Supplemental Materials and Methods

Abs, cell lines, and reagent:

Armenian hamster antibody specific for murine VISTA (clone 13F3) was generated as described before (19). Polyclonal hamster control IgG was from BioXCell (Lebanon, NH). Antibodies αCD3 (2C11), αCD28 (PV-1), αCD4 (GK1.5), αCD8 (53-6.7), αCD11b (M1/70), αF4/80 (BM8), αCD11c (N418), αNK1.1 (PK136), αGr1 (RB6-8C5) were purchased from Ebioscience. LPS (Sigma), recombinant murine IFN-γ (Peprotech), human IL-2 (Peprotech), murine GM-CSF (Peprotech) were used at indicated concentrations. Melanoma cell line B16BL6 was as described (3). B16F10 expressing chicken ovalbumin (B16OVA) was generated as described before (9). Bladder tumor MB49 was obtained from Dr. Matzinger (21).

In vitro generation of induced Tregs (iTregs) and analysis of their suppressive function

Naïve CD4+ T cells ($CD25^{-}Foxp3GFP^{-}CD62L^{hi}$) were sorted from naïve splenocytes and simulated with plate-bound anti-CD3 (2C11, 2.5 μg/mL), recombinant human TGFβ (2 ng/mL) , 50 units/ml human IL-2 (PeproTech), in the presence of either plate-bound control protein or VISTA-Ig fusion protein (5 μg/mL), in RPMI medium 1640 supplemented with 10% FBS, 10 mM Hepes, 50 μM 2-mercaptoethanol, penicillin/streptomycin/l-glutamine. $Foxp3GFP^{+}$ induced Tregs (iTregs) were sorted on day+5 based on GFP expression, and their suppressive function was examined in a suppression assay (9). Briefly, congeneically mismatched naïve CD4 responder T cells were CFSE-labeled and stimulated with irradiated spleen APCs (2000 rads), polyclonal stimuli (anti-CD3, 5 μg/mL) in the presence of graded numbers of $Foxp3GFP^{+}$ iTregs. CFSE dilution profile was analyzed after 3 days by flow cytometry.

Tumor-mediated induction of iTregs

Assay was as described previously (9). Briefly, congeneically-marked naïve OTII $CD4^+$ T cells ($Foxp3GFP^-$ $Vbeta\text{-}5^+$ $CD25^-$ $CD62L^{hi}$) were isolated from naïve mice using $CD4^+$ T cell isolation kit (Miltenyi) and flow sorted. Cells (500,000) were adoptively transferred into B16OVA tumor-bearing mice that were sub-lethally irradiated (300rads). Mice were treated with VISTA mab (clone 13F3) or hamster control IgG every 2 days from day 0 for the entire duration of the experiment. Mice were analyzed when tumors reached 9-10 mm diameter. Cells from tumor-draining lymph node and tumor tissue were harvested and examined for the conversion of naïve OTII cells into $Foxp3GFP^+$ iTregs by flow cytometry.

Generation of bone-marrow derived DCs

Bone marrow derived DCs were differentiated as described (Lutz et al., 1999; Son et al., 2002) with some modifications. Briefly, on day 0, bone marrow cells were isolated from tibia and femur by flushing with 27G needle. After red blood cell lysis, $1\text{-}2\times10^6$ bone-marrow cells were resuspended in 1 mL complete RPMI 1640 medium containing 10 ng/mL recombinant murine GM-CSF and 10 ng/mL IL4 (Peprotech). On day+2, the floating cells were gently aspirated off and medium was refreshed. Cells were cultured for a total of 8 days, with medium refreshed on day+4 and day +6. On day +8, loosely adherent cells (70-80% CD11c+) were collected and were further maturated in the presence of 10 μg/mL LPS (Sigma) for 5-6 hrs before used as APCs.

Tumor inoculation, vaccine administration, and analysis of tumor infiltrating leukocytes

MB49 (300,000), B16OVA (120,000), and B16BL6 (18,000) tumor cells were inoculated on the right flank of female mice. For the PTEN/BRAF melanoma model, tumors were induced by intradermal injection of 10μl tamoxifen (10 μl dissolved in DMSO) on the lower back. Tumor size was measured by a caliper every 2-3 days. For MB49, B16OVA and B16BL6 tumors, due to the relative uniform shape and height of these transplantable tumors, tumor size is calculated by

multiplying the two perpendicular diameters (length x width) and shown as mm^2. For the PTEN/BRAF inducible melanoma model, tumors typically grew with irregular and diverse height, therefore tumor size was measured as height x length x width and shown as mm^3 .

For prophylactic anti-VISTA mab treatment, mice were treated every 2 days with 300 μg antibody subcutaneously, starting on day 0 for the entire duration of the experiment. For therapeutic treatment, mice were treated with subcutaneous injection of 300μg mab every day for 10 days on day+2 or day+7 post tumor inoculation, followed by continuous antibody injection every 2 days for the entire duration of the experiment.

Tumor vaccine consisted of CD40 agonistic antibody FGK (100μg), LPS (30μg), polyI:C (100μg), CpG (ODN1826, 30μg), Gardiquimod (30μg), tumor antigen peptide TRP1 (106-130) (100μg) and a mutated TRP2 peptide DeltaV-TRP2 (180-188) (100μg) (22-24). For treating 2-day tumors, 1x dose of vaccine mixture was applied with split injections of intraperitoneal injection and subcutaneous injection on the opposite flank of the tumor site. A prime-boost vaccine regime was applied for treating 7-day established B16 melanoma. Briefly, vaccine mixture was applied on day+7 and day+14, each with split dose injection at 2 sites on the back of the mice. To analyze tumor-infiltrating T cells upon vaccine/anti-VISTA combination treatment, tumor-bearing mice were treated on day+7 with vaccine, or 13F3, or the combination. Half vaccine dosage was applied to allow partial response instead of complete tumor rejection. Tumors from all groups were harvested for analysis around 2 weeks post treatment.

For analysis of tumor-infiltrating leukocyte (TILs) populations, tumors were typically harvested when the control-Ig treated group reached a diameter of 8-10 mm, which correlated with ~18-20 days post tumor inoculation in B16OVA and B16BL6 tumor models, or 25-30 days post tamoxifen induction in the PTEN/BRAF model. Tumors were digested with liberase (0.4 mg/mL)

and Dnase (0.2 mg/mL) for 20 min at 37C. Tumor tissues were mashed with a syringe through 70 um cell strainers. An aliquot of the single cell suspensions was analyzed directly by flow cytometry for the presence of TILs based on markers CD45, CD11b, Gr1, CD11c, CD4 and CD8.

For analyzing TRP1 transgenic $CD4^+$ T cells in B16 tumor-bearing mice, mice were inoculated with 40,000 B16 tumor cells and were treated with control-Ig or VISTA mab (300 μg) subcutaneously on the same flank as the tumor site every 2 days starting from day +1. On day+7 when tumors reached ~4mm diameter, purified TRP1 cells (25,000) were adoptively transferred into sub-lethally irradiated (300rads) tumor-bearing mice. Mice were continuously treated with antibodies and the presence of TRP1 cells in tumor tissue was analyzed on day+10 post adoptive transfer.

For TIL cytokine analysis in the B16OVA model, TILs were enriched via a 40%-80% percoll gradient (run 1500 rpm for 20 min with no brake), and harvested from the middle layer of the gradient. TILs were re-stimulated overnight in complete IMDM medium with 10% FBS, 2 mM L-glutamine, 50uM 2-mercaptoethanol, 1% penicillin-streptavidin, 1 mM nonessential amino acids, human IL2 (5 units/mL Peprotech), 1x monensin (ebioscience), 1x brefeldin A (biolegend), OVA peptide OVA323-339 (10 μg/mL) and OVA257-264 (2.5 μg/mL) (Anaspec).

For TIL cytokine analysis in the B16BL6 model, TILs were enriched via percoll gradient as described above. TILs were re-stimulated overnight in complete IMDM medium supplemented with supplemented with 10% FBS, 2 mM L-glutamine, 50uM 2-mercaptoethanol, 1% penicillin-streptavidin, 1 mM nonessential amino acids, human IL2 (5 units/mL Peprotech), 1x monensin (ebioscience), 1x brefeldin A (biolegend), tumor specific antigen peptide TRP1 (5μg/mL), gp100 (1 μg/mL), TRP2 (1μg/mL), and 150,000 LPS-matured BMDCs. TRP-2 peptide sequence is

H2N-SVYDFFVWL-OH; TRP-1 peptide sequence is SGHNCGTCRPGWRGAACNQKILTVR; mouse gp100 peptide sequence is H2N-EGSRNQDWL-OH.

For CD107 staining, both CD107a-FITC and CD107b-FITC (ebioscience) were added to the medium during overnight culture. For intracellular cytokine analysis, TILs were harvested after overnight culture, and stained with surface lineage markers such as CD4 and CD8. Cells were fixed in the presence of 1% formaldehyde for 20 min at RT, and then permeabilized with 0.5% saponin buffer for 10min at RT. Cells were then stained with antibodies specific for IFNγ and granzyme B.

For analyzing cytokine production (i.e. IL12p40 and TNFα) in tumor-infiltrating DCs, tumors of 6-7 mm size were harvested typically around day 10-12 for MB49 and day 14-16 for B16 tumors, and digested into single cell suspensions as described above. TILs were enriched via percoll gradient, and cultured for 16hrs in complete IMDM medium in the presence of 1x monensin/brefeldin A as described above, supplemented with 1ng/mL GM-CSF. Cells were then harvested and analyzed by flow cytometry.