of the other groups. The data were reproduced in a second independent experiment.
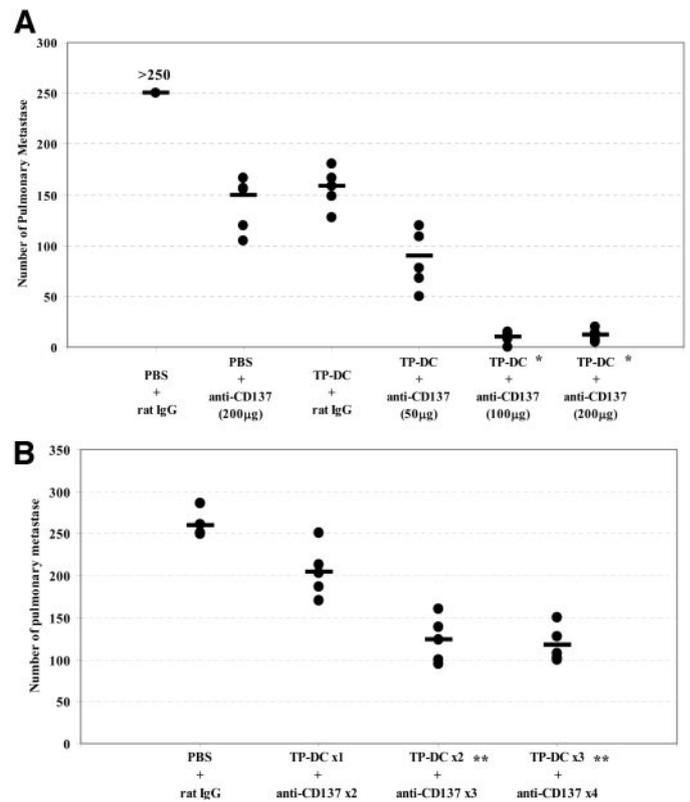

A

B

Fig. 5. *A*, dose dependence of antitumor effect of anti-CD137 administration in addition to TP-DC immunization. Mice with 3-day–old pulmonary metastases were treated intraperitoneally with either 50, 100, or 200 μg of anti-CD137 on days 1 and 4 after TP-DC immunization on day 0. Control group received either PBS + rat IgG (200 μg), PBS + anti-CD137 (200 μg), or TP-DC + rat IgG (200 μg). *, $P < 0.001$ *versus* all other groups. *B*, continued treatment with TP-DC + anti-CD137 augments antitumor efficacy. Mice with 10-day–old pulmonary metastases were treated with either TP-DC immunization $\times$ 1 (day 10) and anti-CD137 administration $\times$ 2 (day 11 and 14), TP-DC immunization $\times$ 2 (day 10 and 14) and anti-CD137 administration $\times$ 3 (day 11, 14, and 17), or TP-DC immunization $\times$ 3 (day 10, 14, and 17) and anti-CD137 administration $\times$ 4 (day 11, 14, 17, and 18). **, $P < 0.001$ *versus* all other groups.

anti-CD137 and TP-DCs in generating an immune response within the host to reject advanced tumor burdens.

In the lung tumor models, we examined the effect of titrating the dose on anti-CD137 and maximizing therapy. In Fig. 5*A*, mice with 3-day–old lung metastases were treated with a single vaccination of TP-DCs and given either 50, 100, or 200 μg of anti-CD137. The lower dose of 50 μg was found to be less effective than the higher doses. In the 10-day–old lung model, the number of TP-DC vaccinations as well as multiple dosing of anti-CD137 (100 μg) was evaluated. As shown in Fig. 5*B*, two TP-DC vaccinations + three doses of anti-CD137 resulted in better antitumor reactivity than one TP-DC vaccination + two doses of anti-CD137. Furthermore, increasing the number of vaccinations and doses of anti-CD137 did not improve the therapeutic effect.

Using the subcutaneous tumor model, we examined the impact of combined TP-DC therapy + anti-CD137 on survival. Mice were inoculated with MCA 205 subcutaneously and treated with different combinations of therapy as described in Materials and Methods. Therapy with anti-CD137 + PBS or TP-DCs + rat IgG had no effect on the growth of subcutaneous tumors compared with control animals (Fig. 6*A*). However, the combined treatment of TP-DCs + anti-CD137 resulted in a significant reduction of tumor growth. This translated to significantly improved survival, as shown in Fig. 6*B*.

We went on to examine the effect of TP-DCs + anti-CD137 on minimal residual disease after surgical resection. B16-BL6 melanoma

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cells were inoculated subcutaneously in the flanks of mice. On day 10, the tumors were resected, and mice received two TP-DC vaccinations and anti-CD137 as described in Fig. 7. Local tumor recurrence was the primary end point. By day 40, the group treated with TP-DCs + anti-CD137 had the lowest recurrence rate compared with the groups receiving monotherapy or control treatment (Fig. 7A). This group also had the best survival rate compared with the other groups (Fig. 7B).

Polarization Effect of Anti-CD137 during TP-DC Vaccination in Shifting Tumor-reactive T Cells Toward a Type 1 Phenotype. To investigate the mechanisms involved in the augmented therapeutic efficacy demonstrated by TP-DC + anti-CD137 administration, we examined the immune function of T cells from VPLNs and spleens of treated mice. Mice with 3-day–old MCA 205 pulmonary metastases were inoculated with TP-DC and anti-CD137 as described in Fig. 8. VPLNs and spleens were harvested on day 9 for immune function analysis. Anti-CD137 administration alone resulted in no differences in interferon (IFN)-γ or IL-4 released by VPLNs or splenocytes compared with control animals. TP-DC administration alone resulted in the production of a modest amount of IFN-γ and IL-4 by both VPLNs and splenocytes in response to MCA 205 tumor cells. There was a significant increase in the amount of IFN-γ released by these cell populations from mice that received both TP-DCs and anti-CD137 (Fig. 8A). This response was immunologically specific. By

Fig. 7. Combined therapy with TP-DC + anti-CD137 mAb resulted in lower local recurrence rates and improved survival after surgical resection of subcutaneous tumors. B6 mice were inoculated subcutaneously with 1×10^6 B16-BL6 tumor cells in the flank. Ten days later, the tumors were surgically excised. TP-DCs (10^6) were administered on days 13 and 17 along with anti-CD137 (100 μg) on days 14 and 17. Control groups received PBS + rat IgG, TP-DC + rat IgG, or PBS + anti-CD137. Each group consisted of 10 mice. *A*, percentage of local recurrence. *, $P < 0.004$ *versus* PBS + rat IgG and PBS + anti-CD137. *B*, survival of animals. *, $P < 0.004$ *versus* PBS + rat IgG and PBS + anti-CD137.
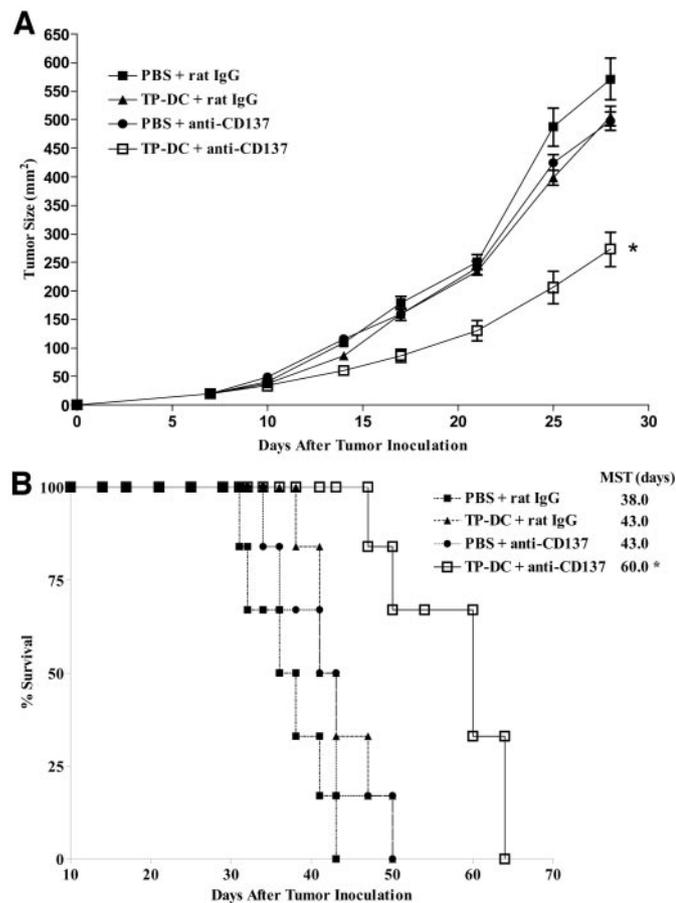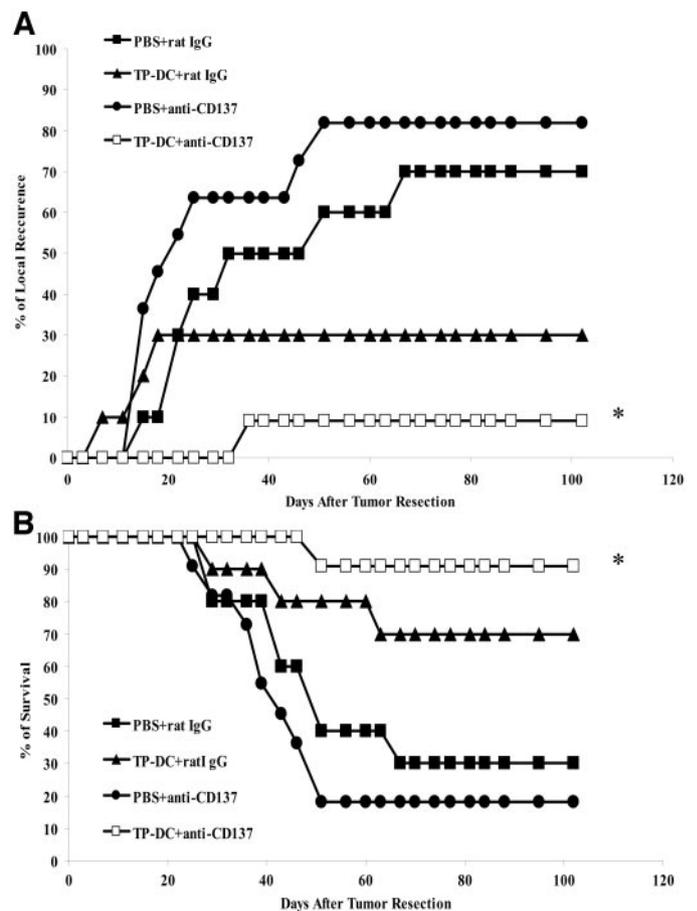

Fig. 6. Anti-CD137 mAb administration augmented antitumor reactivity of TP-DC immunization in established 7-day subcutaneous tumor and prolonged survival. B6 mice were inoculated subcutaneously with 2×10^5 MCA 205 tumor cells on day 0. Mice bearing 7-day tumors were treated either with PBS or TP-DC immunization on day 7 followed by either rat IgG or anti-CD137 administration on days 8 and 11. *A*. Data are reported as the average tumor area $\pm$ SE of six mice per group. *, $P < 0.001$ for TP-DC + anti-CD137 *versus* all other groups. *B*. Survival was monitored over time after tumor inoculation, and the median survival time (*MST*; in days) was determined. *, $P < 0.0001$ for TP-DC + anti-CD137 *versus* all other groups.

contrast, the administration of anti-CD137 in conjunction with TP-DCs resulted in significantly decreased IL-4 release (Fig. 8B).

TP-DC and Anti-CD137 Combined Treatment Enhances the Generation of Both CD4$^+$ CD62L^{low} and CD8$^+$ CD62L^{low} Cells. To further characterize the host lymphoid response to TP-DC + anti-CD137 therapy, we evaluated the expression of CD62L (L-selectin) on CD4$^+$ and CD8$^+$ VPLN cells. Mice were treated identically to the protocol described above, and VPLN cells were harvested 9 days after intravenous tumor infusion. Using three-color staining, CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells were gated to evaluate CD62L expression. TP-DC administration without anti-CD137 increased the percentage of CD62L^{low}-expressing CD4$^+$ cells from 17% to 27%. The addition of anti-CD137 further increased the percentage of CD62L^{low}-expressing CD4$^+$ cells to approximately 34%. A similar but more pronounced effect in increasing the number of CD62L^{low}-expressing cells was observed with the CD8$^+$ T-cell subset. TP-DC vaccination without anti-CD137 increased the percentage of CD62L^{low}-expressing CD8$^+$ cells from 10% to 26%. The addition of anti-CD137 further increased the percentage of CD62L^{low}-expressing CD8$^+$ cells to 52%. We consider this a significant observation because it has previously been reported that effector T cells capable of mediating tumor rejection *in vivo* are concentrated in the CD62L^{low}-expressing cell populations (23, 24).
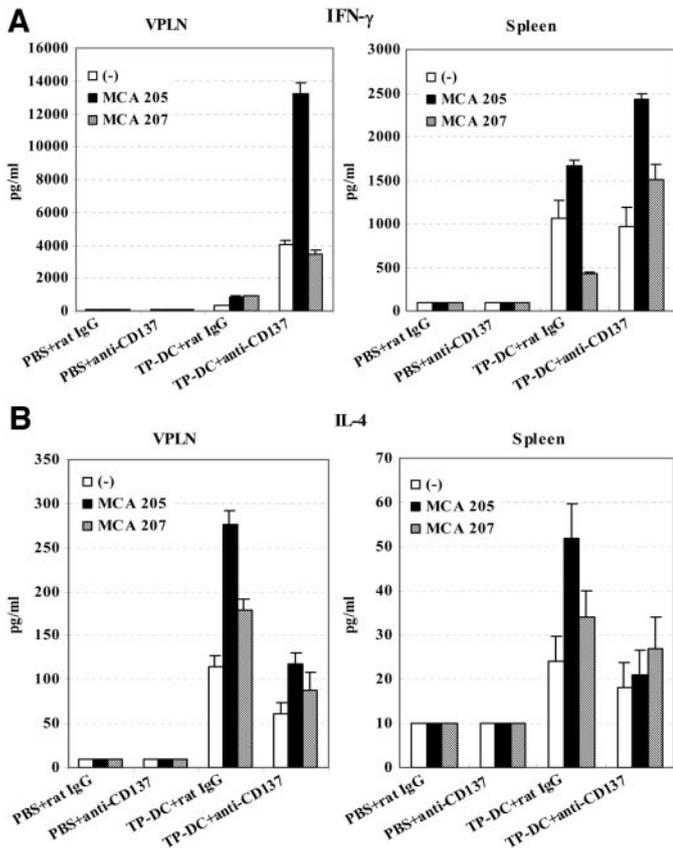
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Fig. 8. Anti-CD137 mAb administration enhanced IFN-γ secretion and decreased IL-4 secretion in conjunction with TP-DC immunization. IFN-γ (*A*) and IL-4 (*B*) secretion of lymphoid cells in response to tumor stimulation. B6 mice bearing established 3-day–old pulmonary metastases received immunization with PBS, UP-DCs, or TP-DCs on day 3 and intraperitoneal administration of rat IgG (100 μg) or anti-CD137 (100 μg) on days 4 and 7. VPLN cells and splenocytes (1×10^6 cells) were harvested on day 9 and cocultured with irradiated MCA 205 cells or MCA 207 cells (specificity control). After 24 hours, supernatants were collected for cytokine analysis. These data are representative of three independent experiments.

Identification of the Effector Cells Mediating Tumor Regression *In vivo*. We sought to identify the effector cells involved in the tumor rejection response associated with TP-DC + anti-CD137 therapy by using mAbs to deplete NK, CD4+, and CD8+ cells or by using KO mice. In antibody depletion experiments, the antitumor efficacy of TP-DC + anti-CD137 combined therapy was completely abrogated by the depletion of CD8+ T cells (Fig. 9*A*). Partial abrogation was observed by the depletion of CD4+ cells or NK cells. Use of KO mice devoid of NK, CD4+, or CD8+ cells confirmed this observation (Fig. 9*B*). The antitumor activity was completely abrogated in CD8 KO hosts and IL-15 KO hosts and partially abrogated in the CD4 KO mice. We concluded that the complete abrogation in the IL-15 KO mice was related to the absence of NK cells as well as an approximately 50% reduction of CD8+ cells in the IL-15–deficient mice compared with the wild-type host (data not shown). These data, taken together, indicate that CD8+ T cells are essential in the antitumor activity of TP-DC + anti-CD137, whereas NK cells and CD4 cells also play substantial roles.

Anti-CD137 Boosts the Proliferation of Antigen-reactive T Cells Induced by TP-DCs. Table 1 summarizes the cell yield obtained from VPLNs performed in four separate experiments. VPLNs were harvested 6 days after DC vaccination on day 0 followed by anti-CD137 administration on days 1 and 4. The administration of anti-CD137 alone did not result in an increase in the number of VPLN cells. The administration of UP-DCs or TP-DCs alone increased the

cell yield of VPLNs compared non–DC-treated mice, but the values were not significantly different from each other. The combination of anti-CD137 and UP-DCs resulted in a VPLN cell yield that was not different from that seen with UP-DCs alone. However, the combination of anti-CD137 + TP-DCs resulted in a significantly higher cell yield in the VPLNs compared with TP-DCs alone or any other control groups. This suggested that the *in vivo* administration of anti-CD137 increased cell proliferation of antigen-reactive lymphoid cells that may have been sensitized by the TP-DC vaccine.

To explore this observation further, we used the OT-1 model that had a defined antigen-reactive T-cell population to OVA. As described in Materials and Methods, OT-1 CD8+ T cells were isolated, labeled with CFSE, and adoptively transferred intravenously into wild-type B6 mice on day −3. Subsequently, on day 0, the recipient mice were immunized with UP-DCs or DCs pulsed with B16-OVA tumor lysate. Groups of mice were given anti-CD137, and VPLN cells were harvested on day 6 for analysis of proliferation.
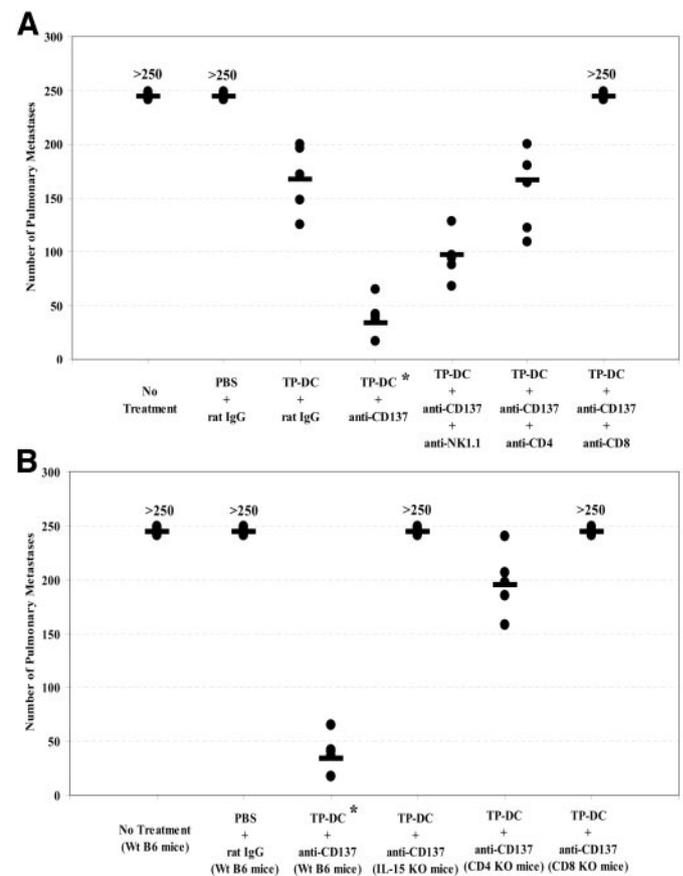

Fig. 9. TP-DC and anti-CD137 mAb tumor immunity was mediated by NK cells, CD8+ cells, and CD4+ cells. Treatment of 3-day–old MCA 205 pulmonary metastases using neutralizing antibody (*A*) or in KO mice (*B*) was as described in Materials and Methods. *, $P < 0.001$ *versus* all other groups.

Table 1 *Cell yield in vaccine-primed lymph nodes 6 days after inoculation*

	No. of cells ($\times 10^6$ per LN)				
Groups	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean ± SE
PBS + rat IgG	4.3	0.9	4.2	5.5	3.7 ± 2.0
PBS + anti-CD137	3.0	0.2	7.4	2.6	3.3 ± 3
UP-DC + rat IgG	23.0	10.0	22.6	20.0	18.9 ± 6.1
UP-DC + anti-CD137	24.0	20.0	20.0	17.5	20.4 ± 2.7
TP-DC + rat IgG	24.0	12.5	24.5	24.0	21.3 ± 5.8
TP-DC + anti-CD137	30.0	30.0	47.5	37.0	36.1 ± 8.3

Abbreviation: Exp., experiment.

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Fig. 10. Anti-CD137 enhanced the proliferation of antigen-reactive T cells induced by TP-DCs. OT-1 CD8$^+$ T cells were isolated, labeled with CFSE, and adoptively transferred intravenously into wild-type B6 mice on day −3. The recipient mice were immunized with UP-DCs or B16-OVA TP-DCs on day 0 and received injection with rat IgG or anti-CD137 mAb on days 1 and 4. Groups of mice were given anti-CD137, and VPLN cells were harvested on day 6 for analysis of proliferation. These data are representative of two independent experiments.
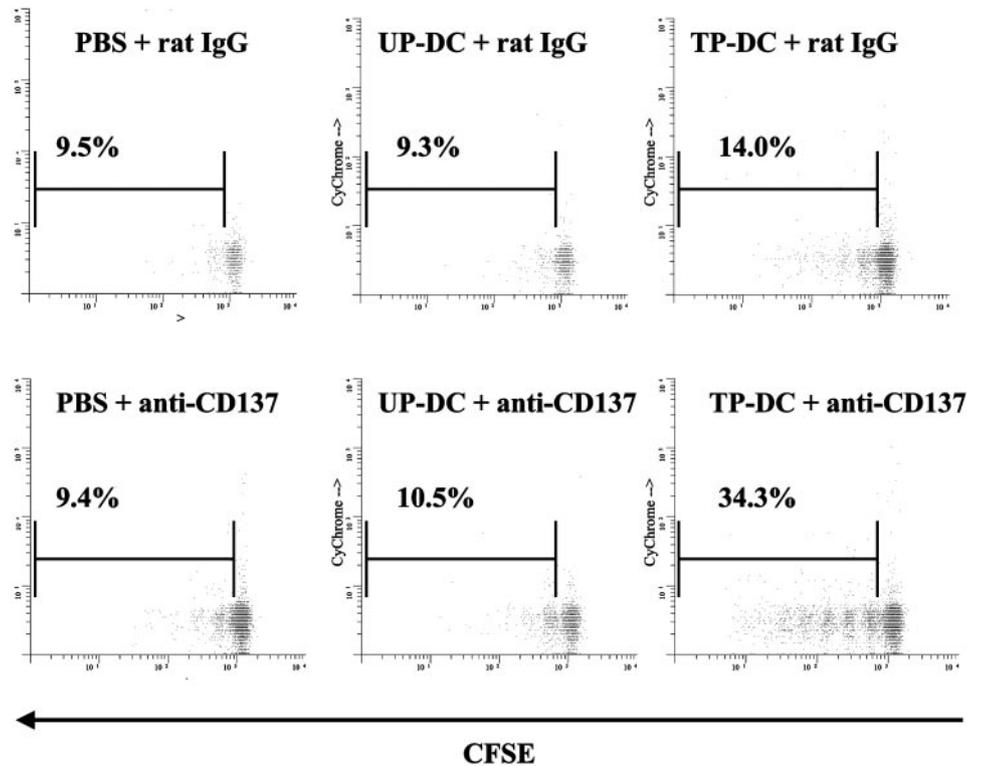
Proliferation of the adoptively transferred OT-1 cells was assessed by measuring CFSE profiles of the VPLN cells using flow cytometry (Fig. 10). Mice given PBS, anti-CD137 alone, UP-DCs alone, or UP-DCs + anti-CD137 demonstrated minimal evidence of proliferation of OT-1 CD8$^+$ T cells (~10%). There was a minor increase in the number of CFSE-positive cells after TP-DC (14%). However, the combination of anti-CD137 + TP-DC resulted in significant proliferation of CFSE-labeled OT-1 cells in the VPLNs (~ 34%). In addition, TP-DC + CD137 treatment increased the percentage of CD62L^{low}-expressing CFSE-labeled OT-1 cells (26%) compared with TP-DCs alone (11%), anti-CD137 alone (10%), and other control groups (≤10%).

Anti-CD137 Monoclonal Antibody Administration Polarizes T Cells Activated by TP-DCs Toward the Type 1 Phenotype via Natural Killer Cells. As with the MCA 205 model, we found that anti-CD137 administration resulted in polarizing the immune response to tumor-associated antigen in the OT-1 model. As shown in Fig. 11A, the administration of anti-CD137 + TP-DC significantly enhanced IFN-γ secretion by VPLNs and spleen cells to OVA compared with TP-DC + rat IgG. By contrast, antigen-stimulated IL-4 release by both cell populations was significantly reduced to undetectable levels when anti-CD137 was given in addition to TP-DC vaccination (Fig. 11B).

We proceeded to examine the effect of depleting mAb to CD4 cells and NK cells in this experimental model. As described in Fig. 11A and B, depletion of host CD4$^+$ T cells significantly reduced IFN-γ secretion induced by anti-CD137 + DC but did not alter IL-4 production. However, after depletion of host NK1.1$^+$ cells, the enhanced IFN-γ secretion and the decreased IL-4 secretion due to the use of anti-CD137 + DC were abrogated to levels comparable with those induced by DC vaccination alone. These results indicate that the antitumor efficacy of anti-CD137 + TP-DC combined therapy was associated with T-cell polarization toward a type 1 pattern mediated by NK cells, in addition to CD4$^+$ T-cell help.

DISCUSSION

We sought to determine whether anti-CD137 administration would be an effective adjuvant with DC-based vaccines and to evaluate what mechanisms were involved. An initial observation we made in our studies was that CD137 was up-regulated on a subset of VPLN T cells 3 days after TP-DC vaccination. This was not seen with the administration of UP-DCs, indicating that the pulsing with tumor lysate induced a functional difference in the DCs that mediated the activation of T cells in the VPLNs. Little information is available regarding the effects of tumor lysate uptake on DC function or biology. Grolleau *et al.* (25) recently used microarray analysis to identify gene expression differences between UP-DCs and TP-DCs in the murine system and found a broad spectrum of immunomodulating genes that are altered by lysate pulsing. The mechanisms by which TP-DCs up-regulate CD137 on VPLN T cells remain to be elucidated.

We postulated that anti-CD137 administration may potentiate the antitumor reactivity of TP-DC therapy. Subsequent experiments demonstrated that administration of anti-CD137 enhanced the antitumor reactivity of TP-DC vaccines in both weakly (*i.e.,* MCA 205) and poorly (*i.e.,* B16-BL6) immunogenic tumor models. The combined therapy of anti-CD137 + TP-DC resulted in more effective therapy of advanced disease and improved survival and was effective in the postoperative setting in treating residual microscopic disease.

We found that anti-CD137 administration polarized the antitumor reactivity of T cells to tumor antigen toward a type 1 response (*i.e.,* IFN-γ) and away from a type 2 response (*i.e.,* IL-4). This was observed in the VPLNs and spleens of vaccinated mice. The results of this study extend our previous observations regarding the ability of anti-CD137 to polarize T-cell reactivity during *in vitro* culture (26). In that report, we examined the effects of anti-CD3, anti-CD28, and anti-CD137 on activating tumor-draining lymph node (TDLN) cells *in vitro*. Anti-CD3, with or without anti-CD28, up-regulated CD137
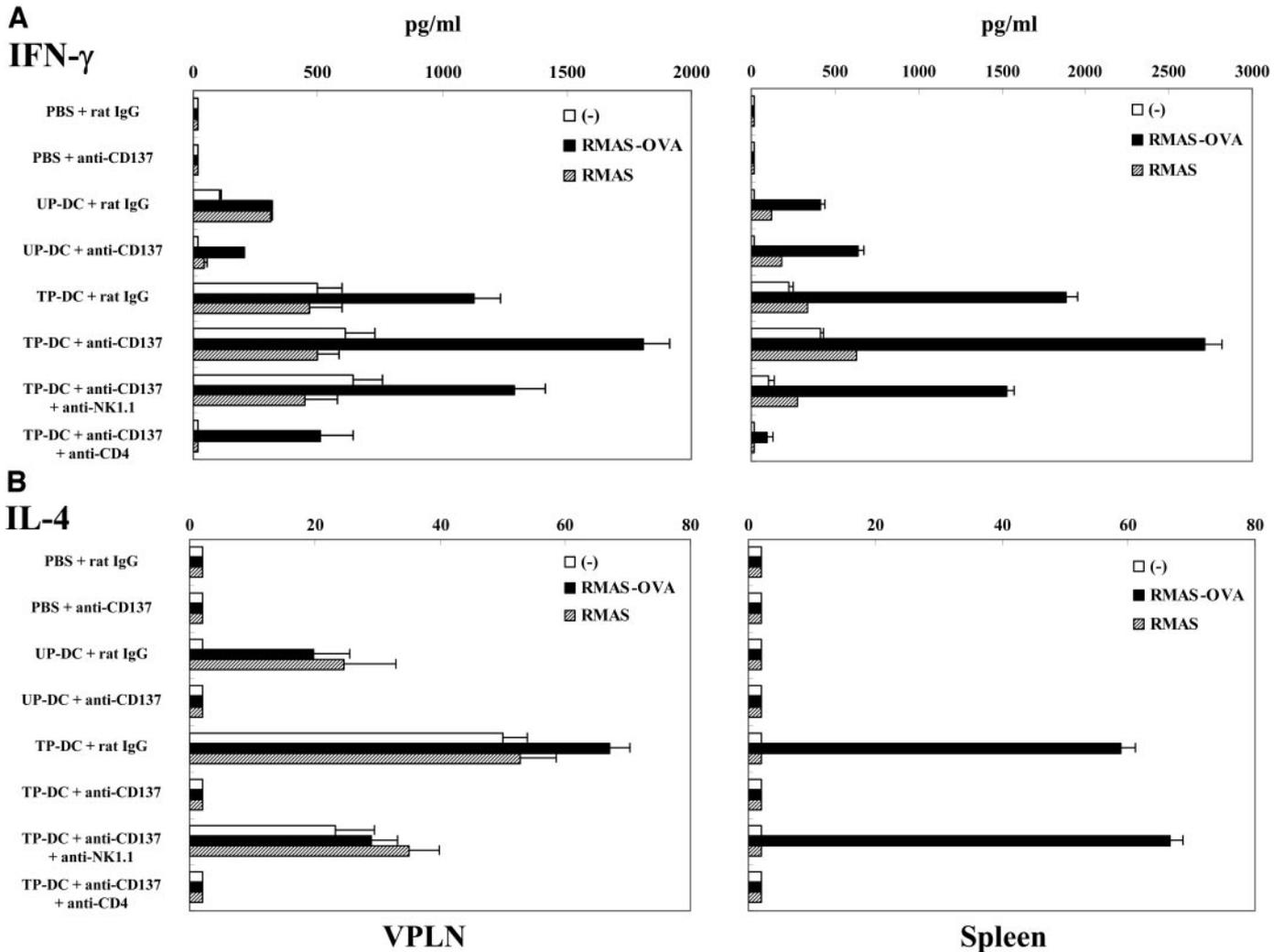
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Fig. 11. Anti-CD137 mAb administration enhanced IFN-γ secretion and decreased IL-4 secretion in conjunction with TP-DC immunization. Anti-CD137 administration polarized T cells activated by TP-DCs toward a type 1 phenotype via NK cells. IFN-γ (*A*) and IL-4 (*B*) secretion of lymphoid cells in response to OVA peptide stimulation. B6 mice transfused with OT-1 transgenic T cells on day −3 were inoculated subcutaneously with PBS, UP-DCs, or B16-OVA TP-DCs on day 0 and given rat IgG or anti-CD137 intraperitoneally on days 1 and 4. VPLN cells and splenocytes were harvested on day 6 and cocultured with irradiated RMAS cells in the presence or absence of OVA peptide. After 24 hours, supernatants were collected for cytokine analysis. To deplete the host CD4+ or NK1.1+ lymphocyte subset, mice received intraperitoneal injection with GK 1.1 (400 μg) or PK136 (400 μg), respectively, on day −2.

expression on TDLN cells. The subsequent addition of anti-CD137 polarized the type 1 *versus* type 2 response of the TDLN cells and augmented their therapeutic efficacy in the adoptive immunotherapy of established tumor.

The *in vivo* antitumor reactivity of anti-CD137 + TP-DC vaccination was mediated by CD8+, CD4+, and NK cells. Among these lymphoid cells, CD8+ T cells manifested the most significant antitumor effect as compared with CD4+ and NK cells. Other reports examining anti-CD137 alone as an antitumor therapy have also found CD8+ cells to be critical (15, 27). The major role of CD8+ cells as effectors may relate to the relatively greater expression of CD137 on CD8+ cells compared with CD4+ cells after T-cell receptor engagement (28). The depletion of CD4+ or NK cells partially abrogated the antitumor response of therapy, which suggested that these populations may have an interactive role with CD8+ T cells. In adoptive immunotherapy models, CD4+ tumor reactive cells have been shown to provide help to CD8+ cells by releasing IL-2 (22). Wilcox *et al.* (29) recently reported that CD137-stimulated NK cells promote the expansion of CD8+ cytolytic T cells *in vitro*, thus providing another form of help.

We consistently found that TP-DC vaccines + anti-CD137 resulted in a greater cell yield from the VPLNs compared with UP-DC vaccine given with or without anti-CD137. To further investigate the mechanisms of this effect, we used the OT-1 transgenic model. We found that the increased number of cells in the LNs draining TP-DCs was due to the preferential accumulation of antigen-reactive OT-1 cells. These cells also demonstrated increased proliferation compared with OT-1 cells found in LNs draining UP-DCs. In addition, we found that the administration of anti-CD137 caused increased survival and proliferation of antigen-reactive cells (*i.e.,* OT-1 CD8+ cells). Several groups have reported previously that anti-CD137 can promote survival of CD8+ T cells (30–34). The induced proliferation of CD8+ cells by anti-CD137 is supported by some investigators (30–33) but not by others (34). The majority of studies and our data would support the former.

The polarization effect of anti-CD137 was also observed in the OT-1 model. The depletion of NK cells, but not CD4+ cells, reversed the polarization effect of the cytokine response of both VPLNs and splenocytes. These findings demonstrate a novel interaction between anti-CD137–activated NK cells and T cells. The cross-talk between NK cells and CD8+ cells that promotes expansion and increased lytic activity as reported by Wilcox *et al.* (29) may be related to the

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cytokine milieu seen in our studies; namely, the down-regulation of type 2 cytokines.

In summary, there are several salutary effects of anti-CD137 when administered in conjunction with tumor vaccines. These include polarization of tumor-reactive T-cell responses toward a type 1 profile, increased survival and proliferation of activated T cells, and activation of NK cells that interact with the adaptive immune system in the polarization process. This provides a useful tool to break immunologic "ignorance" to poorly immunogenic tumors, making the combined therapy using this antibody and TP-DC vaccine more effective.

ACKNOWLEDGMENTS

REFERENCES

1. Mayordoma JI, Zorina T, Storkus WJ, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumor immunity. Nat Med 1995;1:1–6.
2. Fields RC, Shimizu K, Mule JJ. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proc. Natl Acad Sci USA 1998;95:9482–7.
3. Boczkowski D, Nair SK, Nam JH, Lyerly HK, Gilboa E. Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. Cancer Res 2000;60:1028–34.
4. Lambert LA, Gibson GR, Maloney M, et al. Intranodal immunization with tumor lysate-pulsed dendritic cells enhances protective anti-tumor immunity. Cancer Res 2001;61:641–6.
5. Gatza E, Okada CY. Tumor cell lysate-pulsed dendritic cells are more effective than TCR Id protein vaccines for active immunotherapy of T cell lymphoma. J Immunol 2002;169:5227–35.
6. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4:328–32.
7. Hsu FJ, Benike C, Fagnoni F, et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med 1996;2:52–8.
8. Morse MA, Deng Y, Coleman D, et al. A phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clin Cancer Res 1999;5:1331–8.
9. Banchereau J, Palucka AK, Dhodapkar M, et al. Immune and clinical responses in patients with metastatic melanoma to CD34+ progenitor-derived dendritic cell vaccine. Cancer Res 2001;61:6451–8.
10. Chang AE, Redman B, Whitfield JR, et al. A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. Clin Cancer Res 2002;8:1021–32.
11. Kwon BS, Tan KB, Ni J, et al. A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. J Biol Chem 1997;272:14272–6.
12. Wilcox RA, Chapoval AI, Gorski KS, et al. Expression of functional CD137 receptor by dendritic cells. J Immunol 2002;168:4262–7.
13. Takahashi C, Mittler RS, Vella AT. Differential clonal expansion of CD4 and CD8 T cells in response to 4-1BB ligation: contribution of 4-1BB during inflammatory responses. Immunol Lett 2001;76:183–91.
14. Lee HW, Park SJ, Choi BK, et al. 4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1. J Immunol 2002;169:4882–8.
15. Melero I, Shuford WW, Newby SA, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat Med 1997;1997;3:682–5.
16. Wilcox R, Flies D, Zhu G, et al. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. Clin Investig 2002;109:651–9.
17. Barth RJ, Bock SN, Mulé JJ, Rosenberg SA. Unique murine tumor-associated antigens identified by tumor infiltrating lymphocytes. J Immunol 1990;144:1531–7.
18. 18. Tanigawa K, Takeshita N, Eickhoff GA, Shimizu K, Chang AE. Antitumor reactivity of lymph node cells primed in vivo with dendritic cell-based vaccines. J Immunother 2001;24:493–501.
19. Geiger JD, Wagner PD, Shu S, Chang AE. A novel role for autologous tumor cell vaccination in the immunotherapy of the poorly immunogenic B16-BL6 melanoma. Surg Oncol 1992;1:199–208.
20. Catipovic B, Talluri G, Oh J, et al. Analysis of the structure of empty and peptide-loaded major histocompatibility complex molecules at the cell surface. J Exp Med 1994;180:1753–61.
21. Wexler H. Accurate identification of experimental pulmonary metastases. J Natl Cancer Inst (Bethesda) 1966;36:641–5.
22. Aruga A, Aruga E, Cameron MJ, Chang AE. Different cytokine profiles released by CD4+ and CD8+ tumor-draining lymph node cells involved in mediating tumor regression. J Leukocyte Biol 1997;61:1–10.
23. Kagamu H, Touhalisky JE, Plautz GE, Krauss JC, Shu S. Isolation based on L-selectin expression of immune effector T cells derived from tumor-draining lymph nodes. Cancer Res 1996;56:4338–42.
24. Kagamu H, Shu S. Purification of L-selectin^low cells promotes the generation of highly potent CD4 antitumor effector T lymphocytes. J Immunol 1998;160:3444–52.
25. Grolleau A, Misek D, Kuick R, Hanash S, Mulé J. Inducible expression of macrophage receptor marco by dendritic cells following phagocytic uptake of dead cells uncovered by oligonucleotide arrays. J Immunol 2003;171:2879–88.
26. Li Q, Carr A, Ito F, Teitz-Tennenbaum S, Chang A. Polarization effects of 4-1BB during CD28 costimulation in generating tumor-reactive T cells for cancer immunotherapy. Cancer Res 2003;63:2546–52.
27. Miller RE, Jones J, Le T, et al. 4-1BB-specific monoclonal antibody promotes the generation of tumor-specific immune responses by direct activation of CD8 T cells in a CD40-dependent manner. J Immunol 2002;169:1792–800.
28. Taraban VY, Rowley TF, O'Brien L, et al. Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses. Eur J Immunol 2002;32:3617–27.
29. Wilcox R, Tamada K, Strome S, Chen L. Signaling through NK cell-associated CD137 promotes both helper function for CD8+ cytolytic T cells and responsiveness to IL-2 but not cytolytic activity. J Immunol 2002;169:4230–6.
30. Halstead ES, Mueller YM, Altman JD, Katsikis PD. In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses. Nat Immunol 2002;3:536–41.
31. Cooper D, Bansal-Pakala P, Croft M. 4-1BB (CD137) controls the clonal expansion and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity. Eur J Immunol 2002;32:521–9.
32. Cannons JL, Lau P, Ghumman B, et al. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. J Immunol 2001;167:1313–24.
33. Laderach D, Movassagh M, Johnson A, Mittler RS, Galy A. 4-1BB co-stimulation enhances human CD8+ T cell priming by augmenting the proliferation and survival of effector CD8+ T cells. Int Immunol 2002;14:1155–67.
34. May K Jr, Chen L, Zheng P, Liu Y. Anti-4-1BB monoclonal antibody enhances rejection of large tumor burden by promoting survival but not clonal expansion of tumor-specific CD8+ T cells. Cancer Res 2002;62:3459–65.

Anti-CD137 Monoclonal Antibody Administration Augments the Antitumor Efficacy of Dendritic Cell-Based Vaccines

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