Supplementary Information

Accumulation of Memory Precursor CD8 T Cells in Regressing Tumors Following Combination Therapy with Vaccine and Anti-PD-1 Antibody
Supplemental Materials and Methods

**Antibodies and adjuvants:** Monoclonal antibodies (mAbs) used for *in vivo* cell depletion (Anti-CD4, clone GK1.5 and anti-CD8, clone 2.43) and purified anti-PD-1 antibody (G4 clone) were obtained from the Antibody Core Facility at Mayo Clinic (Rochester, MN). Hamster IgG (isotype control) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Complete Freund’s Adjuvant (CFA) and Incomplete Freund’s Adjuvant (IFA) were purchased from Sigma-Aldrich (St. Louis, MO.).

**Flow cytometry:** All the staining antibodies for flow cytometry were obtained from eBioscience except the following CD62L-PE, CD8-APCCy7, CD8-PerCP, CD4-PE (BD pharmeden); and Tbet-APC (Biolegend). For serum antibody analysis, cultured TUBO cells were harvested and incubated at 4°C for 30 min with 1:100 dilution of primary antibody (from different groups of mice) and washed three times, followed by secondary labeling with anti-mouse IgG FITC (eBioscience) at 4°C for 30 min. For the analysis of neu expression on TUBO cells grown *in vivo*, TUBO cells were harvested from different groups of tumor-bearing animals. Staining for surface neu expression was performed using 7.16.4. anti-neu antibody followed by FITC conjugated rat anti-mouse IgG or with FITC-conjugated rat anti-mouse IgG alone. Samples were run on a BD LSR II and analyzed using flowjo software.

**Intracellular cytokine staining:** For intracellular cytokine staining, tumor infiltrating lymphocytes (TILs) were obtained from tumors (35-38 days post tumor implantation) using lympholyte M gradient. TILs were stimulated with peptide pulsed P815 cells for 8 hrs in the presence of golgistop (BD biosciences) and then fixed and stained for intracellular cytokine production using Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences). For
intracellular interleukin-12 (IL-12) staining TILs were obtained as described above. These TILs were stained for surface CD11c, CD45 and then fixed and stained directly (i.e. without *in vitro* stimulation) for intracellular IL-12 using the Cytofix/Cytoperm Fixation/Permeabilization kit.

**Serum MCP-1 Enzyme-linked Immunosorbent assay (ELISA).** Serum MCP-1 was detected using sandwich ELISA that was performed using mouse CCL2 (MCP-1) ELISA Ready-SET-Go kit (Affymetrix, Inc. San Diego, CA) as per the manufacturer’s instructions. Briefly, plates were coated with 100μl/well of purified anti-mouse CCL2 (MCP-1) antibody and incubated overnight at 4°C. Following this, plates were washed and blocked at room temperature (RT) for 1 hour using ELISA diluent. After blocking and washing, mouse sera that was collected on day 35 post tumor challenge from different groups were added at a 1:10 dilution (100μl/well) in duplicates and incubated for overnight at 4°C followed by incubation for 1 hour at RT using biotinylated anti-mouse CCL2 antibody. After washing, 100μl/well of Avidin-HRP was added and plates were incubated at RT for 30 mins. The plates were developed using tetramethylbenzadine (TMB), which was stopped by addition of 1M phosphoric acid. The plates were read at 450nm on a Spectra Max Plus 384 (MDS Analytical Technologies, Sunnyvale CA). Standard curves prepared using recombinant mouse CCL2 (MCP-1) protein that was provided with the kit, were used to convert the optical density signal into estimated serum MCP-1 concentration.

**In vitro blockade of PD-1 on TUBO cells.** The effect of blockade of PD-1 on the growth of TUBO cells was determined in *in vitro* culture experiment. TUBO cells were seeded on a six well plate at a concentration of 1x10⁶ cells/well. Cells were allowed to settle for an hour and were then treated with 10μg/mL Syrian Hamster IgG or 10μg/mL anti-PD1 Ab or left untreated
for next 24 hours. After incubation, cells were harvested using Trypsin-EDTA, pelleted, and counted on a hemocytometer.
Fig. S1. *In vitro* blockade of PD-1 on TUBO cells does not inhibit cell growth. TUBO cells cultured in 6-well plate were treated with different conditions (anti-PD1 ab, isotype ab and no treatment) and effect of PD-1 blockade on tumor cell growth was determined. Shown is the data representative of number of TUBO cells recovered from each well after culturing for 24 hours under different conditions. Data is shown as mean (±s.e.m) and representative of three replicates.
Fig. S2. **CD4 T cells and CD4 Tregs in TUBO TILs.** Tumors from different treatment groups were harvested on day 35-38 post tumor implantation, tumor-infiltrating lymphocytes (TILs) were isolated from these tumors as described in materials and methods. TILs were stained for surface markers CD45, CD4, CD25 and intracellular Foxp3 (FoxP3 staining kit). Shown are the mean (± s.e.m, n=4) levels of tumor-infiltrating CD4 T cells (A) and CD4 Tregs (B) in treated and control animals. Data is representative of four independent experiments.
**Supplementary Figure S3**

**A**

![Graph A](image)

**B**

![Graph B](image)

**Fig. S3. Combination therapy enhances the antigen-specific function of tumor-infiltrating lymphocytes.** TILs responses against β-catenin (A) and legumain (B) were assessed by intracellular cytokine staining for IFN-γ. TILs were harvested from the tumors of mice between 35-38 days following tumor challenge, stimulated _ex vivo_ and stained for flow cytometry analysis. Shown are the means of the (± s.e.m) six samples pooled from 3 independent experiments.
**Fig. S4. Antigen-specific production IL-5 and IL-4 by tumor-infiltrating CD8 T cells.** The cytokines IL-5 (A) and IL-4 (B) accumulated in the supernatant of CD8 TILs stimulated with P815 cells pulsed with N1 peptide at 48hrs were measured using a multiplexed cytokine assay. Shown are the means (± s.e.m) of duplicate samples pooled from 3 mice. Data shown are the representative of one of three experiments with similar results.
Fig. S5. Combination therapy decreased the number of tumor-infiltrating myeloid derived suppressor cells (MDSCs). MDSCs in tumors of different treatment groups were analyzed. (A) shown are the cytometry dot plots (gated on R1) representative of tumor-infiltrating MDSCs (CD11b+Gr-1+) in the different treatment groups. Quadrants were established with isotype controls and the inset values are the percent of R1 gated lymphocytes that fall in that quadrant. Dot plots shown are the representative of one of three separate experiments with similar results. (B) Shown are the mean numbers of tumor-infiltrating MDSCs of total leukocytes in different treatment groups. Data is shown as mean (± s.e.m) and is from three independent experiments.
Fig S6. **PD-1 expression on tumor DCs and splenic CD8 T cells in co-culture:** Tumor DCs (CD11c^+ cells) and purified splenic CD8 T cells from *in vitro* co-culture experiment were analyzed for the expression of PD-1 by staining the cells obtained from co-culture with anti-mouse CD11c-APC, anti-mouse CD8-PerCP and anti-mouse PD1-PE antibodies. Shown is the histogram representative of PD-1 expression on tumor-derived CD11c^+ cells and purified splenic CD8 T cells compared to isotype controls. Data shown is the representative of one of the three experiments with similar results.