Supporting Information

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SI Materials and Methods

Animals, Peptides, and Cell Lines. Male 6- to 8-wk-old C57BL/6 mice were obtained from the National Cancer Institute (NCI) at Frederick, MD, and Ly5.1 C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animal use adhered to National Institutes of Health Laboratory Animal Care Guidelines and was approved by the NCI Animal Care and Use Committee (ACUC).

The peptides SNC9-H8 (STHVNHLHC) (1) and Ovalbumin (OVA)257-264 (SIINFEKL) were synthesized (GenScript). The TRAMP-C2 cell line derived from transgenic adenocarcinoma of mouse prostate (TRAMP) (2). The EL4 cell line derived from the C57BL/6N mouse lymphoma was used as a control for tumor cell specificity of the response. Both cell lines were purchased from American Type Culture Collection.

Tumor Challenge and Treatment Experiments. Groups of 10 mice each were injected s.c. in the flank on day 0 with 5×10^5 TRAMP-C2 cells. On day 7, treatment was initiated only in animals with palpable s.c. tumors. Each mouse received 5 μg of mIL-15 (PeproTech) i.p. daily, five times per week for 3 wk. Selected animals also received anti-mouse-PD-L1 (9G2; BioExpress), anti-CTLA-4 (UC10-4F10-11; BioExpress), or both. Isotype IgGs (rat IgG or hamster IgG) were administered along with mIL-15 as controls. One group of animals received anti-IL-10 (JES5-2A5; BioExpress) along with mIL-15. Another group was given a pan-neutralizing anti-mouse TGF-β1, 2, 3 mAb (1D11) that was a gift of the Genzyme Corporation with IL-15. The dose and dosing schedule used with these antibodies was 100 μg per injection administered twice weekly for 3 wk. Control mice received PBS injections 100 μL/d using the same dosing schedule as for mIL-15. In vivo depletion of CD25-expressing cells was accomplished using PC61 (BioExpress) 400 μg per mouse, administered i.p. 4 d before inoculation of TRAMP-C2 tumor cells. Mice with large tumors were humanely killed using prospective ACUC-approved criteria to generate survival curves.

In Vivo $CD8^+$ T Cell or NK Cell Depletion. Groups of mice ($n = 8$) were injected i.v. with 5×10^5 TRAMP-C2 cells on day 0 and then received mIL-15 as described above. Groups of animals received 200 μg of either rat anti-mouse-CD8 (clone 2.43; BioExpress) or 50 μL per injection anti-asialo-GM1 (Wako) i.p. beginning on days 0 and 1, and then 3 times per week for 3 wk. Isotype control antibodies including rat IgG (clone LTF-2; BioExpress) or rabbit IgG were given at the same dosages to set up as controls. Tumor sizes were measured routinely.

Cytokine Secretion and Cytotoxicity Assays. Splenic $CD8^+$ T cells from treated animals were purified using magnetic beads (Miltenyi Biotec) and cocultured with SNC9-H8 peptides for 72 h in association with irradiated (3,000 rad) syngeneic splenocytes to function as APCs. To set up a control experiment, the same concentration of the OVA peptide was used as a control peptide. IFN-γ was measured by ELISA (R&D Systems). Cytokine concentrations in supernatants were interpolated from the linear portion of ELISA standard curves.

To perform cytotoxicity assays and verify the specificity of the lysis activity, EL4 tumor cells were used as nonrelevant target cells for control of tumor specificity of the response. On day 21 after tumor challenge splenic cells were cultured with irradiated (100 Gy) TRAMP-C2 tumor cells or EL4 cells at a 50:1 ratio. Recombinant hIL-2 was added to yield a concentration of 10–15 U/mL. After 4 d of culture, cytotoxicity assays were performed: effector (E) cells from each group were cultured with 10^4 TRAMP-C2 or EL4 target (T) cells per well in triplicate at various E/T ratios and incubated at 37 °C for 4 h. Cytotoxic activity was measured by LDH release using CytoTox 96 Non-Radioactive Cytotoxicity Assays (Promega). Percentage cytotoxicity was calculated as $100 \times [(\text{experimental release}) - (\text{effector spontaneous release}) - (\text{target spontaneous release})]/[(\text{target maximum release}) - (\text{target spontaneous release})]$.

IFNγ Secretion by Tumor-Infiltrating Lymphocytes (TILs). TRAMP-C2 tumors were dissected from mice and cut into small pieces, then incubated with a solution containing 0.2 mg/mL DNase and 1.67 WunschU/mL Liberase (Roche) in unsupplemented RPMI 1640. Tumors were incubated at 37 °C for 30 min with shaking, disaggregated, passed through a 40-μm filter, and washed several times. A Ficoll gradient was performed to eliminate dead cells and tumor debris. Alive TILs were seeded into 96-well plates and cocultured with SNC9-H8 peptides for 72 h in association with irradiated (3,000 rad) syngeneic splenocytes to function as APCs. IFN-γ was measured by ELISA (R&D Systems). The cytokine concentration in supernatants was interpolated from the linear portion of the ELISA standard curves.

$CD4^+CD25^+$ T Cell Suppressor Assay. Splenic $CD4^+CD25^+/CD4^+CD25^-$ T cells from tumor-bearing animals were purified with $CD4^+CD25^+$ isolation kits, and naïve splenic $CD8^+$ T cells were isolated with CD8 T cell isolation kits according to the manufacturer's protocols (Miltenyi Biotec). Purities of cells were greater than 95%. $CD4^+CD25^-$ T cells (2×10^5/well) or naïve $CD8^+$ T cells (2×10^5/well) were cocultured with $CD4^+CD25^+$ T cells from each treated group at different ratios as described with 1 μg/mL of plate-bound anti-CD3 stimulation for 72 h, with ^{3}H-TdR (1 μCi/well) added to culture systems during the last 6 h to measure cell proliferation.

Carboxyfluorescein Succinimidyl Ester (CFSE) Labeling $CD8^+CD122^-$ T Cells and Cell Proliferation Tracking. To evaluate inhibition of $CD8^+CD122^-$ proliferation mediated by $CD8^+CD122^+$ T cells, splenic $CD8^+CD122^+/CD8^+CD122^-$ T cells were first purified with negative selection to deplete non-CD8 T cells (Miltenyi Biotec). $CD8^+$ T cells were incubated with biotin-labeled anti-CD122 antibody (eBioscience) and positively selected by Strepavidin-conjugated magnetic beads with magnetic columns (Miltenyi Biotec).

Naïve $Ly5.1^+$ $CD8^+CD122^-$ T cells (3×10^5/well) were labeled with CFSE (Invitrogen) and stimulated with plate-bound anti-CD3 (2.5 μg/mL) along with soluble anti-CD28 (2.5 μg/mL) for 48 h. Cells in selected wells were cocultured with $CD8^+CD122^+$ T cells (1×10^5/well) purified from tumor-bearing treated animals. Harvested cells were stained with anti-mouse Ly5.1, and cell differentiation was measured with flow cytometry.

Statistical Analysis. Kaplan-Meier nonparametric regression analysis was performed to assess survival times of tumor-bearing animals, with significance of differences determined by the log–rank test using JMP statistical software (SAS Institute). The ANOVA test was performed to compare tumor volumes using JMP statistical software. Comparisons of cytokine secretion among groups and absolute numbers of specific cell subsets were analyzed using an unpaired Student's t test. A value of $P < 0.05$ was considered significant.

1. Fassò M, et al. (2008) SPAS-1 (stimulator of prostatic adenocarcinoma-specific T cells)/SH3GLB2: A prostate tumor antigen identified by CTLA-4 blockade. *Proc Natl Acad Sci USA* 105:3509–3514.

2. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM (1997) Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Res* 57:3325–3330.

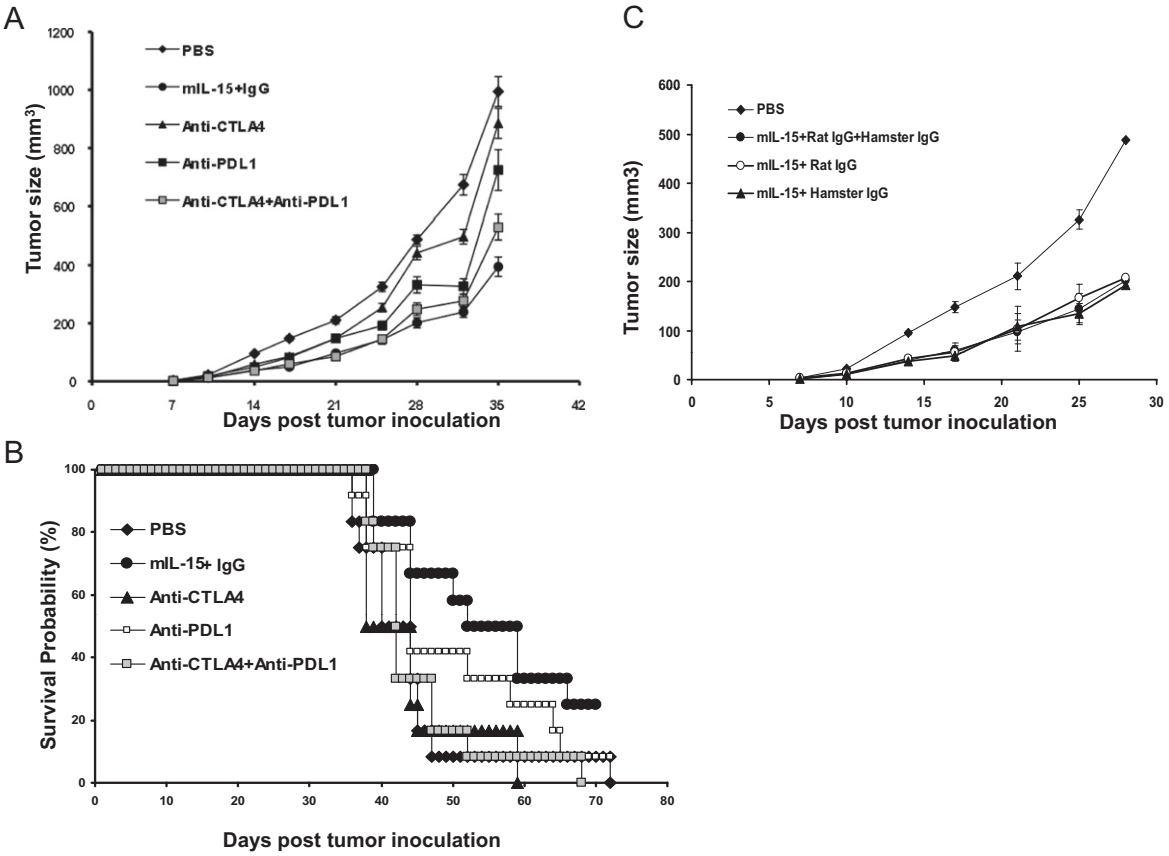

Fig. S1. Single antibody or the combination of anti-PD-L1 and anti-CTLA-4 antibodies did not provide mice effective protection compared with IL-15 treatment alone against TRAMP-C2 tumors. (*A*) Tumor growth curves illustrate the in vivo growth of TRAMP-C2 prostate tumor with the different treatments. The tumor sizes were represented by means ± SEM, $n = 10$. Data are representative of three independent experiments. (*B*) Kaplan-Meier survival curves illustrating tumor-bearing animal survival after the different treatments. Data are representative of three independent experiments and $n = 10$. (*C*) Isotype of the IgGs did not affect the therapeutic effect mediated by IL-15 against tumor growth. Rat IgG and/or hamster IgG were injected at 100 μg per mouse, twice weekly for 3 wk along with IL-15. Tumor size was measured twice weekly. PBS-treated animals served as controls. Growth curves illustrate in vivo growth rates of TRAMP-C2 prostate tumors associated with different treatments. Tumor sizes shown represent means ± SEM, $n = 8$.

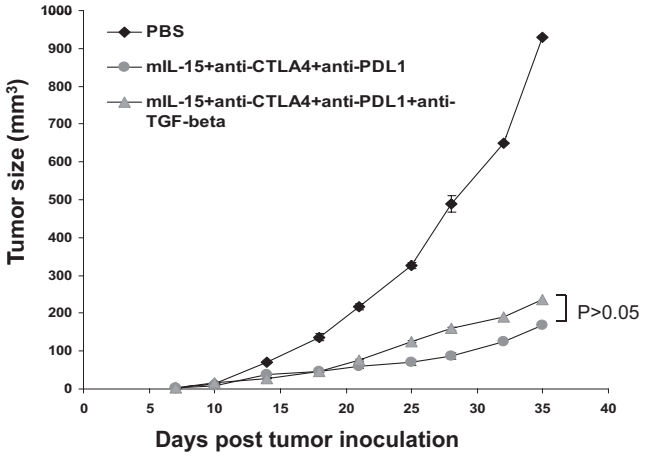

Fig. S2. Administration of anti-TGF-β along with triple combination treatment (mIL-15 combined with anti-CTLA-4 and anti-PD-L1 antibodies) did not provide mice more protection against TRAMP-C2 tumors compared with triple combination treatment alone. The addition of anti-TGF-β to the triple combination did not inhibit tumor growth beyond that achieved with triple combination treatment alone. The tumor sizes are represented by the means ± SEM, $n = 10$. Data are representative of three independent experiments.

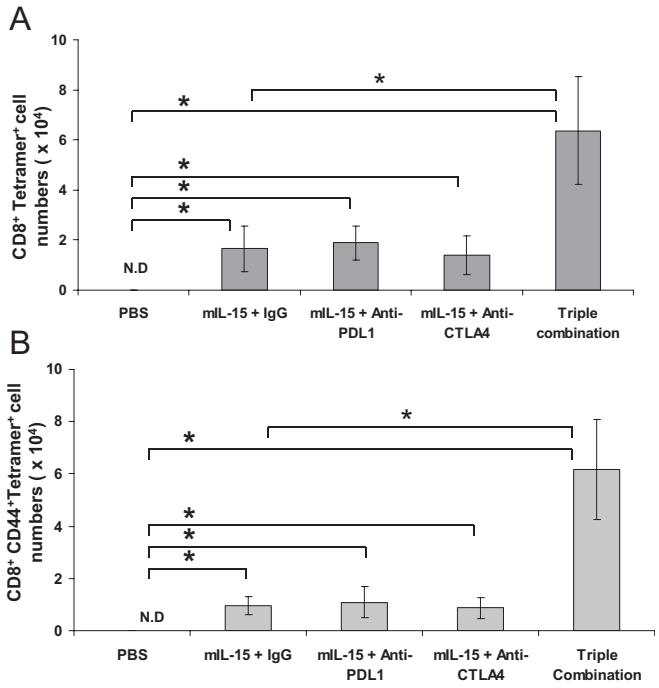

Fig. S3. IL-15 treatment with simultaneous blockade of CTLA-4 and PD-L1 was associated with an increase of numbers of tumor-specific CD8 T cells. On day 21, splenocytes derived from treated tumor-bearing mice were stained with PE-conjugated SPAS-1-tetramer, APC-anti-CD8, and PE-Cy5.5-CD44 antibodies. (*A*) Absolute numbers of tetramer$^+$-CD8 cells. (*B*) Absolute numbers of tetramer$^+$-CD8$^+$CD44^{high} T cells. Data represent five independent experiments. *$P < 0.05$.

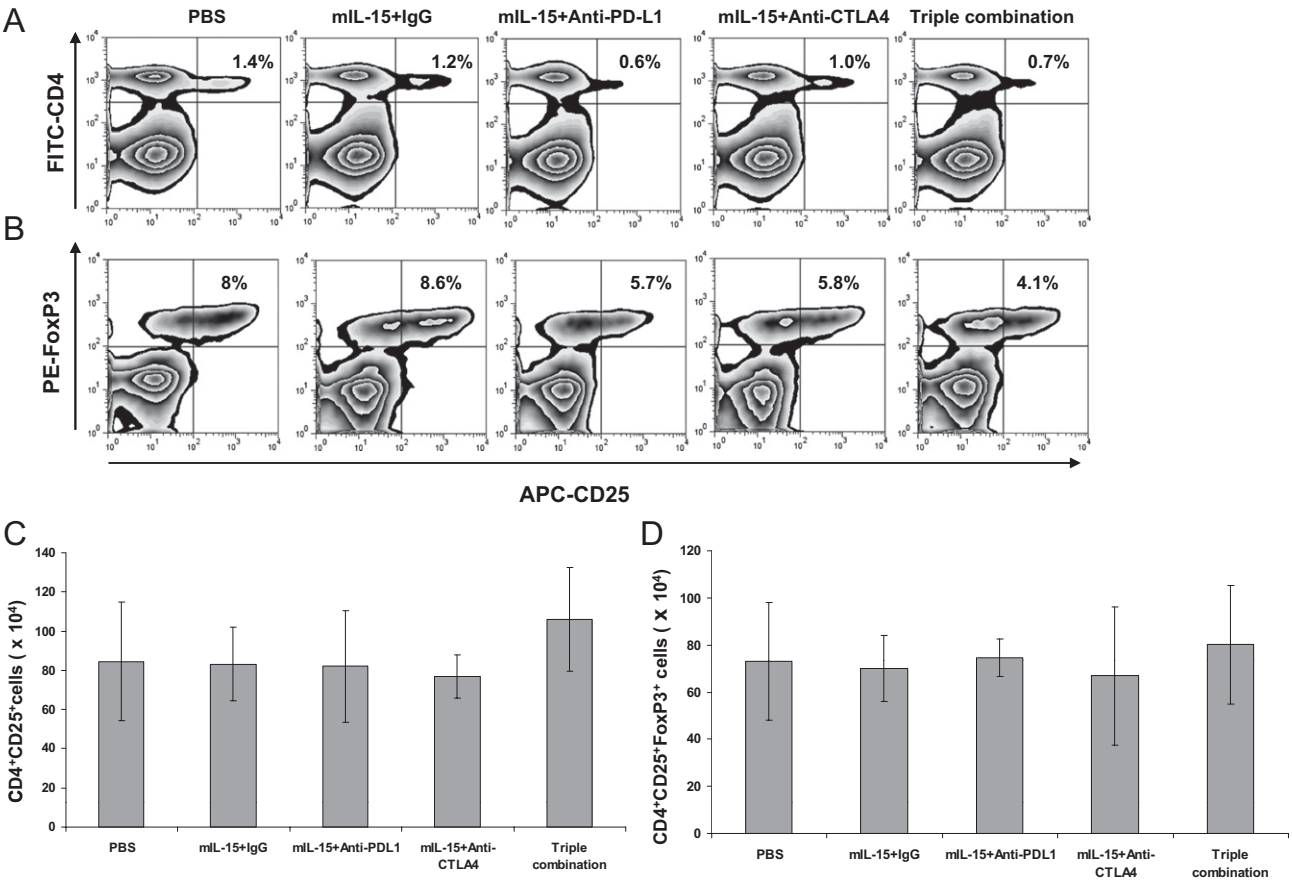

Fig. S4. IL-15 treatment with simultaneous blockade of CTLA-4 and PD1/PDL1 did not affect the numbers of $CD4^+CD25^+$ regulatory T cells (Tregs). On day 21, splenocytes from tumor-bearing animals receiving different treatments were stained with Phycoerythrin (PE)-anti-FoxP3 intracellularly, along with APC-anti-CD25 and FITC-anti-CD4 antibodies. (*A*) Percentage of cells coexpressing CD4 and CD25, gated by forward and side scatter; (*B*) percentage of $CD25^+FoxP3^+$ T cells, based on the gated $CD4^+$ T cells. (*C*) Absolute numbers of $CD4^+CD25^+$ Tregs in each group. (*D*) Absolute numbers of $CD4^+CD25^+FoxP3^+$ Tregs in each group. Data represent three independent experiments, with each group containing 8–10 mice.

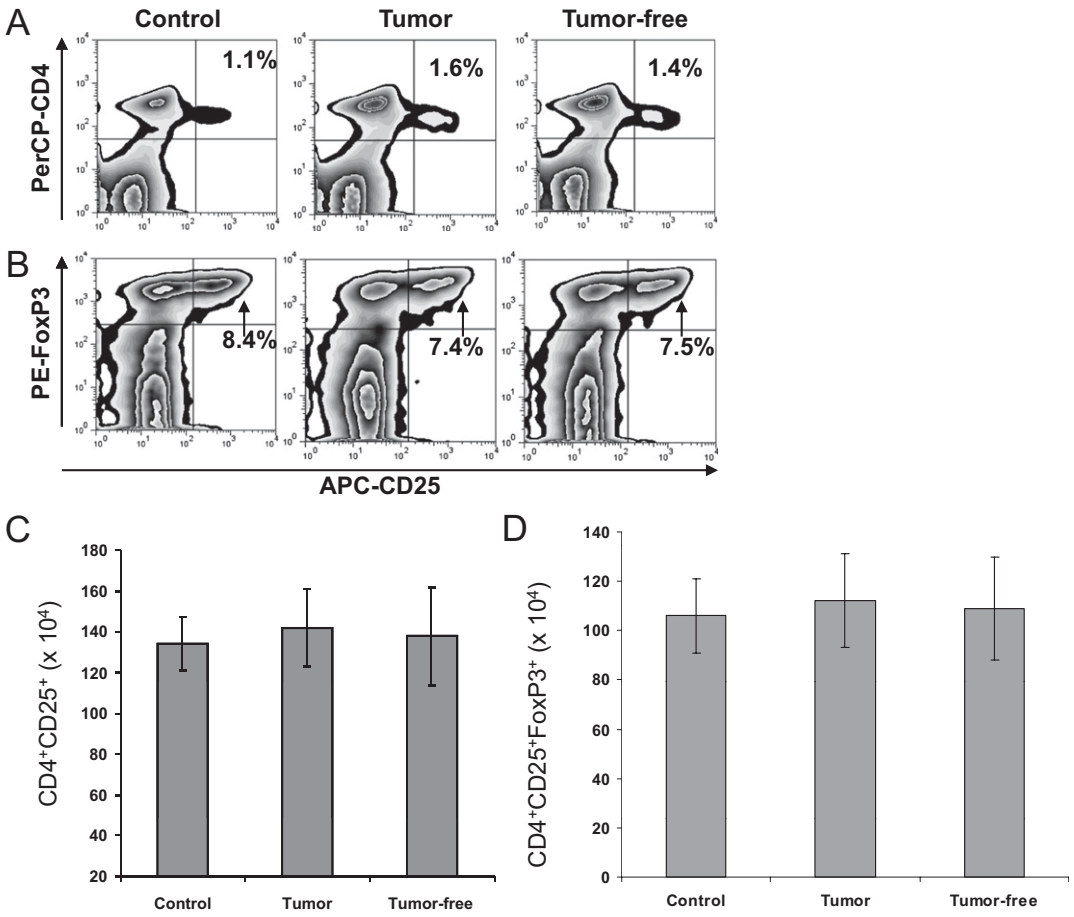

Fig. S5. TRAMP-C2 rechallenge against triple combination-treated animals did not affect the numbers of $CD4^+CD25^+$ Treg cells. At the termination of the study, splenocytes from rechallenged animals were stained with PE-anti-FoxP3 intracellularly, along with APC-anti-CD25 and PerCP-anti-CD4 antibodies. (*A*) Percentage of cells coexpressing CD4 and CD25, gated by forward and side scatter; (*B*) percentage of $CD25^+FoxP3^+$ T cells, based on the gated $CD4^+$ T cells. (*C*) Absolute numbers of $CD4^+CD25^+$ Tregs in each group. (*D*) Absolute numbers of $CD4^+CD25^+FoxP3^+$ Tregs in each group. Data represent two independent experiments, with each group containing three mice.

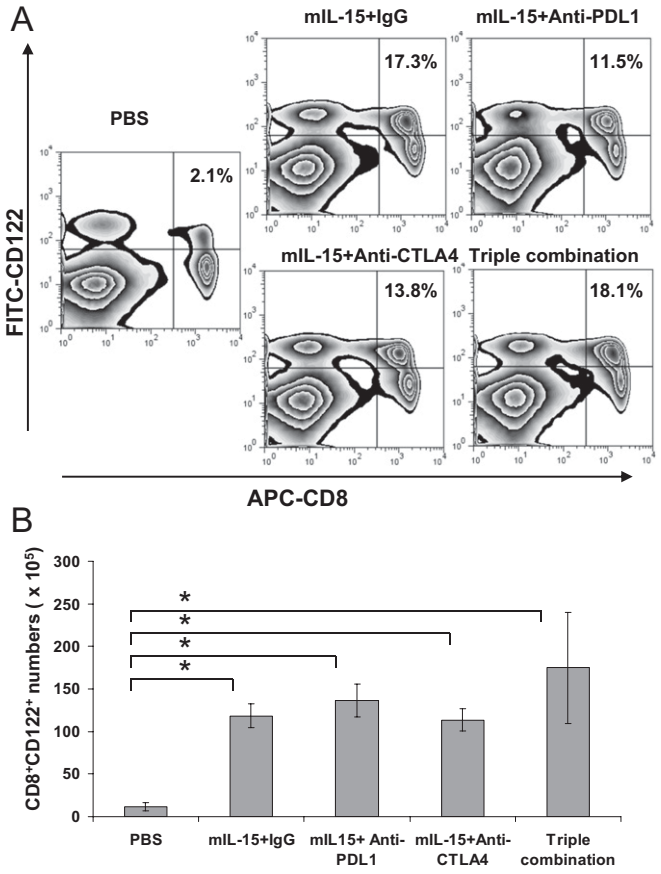

Fig. S6. IL-15 treatment increased both the percentage and absolute number of $CD8^+CD122^+$ cells. Splenocytes from treated tumor-bearing animals were stained with APC-anti-CD8 and FITC-anti-CD122. Representative data are shown in *A*. Absolute numbers of $CD8^+CD122^+$ T cells are summarized in *B*.

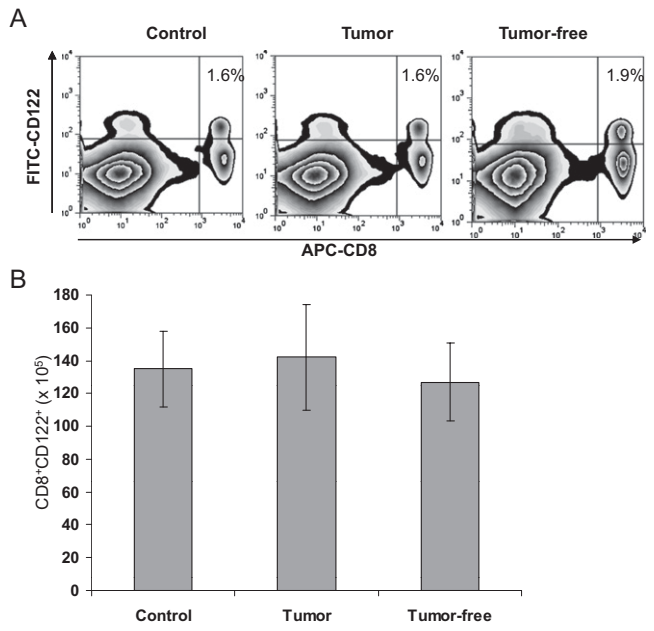

Fig. S7. TRAMP-C2 rechallenge against triple combination-treated animals did not affect the numbers of $CD8^+CD122^+$ T cells. At the termination of the study, splenocytes from rechallenged animals were stained with APC-anti-CD8 and FITC-anti-CD122 antibodies. (*A*) Percentage of cells coexpressing CD8 and CD122, gated by forward and side scatter; (*B*) absolute numbers of $CD8^+CD122^+$ T cells in each group. Data represent two independent experiments, with each group containing three mice.

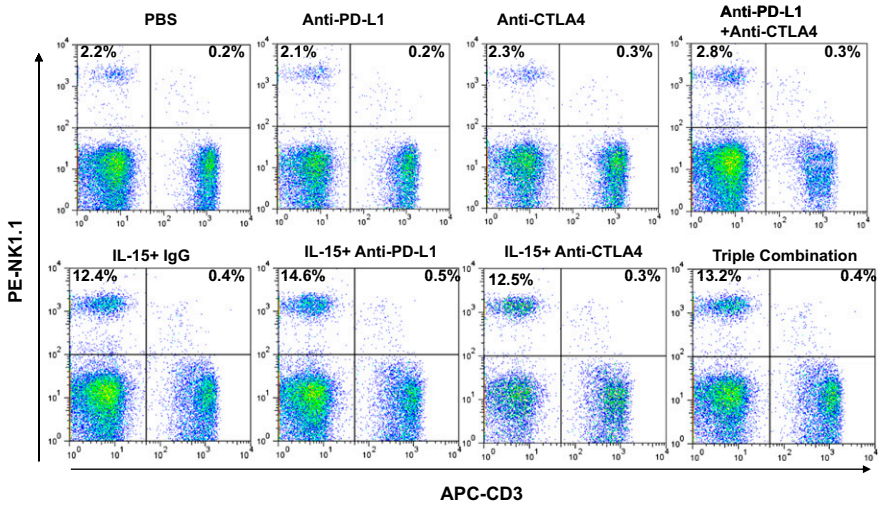

Fig. S8. IL-15 treatment was associated with an increase of NK1.1$^+$ cells in the spleens of TRAMP-C2 tumor-bearing animals. On day 21, splenocytes derived from tumor-bearing animals receiving different treatments were stained with PE-anti-NK1.1 and APC-anti-CD3. Values presented represent the percentage of NK1.1$^+$ cells in the total cell population.

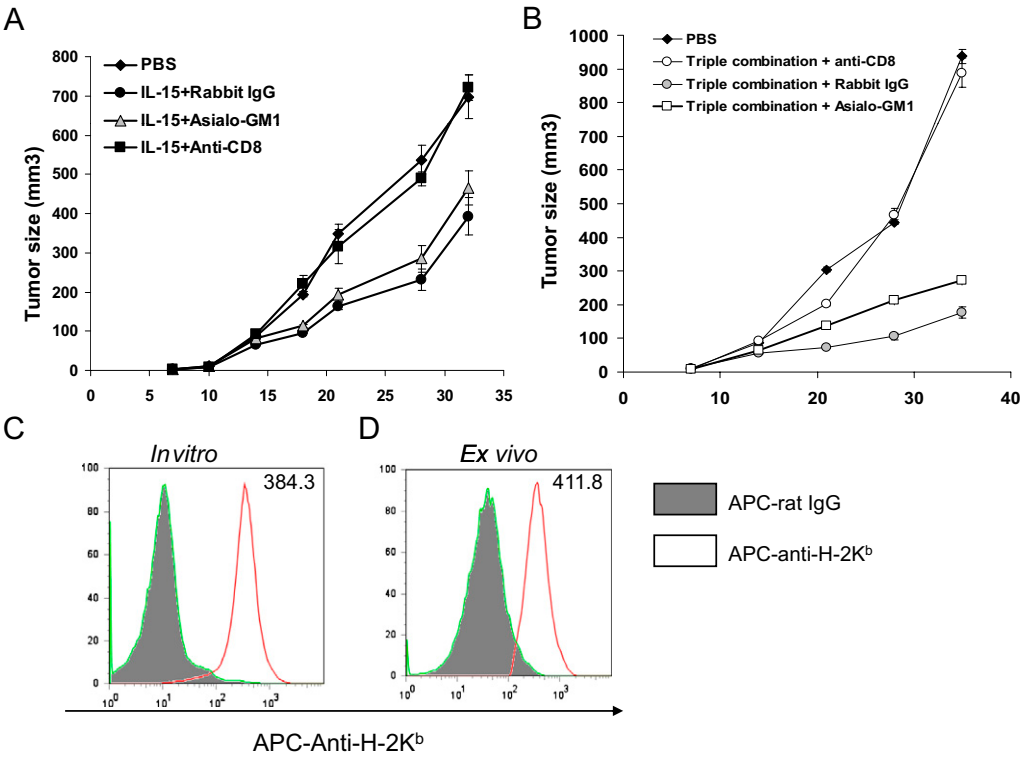

Fig. S9. CD8$^+$ T cells instead of NK cells were involved in the antitumor response to TRAMP-C2 mediated by IL-15. Growth curves illustrate in vivo growth rates of TRAMP-C2 prostate tumors associated with mIL-15 (*A*) or triple combination (*B*) treatment. Seven days after tumor inoculation, mice with palpable tumors received 5 μg/d of mIL-15 injected 5 times per week for 3 wk. Anti-PDL1 plus anti-CTLA4 were injected at 100 μg per mouse, twice weekly for 3 wk. Selected groups of mice were administrated 50 μL per mouse anti-asialo-GM1 or rabbit IgG to deplete NK cells on days 0 and 1, and then three times per week for 3 wk. Anti-CD8 antibody (200 μg per injection) was provided to deplete CD8 T cells with the same schedule as anti-asialo-GM1. PBS-treated animals served as controls. Data represent two independent experiments. Tumor sizes shown represent means ± SEM, $n = 8$. (*C* and *D*) Expression of MHC class I on TRAMP-C2 cells was analyzed by flow cytometry using APC-anti-mouse-H-2K^b (open area). An antibody of the same isotype was used as a negative control (filled area). MHC class I Mean Fluorescence Intensity (MFI) of TRAMP-C2 cells either (*C*) in vitro or (*D*) obtained ex vivo from tumor-bearing animals treated with PBS was shown.