Supplementary Methods

Blocking antibodies and peptides. Rat anti-mouse PD-1 (29F.1A12, rat IgG2a, *k*), PD-L1 (10F.9G2, rat IgG2b, *k*), and PD-L2 (3.2, mouse IgG1) have been described (24). Anti-CTLA-4 (clone 9D9, mouse IgG2b) blocking antibody and isotype control antibodies obtained from BioXCell. CT26 tumor antigen peptide AH-1 (SPSYVYHQF) (48), and ID8-VEGF tumor antigen peptide folate receptor (FR) (49) were synthesized by New England Peptides. For depletion of $CD4^+$ or $CD8^+$ T cells, mice were given 500 μg of anti-mouse CD4 antibody (GK1.5, rat IgG2b; BioXCell) or CD8 antibody (53-6.7, rat IgG2a; BioXCell) i.p. on days -1 and 0 before CT26 tumor inoculation. Tumor size was measured using a caliper.

Isolation of TILs. Thirteen to 15 days after CT26 and 25-30 days after ID8-VEGF tumor challenge, mice were euthanized, and tumor leukocytes were isolated by dissociating tumor tissue in the presence of 2.5 mg/ml collagenase D for 20 min before centrifugation on a discontinuous percoll gradient (GE Healthcare). Tumor leukocyte suspensions were mixed with αCD8 microbeads (Miltenyi Biotec) and separated into $CD8^+$ T cells by positive selection on a MACS column. $CD8^+$ T cell populations were confirmed to contain >95% $CD8^+$ T cells. In some experiments, these $CD8^+$ T cells were purified on a FACSAria (BD Biosciences) sorter after staining with anti-CD8 antibody (purity >95%). The total number of infiltrating $CD8^+$ T cells/gram of tumor was obtained by multiplying the percentage of $CD8^+$ T cells by the total number of lymphocytes obtained from Ficoll gradient and dividing that number by 100 and by the weight of the tumors.

$CD4^+CD25^+$ T cells were purified using a Treg isolation kit (Miltenyi Biotec) (purity > 90%). In some experiments, the $CD4^+CD25^+$ T cells were purified on a FACSAria (BD

Biosciences) after staining with anti-CD4 and anti-CD25 antibodies (purity >95%).

Flow cytometry and intracellular cytokine staining. Lymphocytes were isolated from tumor, spleen, and lymph nodes. The antibodies to CD8 (53-6.7), CD4 (RMP4-5), CD25 (PC61), CD62L (MEL-14), CD11a (2D7), Ki-67 (B56), Bcl-2 (3F11), phospho-T-bet (O4-46), IFN-γ (XMG 1.2), TNF-α (MP6-XT22), IL-2 (JES6-5H4) CD107a (1D4B), and CD107b (ABL093) were purchased from BD Biosciences. Staining antibodies to CD127 (A7R34), PD-1 (RMP 1–30), 2B4 (C244F4), GITR (DTA-1), ICOS (7E.17G9), phospho-eomesodermin (Eomes; Dan11mag), and the Foxp3 staining kit (FJK-16s) were obtained from eBioscience. The antibodies to CD44 (IM7), LAG-3 (C9B7W), TIM-3 (B8.2C12), FR-4 (12A5) were obtained from Biolegend. Granzyme B (Caltag) and CTLA-4 (Southern Biotech) staining antibodies were also used.

For intracellular staining, T cells from tumor, spleen, and lymph nodes were stimulated with AH-1 (CT-26) or FR-α (ID8-VEGF) peptides for 5h in the presence of brefeldin-A. After fixation and permeabilization, the cells were stained with IFN-γ, TNF-α, and IL-2 antibodies. To detect degranulation, CD107a and CD107b antibodies were added during stimulation. Cells were analyzed on a Canto II or LSR II flow cytometer (BD Immunocytometry Systems). Dead cells were removed by gating on Live/Dead Aqua (Invitrogen), and data were analyzed with FlowJo v.8.8 (TreeStar).

Cytokine bead array analysis. Leukocytes from tumor, spleen, and lymph nodes from mice treated with αPD-1, αPD-L1, αCTLA-4, or isotype antibodies were cultured directly *ex vivo* for 48h. Then the supernatants were collected and cytokines (IFN-γ, TNF-α, IL-6, IL-10, and IL-12p70) were measured using inflammation kit (BD Biosciences) by

cytokine bead array analysis, and TGF-β and IL-10 was measured by ELISA (BD Biosciences).

***in vitro* Treg suppression assay.** Treg suppression was also determined by ^{3}[H]-thymidine incorporation assays using a scintillation counter after 3 days of culture, pulsing with 0.5 μCi/well [^{3}H] thymidine during the last 8 h.

In other experiments, $CD4^{+}CD25^{hi}$ ($CD4^{+}$Treg), $CD4^{+}CD25^{lo}$ ($CD4^{+}$non-Treg) cells, or $CD8^{+}$ T cells were isolated from TILs of CT26 mice by MACS column selection or FACS sorting. Similarly $CD11c^{+}$ lymphocytes were purified from spleens of naïve wild-type mice. Purified cells were pre-treated with αPD-1, αPD-L1, or isotype control IgG1/IgG2a antibodies at 37°C, 5% CO2 for 4h, as described previously (35). After extensive washing to remove unbound antibodies, the cells were labeled with 1 mM CFSE. T and NK cell-depleted splenocytes from wild-type mice were used as APCs. $CD8^{+}$ T cells sorted from spleens of naïve wild-type mice were used as responder cells. A total of 5 x 10^{4} CFSE-labeled $CD8^{+}$ T responder cells were stimulated with 1.5 x 10^{5} APCs and 1 μg/ml purified αCD3. Where indicated, 10^{5} pretreated (with αPD-1/αPD-L1) Tregs were added to the culture. Four days later, cells were harvested and proliferation was determined by CFSE dilution. Cell supernatant was harvested to test for secretion of IFN-γ.

To determine if reactions with Tregs are mediated by direct contact, a transwell was added to the well containing effector and stimulator cells. Tregs were either in direct contact (C) with the effector cells or were separated by a transwell membrane (T). [^{3}H]-thymidine incorporation into effector T cells in the presence or absence of Tregs.

Phospho-signaling using flow cytometry. Phosphoflow staining was done as described (50-51). Lymphocytes were isolated from tumor and stained with phospho-antibodies. Following surface staining with lineage markers, purified $CD8^+$ T cells were fixed with 2% paraformaldehyde for 10 min at 37°C. After fixation, plates were prechilled on ice for 1 min before slowly adding ice-cold methanol to a final concentration of 90% methanol to permeabilize. Cells were then incubated on ice for 30 min and washed with 1% FCS/PBS buffer. Cells were then blocked with 10% FCS/PBS for 10 min at room temperature and subsequently stained with the phospho-antibodies for 1h at room temperature. After incubation, cells were washed four times with FACS buffer, resuspended in PBS, and analyzed on a flow cytometer.

Statistical analysis. Data were analyzed using Prism 5.0 software (GraphPad). Experiments were repeated at least 3-5 times. The data presenting the differences between the groups were assessed using two-tailed unpaired Student t-tests. $p<0.05$ indicated that the value of the test sample was significantly different from that of the relevant controls.

Supplementary Figure Legends

Supplementary Figure 1. Splenic T cells in CT26-bearing mice express low levels of PD-1 or CTLA-4. Representative and summary data showing the frequency of PD-1 and CTLA-4 expression on $CD8^+$ (A) and $CD4^+$ (B) T cells from spleen of CT26 tumor bearing mice.

Supplementary Figure 2. CT26 and ID8-VEGF tumors upregulates PD-1 and CTLA-4 ligands on tumor and myeloid cells. Expression of PD-L1, PD-L2 and B7.1 on $EpCAM^+$ tumor cells and tumor infiltrating $CD45^+$ myeloid cells of CT26 (A) and ID8-VEGF (B) tumors.

Supplementary Figure 3. *in vivo* depletion of total $CD8^+$ and $CD4^+$ T cells totally abolished CT26 tumor regression. Survival of mice inoculated with 0.5 x 10^6 CT26 tumor cells and injected i.p. with αCD4 (GK1.5, 500 μg) or αCD8 antibodies (19/178, 500 μg) day -1 and 0 (resulting in >90% of $CD4^+$ or $CD8^+$ T-cell depletion, respectively). Tumor size of >200mm^3 was defined as lack of survival. Each curve is representative of 3 independent experiments of 12 mice per group.

Supplementary Figure 4. Combining αCD25 antibody with PD-1 and CTLA-4 blocking antibodies did not show additional benefit in tumor rejection. Survival of mice inoculated with 0.5 x 10^6 CT26 tumor cells and injected i.p. αCD25 antibody (PC61, 250 μg) 3 days following tumor inoculation. Tumor size of >200mm^3 was defined as lack of survival. Each curve is representative of 3 independent experiments of 12 mice per group (ns, p value not significant).

Supplementary Figure 5. Lymphocyte infiltration into the tumor and lymph nodes of CT26 mice. BALB/c mice were subcutaneously injected with 0.5 x 10^6 CT26 tumor cells. The mice were treated i.p. with αPD-L1 and αCTLA-4 in combination on days 3, 6, 9 after tumor inoculation. TIL were analyzed by flow cytometry on day 15. Absolute numbers of $CD4^+CD25^+Foxp3^+$ Tregs (i), $CD8^+$ T cells (ii), and $CD4^+$ Teffs (iii) per pool of lymph nodes from 12 mice (A) or per cubic millimeter of tumor (B) are shown. All data show $p<0.001$ according to the Mann-Whitney test.