Supplemental Methods

Drugs

The MEK inhibitor trametinib (GSK1120212) and BRAF inhibitor vemurafenib were purchased from MedChem Express (Monmouth Junction, NJ). Both drugs were dissolved in DMSO to make a 10-mM stock solution for *in vitro* studies. The highest DMSO concentration (0.001% DMSO) used for *in vitro* studies was non-toxic to the cells. For *in vivo* studies, trametinib powder (0.5 mg/kg) was dissolved in 0.5% hydroxypropyl methylcellulose and 0.2% Tween-80 (Sigma Aldrich) to make a homogenous suspension and was administered by oral gavage.

Cytotoxicity and Viral Plaque Assays

Human and murine melanoma cells (7.5×10^3) were seeded on 96-well plates, treated with vehicle or MEKi (trametinib) (10 nM for SK-MEL-28, 5 nM for SK-MEL:5, 1.25 nM for SK-MEL-2, and 5 nM for D4M3A). Six to eight hours later, cells were treated with T-VEC at the indicated MOI. After 5 days (SK-MEL-28, SKMEL:5, SK-MEL-2) or 3 days (D4M3A) of incubation, MTS assay was performed following manufacturer's instruction (Promega)**.** For plaque assays, cells were plated and treated with T-VEC alone or T-VEC and trametinib as above. Cells were treated with trametinib 6-8 h before T-VEC infection. For T-VEC infection, the virus was diluted using RPMI and seeded over a cell monolayer at the indicated MOI for 2 hours (plates were gently rocked every 15 min to ensure even spread of virus). Whole cell lysates were collected at indicated times and viral titers obtained by plaque assay on a monolayer of Vero cells. Each experimental condition was performed in triplicate and all experiments were conducted three times.

Immunoblotting

Total cell lysates were obtained from human and murine melanoma cells and 40 µg of lysate was loaded onto an SDS-PAGE gel, electrophoresed, and transferred to a PVDF membrane. Antibodies against HSV-1 glycoprotein D (gD) (1:50) (Genetex), cleaved PARP (1:50), total ERK1/2 (1:100) and phospho ERK1/2 (1:100) (Cell Signaling Technologies) were used.

Lumacyte Analysis

SK-MEL-28 cells were seeded into 24-well plates at a density of 500,000 cells/mL and infected with T-VEC or treated with trametinib or both as described above. At the specified time points, cells were detached from the wells using TrypLE (Thermo Fisher Scientific), resuspended in culture medium and then analyzed using a Radiance instrument (LumaCyte). The threshold velocity (which in this case defines the infection metric), was calculated based on the velocity at which approximately 5% of the control cells have a velocity above the infection metric. This is a similar to gating for fluorescence in flow cytometry. A principal component analysis (PCA) was performed using XLStat. Input data included the infection metric, average velocity, and average size of each sample. This combination resulted in components (F1 and F2) that represented the largest possible variance in the data.

Murine treatment studies

All animal experiments were approved by Rutgers Institutional Animal Care and Usage Committee. For survival experiments mice were monitored for tumor-growth and euthanized before tumors reached 400 mm^2. Kaplan-Meier curves were used to calculate survival. Mice were weighed twice a week and no weight loss was observed during the treatment.

For xenograft melanoma models, SK-MEL-28 cells (8×10^6) were injected into the right flank of NSG mice in 100 µl PBS. Mice were treated with either mT-VEC (1×10^5 pfu) or sterile water via intratumoral injection on days 35, 40, and 45. Trametinib (MEKi; 0.5 mg/kg) or vehicle control was given on days 35-43 via oral gavage. Mice in the combination treatment group received both mT-VEC and MEKi at the above doses and schedule. The vehicle control consisted of a mixture of 0.2% Tween 80 and 0.5% hydroxypropyl methylcellulose (HPMC).

For syngeneic melanoma tumor studies, C57BL/6J mice (n = 9/group) were implanted s.c. with 3×10^5 D4M3A murine melanoma cells in the right flank on day 0 and treated with mT-VEC (1×10^6 pfu) or sterile water via i.t. injection on days 15, 19, 22, 26, 29 and 33, and MEKi (0.5 mg/kg) or vehicle (0.2% Tween 80 and 0.5% HPMC was gavaged from days 15-27. For tumor re-challenge studies in mT-VEC and MEKi studies, cured mice (n = 7 from 2 independent experiments) from the combination (mT-VEC + MEKi) group were re-challenged on day 96 with a two-fold increased number of D4M3A cells (6×10^5) in the contralateral (left) flank. Age matched (21 to 22-week-old) naïve mice were implanted as controls (n = 7).

For flow cytometry analysis of tumors from T-VEC and MEKi combination therapy, C57BL/6J mice implanted s.c. with 3×10^5 D4M3A cells on day 0, treated with T-VEC (1×10^6 pfu) or sterile water on days 15, 19 and 22 and MEKi (trametinib; 0.5 mg/kg) or vehicle gavaged orally once daily from days 15-19 (n = 5/group), and euthanized on day 24. Tumors were harvested and FACS was performed as described in the flow cytometry analysis section of methods. Mice that had completely regressed primary tumors and survived long-term were re-challenged with an increased number of D4M3A cells (6×10^5) in the contralateral flank (left) at day 96. For $Batf3^{-/-}$ mouse studies $Batf3^{-/-}$ or C57BL/6J mice were implanted with (3×10^5) D4M3A cells on day 0 and treated with mT-VEC or sterile water via i.t. injection and/or trametinib or vehicle control via oral gavage.

For triple combination studies using mT-VEC + MEKi + αPD-1 antibody, C57BL/6J mice (n = 7/group) were implanted subcutaneously in the right flank with 3×10^5 D4M3A murine melanoma cells on day 0 and treated with mT-VEC (1 x 106 pfu) or sterile water via intratumoral injection on days 15, 19, 22, 26, 29 and 33, MEKi (0.5 mg/kg) or vehicle control on days 15-27 via oral gavage and αPD-1 antibody (clone: RMP1-14, 10 mg/kg) via intra-peritoneal (i.p) injection on days 15, 19, 22, and 26. In addition, groups received doublet combination treatment with mT-VEC and MEKi (and rat IgG isotype control) or triplet therapy with m-TVEC, MEKi and αPD-1 antibody. For re-challenge experiments during triple combination, cured mice (n = 10 from 2 independent experiments) from mT-VEC + MEKi + αPD-1 therapy were re-challenged on day 130 with a two-fold increased number of D4M3A cells (6×10^5) in the contralateral (left) flank. Age matched (26 to 28-week-old) naïve mice were implanted as controls (n = 5). For flow cytometry studies during triple combination, B6 mice (n=6 per group) were implanted subcutaneously in the

right flank with D4M3A cells (3 x 105) on day 0 and treated with mT-VEC ($1x10^6$ pfu) or sterile water via intratumoral injection on days 15, 19 and 22, MEKi (0.5 mg/kg) or vehicle control on days 15-19 via oral gavage and αPD-1 antibody (clone: RMP1-14, 10 mg/kg) via i.p injection on days 15, 19 and 22. In addition, groups received double combination treatment with mT-VEC and MEKi (and rat IgG isotype control) or triple. Tumors were collected on day 24 and flow cytometry analysis of tumor infiltrating lymphocytes was performed as described in supplemental methods.

For triple combination studies in CT26 tumor model, BALB/c mice (n=10 per group) were implanted s.c. in the right flank with CT-26 cells (2×10^5) on day 0 and treated with mT-VEC ($5x10^5$ pfu) or sterile water via i.t. injection on days 8, 12, 15, 19 and 22, MEKi (0.5 mg/kg) or vehicle control on days 8-20 via oral gavage and αPD-1 antibody (clone: RMP1-14, 7.5 mg/kg) via i.p. injection on days 8, 12, 15 and 19. In addition, groups received double treatment with mT-VEC + αPD-1, MEKi + αPD-1, mT-VEC + MEKi and rat IgG isotype control or triple therapy with m-TVEC, MEKi and αPD-1 antibody.

Immune Cell Depletion Studies

For depletion studies, C57BL/6J mice (n = 5/group) were implanted s.c. with 3×10^5 D4M3A murine melanoma cells in the right flank on day 0 and treated with T-VEC (1×10^6 pfu) or sterile water via i.t. injection on days 15, 19, 22, 26, 29 and 33 and MEKi (trametinib; 0.5 mg/kg) or vehicle (0.2% Tween 80 and 0.5% HPMC) gavaged from days 15-27. For depletion of immune cell populations, mice administered via intraperitoneal (i.p.) injection anti-mouse CD8α (clone 2.43; 10 mg/kg), anti-mouse CD4 (clone GK1.5; 10 mg/kg), or clodronate liposomes (first injection 50 mg/kg, followed by 25 mg/kg) on days 12, 15, 18, 21, 25, 28, and 32. Mock group received sterile water (i.t.) + vehicle (0.2% Tween 80 and 0.5% HPMC) + control rat IgG (i.p.) + empty liposomes (i.p.). Isotype group received T-VEC (i.t.) + MEKi (i.p.) + control rat IgG (i.p.) + empty liposomes (i.p.) as above. Anti-CD4 or anti-CD8 group received T-VEC + MEKi + anti-CD4 or anti-CD8 + empty liposomes, whereas Clodronate group received T-VEC + MEKi + control rat IgG + clodronate liposomes.

For flow cytometry analysis in depletion studies C57BL/6J mice implanted s.c. with 3×10^3 D4M3A murine melanoma cells in the right flank on day 0, treated with mT-VEC ($1x10^6$ pfu) or sterile water administered via i.t. injection on days 15, 19, and 22 and MEKi (trametinib; 0.5 mg/kg) or vehicle was gavaged from days 15-19. For depletion of immune cell populations, mice were injected i.p. with anti-mouse CD8α (clone 2.43; 10 mg/kg), anti-mouse CD4 (clone GK1.5; 10 mg/kg), or clodronate liposomes (first injection 50 mg/kg, followed by 25 mg/kg) on days 12, 15, 18, and 21. Tumors were harvested on day 24 and flow cytometry was performed as described in flow cytometry section of methods.

Immunohistochemistry

Tumors were harvested at indicated time points and sections were deparaffinized using Xylene twice for 10 min each, followed by gradual rehydration using 100%, 90% and 70% ethanol treatment (5 min each). Sections were left in distilled water for 10 min, followed by dipping sections in a hematoxylin container for 1 min, washing in tap water for 5 min, dipping in Eosin Y

(1% alcoholic) for 30 s. This was followed by gradual dehydration using 95% ethanol (twice 5 min each) and 100% ethanol (twice 5 min each), treating with Xylene twice for 10 min each, and mounting in Xylene-based media (Cytoseal XYL; Thermo Scientific).

To examine proliferation (Ki67 and pERK1/2), apoptosis (cleaved caspase 3), and T-VEC prevalence (HSV-1 gB) in the SK-MEL-28 xenograft model, NSG mice (n=5) were implanted with human melanoma SK-MEL-28 cells (8 x 10^6) on day 0 and treated with either T-VEC (5 x 10^5 pfu) or sterile water via i.t. injection on days 30 and 34 or trametinib (0.5 mg/kg) or vehicle control on days 30-34 via oral gavage. Mice in the combination group received both T-VEC and trametinib. Animals were euthanized on day 36 and tumors were removed and fixed in 10% formalin for 24-36 h, embedded in paraffin, and 5 μm-sections subjected to immunohistochemistry with indicated antibodies (Key Resources Table, IHC), followed by incubation with appropriate secondary antibodies (Vector Laboratories).

To examine the presence of CD8+ T cells in syngeneic C57BL/6J mice treated with combination (mT-VEC + trametinib) therapy, C57BL/6J mice were implanted with 3 x 10^5 D4M3A cells and treated with mT-VEC (10^6 pfu i.t.) for 3 doses on days 15, 19 and 22 and/or trametinib (0.5 mg/kg) orally once daily on days 15-19. Mice were euthanized on day 24 and tumors were removed and fixed in 10% formalin for 24-36 h, embedded in paraffin, and 5 μm-sections subjected to immunohistochemistry with indicated antibodies (Key Resources Table, IHC), followed by incubation with appropriate secondary antibodies (Vector Laboratories) as described above. For positive cell counting, annotated whole tumor regions were subjected to unsupervised quantification using Visio Pharm quantitative digital pathology software. Positive cell density was computed as positive cell count / mm^2 tissue area for cleaved caspase 3, Ki67, pERK1/2 and CD8. HSV-1 staining was quantified as an average brown staining intensity of HSV-1 glycoprotein (gB) over the tumor area.

Flow Cytometry Analysis

Annexin-V expression was detected on SK-MEL-28 cells after culture for 24 h with or without T-VEC at the indicated MOI and/or trametinib at 5 nM. Cells were centrifuged, counted, re-suspended in FACS buffer (2% inactivated fetal calf serum in PBS), incubated with 7-AAD (BD Biosciences) and FITC-conjugated antibody for 30 min, washed, fixed in 4% paraformaldehyde, washed, re-suspended in FACS buffer, and analyzed using an LSRII flow cytometer (BD Biosciences) and FlowJo software (v.10.4; Tree Star).

For 10-color flow cytometry analyses, flank tumors from treated groups were harvested, mechanically dissociated using a gentleMacs Octo Dissociator (Miltenyi), incubated with collagenase (1 mg/ml, Sigma Aldrich) and DNase I (10 U/ml; Promega) for 30 minutes with rocking at 37°C, mechanically dissociated again, passed through a 40 μm screen, re-suspended in FACS buffer, and stained with fluorochrome-conjugated anti-mouse antibodies, as well as appropriate isotype control antibodies. Fixable live/dead viability Kit (Invitrogen) was used to stain dead cells. We followed a ‘no-wash' sequential staining protocol (BioLegend) to stain dead cells and for surface staining. Intracellular FoxP3 staining was performed following the FoxP3 intracellular staining protocol (BioLegend). For antigen-specific CD8+ T cell determination, tumor samples were stained for 45 min at 4°C with fluorochrome-conjugated MHC-I dextramers

(Immudex) for HSV-1 gB, murine gp100 or murine TRP2 prior to extra-cellular staining and all other steps were followed according to manufacturer's guidelines.

For single-color compensation controls, spleens from naïve C57BL/6J mice were treated with ACK Lysis Buffer (Sigma Aldrich) to lyse red blood cells, and single cells were stained with each of ten fluorescent-conjugated antibodies according to manufacturer's instructions. All samples were analyzed using a BD LSRII flow cytometer. Data were analyzed with FlowJo software (v.10.4; Tree Star). Technicians acquiring and analyzing the data were blinded to the treatments.

Gene Signature Profiles

C57BL/6J mice implanted with D4M3A cells (3×10^5) were treated with mT-VEC (10^6 pfu) via i.t. injection for 3 doses on days 15, 19 and 22 and/or trametinib (0.5 mg/kg) orally once daily on days 15-19. Tumors were harvested on day 24 and total RNA was isolated using a Qiagen RNAeasy kit. Gene expression analysis was performed using the NanoString PanCancer Immune panel. Per sample, 50 ng of total RNA in a final volume of 5 µl was mixed with a 3′ biotinylated capture probe and a 5′ reporter probe tagged with a fluorescent barcode from the custom gene expression code set. Probes and target transcripts were hybridized at 65°C for 12-16 h. Hybridized samples were run on the NanoString nCounter preparation station using the recommended manufacturer protocol, in which excess capture and reporter probes were removed and transcript-specific ternary complexes were immobilized on a streptavidin-coated cartridge. The samples were scanned at maximum scan resolution on the nCounter Digital Analyzer. Data were processed using nSolver Analysis Software and the nCounter Advanced Analysis module. For gene expression analysis data were normalized using the geometric mean of housekeeping genes selected by the GeNorm algorithm.

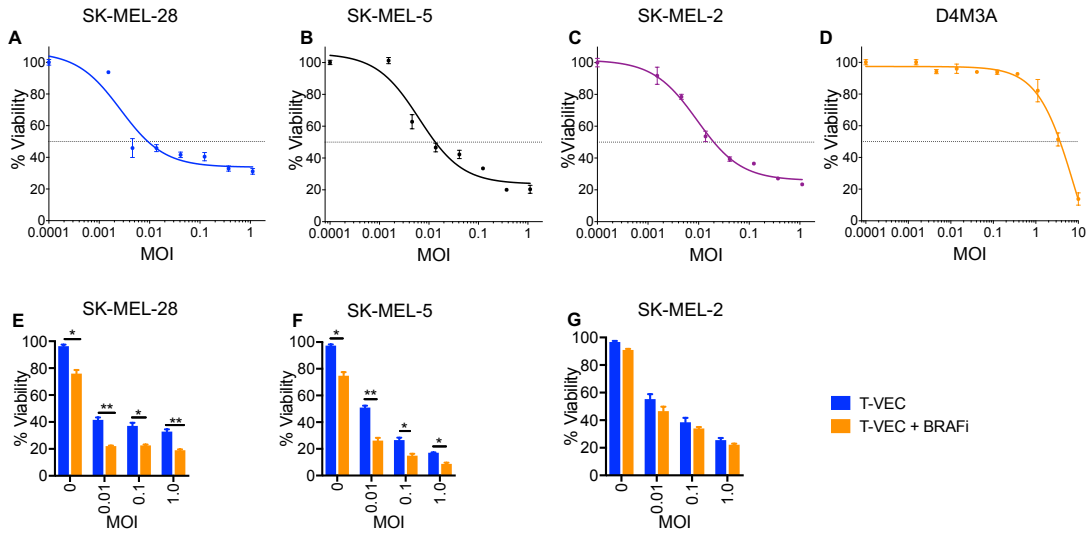

Figure S1. BRAF inhibitors enhance T-VEC cell killing in BRAF mutant melanoma cell lines

Cytotoxic effects of T-VEC in human **(A)** SK-MEL-28, **(B)** SK-MEL-5, **(C)** SK-MEL-2 and mouse **(D)** D4M3A melanoma cell lines. Cells (7.5 x 10^3) were seeded on 96-well plates and treated with T-VEC at the indicated MOI or control (sterile water). After 5 days (SK-MEL-28, SK-MEL-5, SK-MEL-2) or 3 days (D4M3A) of incubation, an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed to measure cell viability. **(E-G)** Cytotoxic effects of T-VEC and vemurafenib (BRAF inhibitor, BRAFi) in human **(E)** SK-MEL-28 , **(F)** SK-MEL-5 and **(G)** SK-MEL-2. Cells (7.5 x 10^3) were seeded on 96-well plates and treated with vehicle or BRAFi (0.5 μM) six to eight hours later, cells were treated with T-VEC at the indicated MOI. After 5 days of incubation, an MTS assay was performed to measure cell viability. This experiment was conducted at least twice with similar results. Data are presented as mean ± SEM and statistical differences between groups were measured by two-tailed student *t* test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Only significant values are indicated.

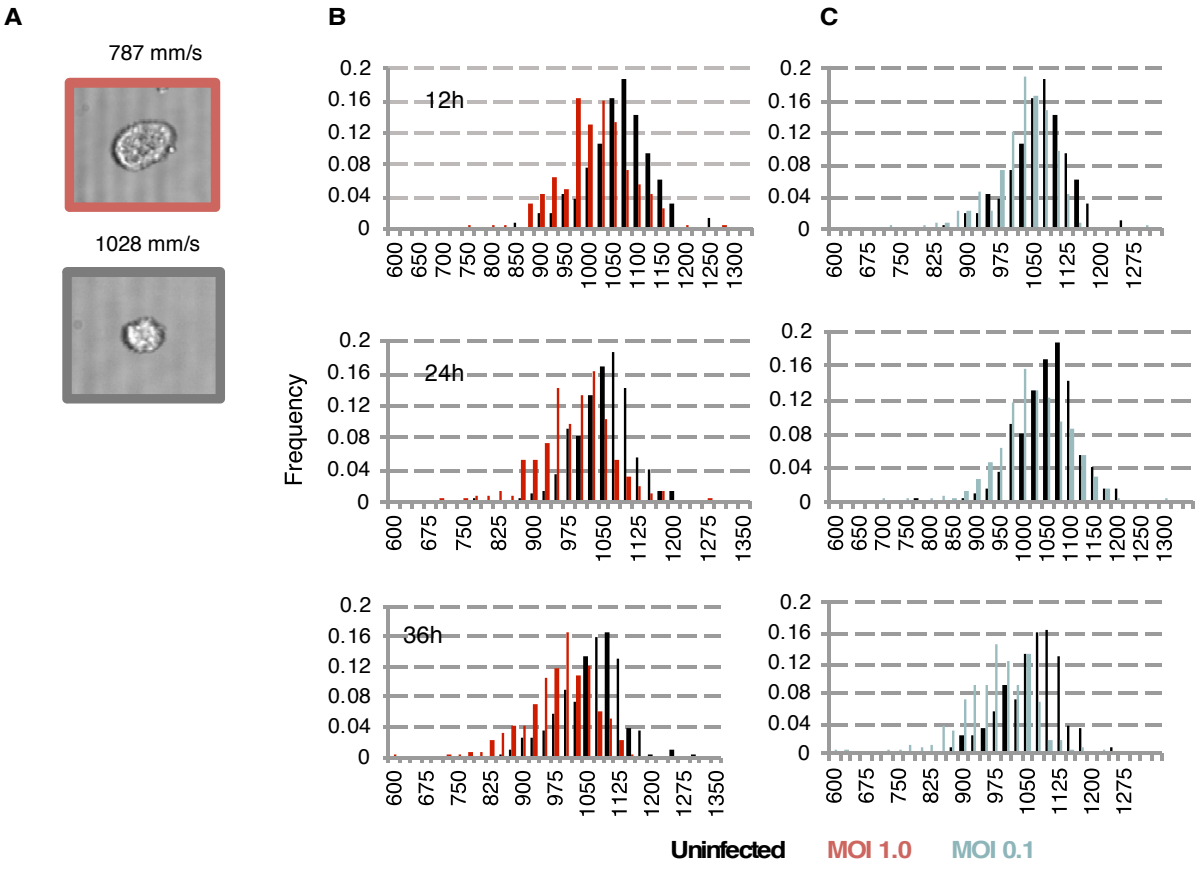

Figure S2. Lumacyte laser flow cytometry analysis

SK-MEL-28 cells were infected with T-VEC at 0.1 MOI and subjected to single cell laser flow cytometry.**(A)** Photomicrograph showing that a virus-infected cell is larger and has a corresponding lower velocity through single cell capillary chamber (787 μm/s; upper panel) compared to un-infected cells that are smaller and have higher velocity (1028 μm/s; lower panel). SK-MEL-28 cells were infected with 1 MOI T-VEC, 0.1 MOI T-VEC or uninfected, and subjected to velocity measurement in single cell capillary chamber. **(B)** Standard velocity histograms generated at 12 h. (top), 24 h. (middle) and 36 hrs. (bottom) for uninfected cells (black bars) or 1 MOI T-VEC (red bars). **(C)** Standard velocity histograms show indicated time points same as **(B)** for uninfected cells (black bars) and 0.1 MOI T-VEC-infected cells (grey bars). These data are calculated using an infection metric as described in the Materials and Methods. This experiment was conducted twice, similar results were obtained.

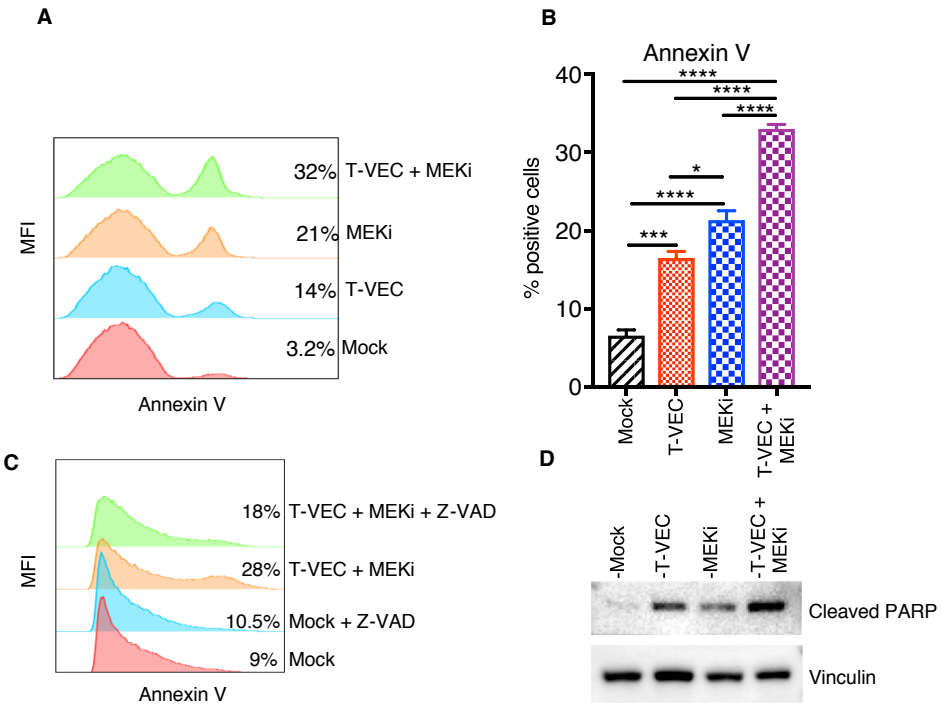

Figure S3. T-VEC and MEK inhibitor induced apoptosis

(A) Flow cytometry analysis of Annexin V staining in SK-MEL-28 cells treated with either T-VEC or MEKi or both. SK-MEL-28 cells were treated with vehicle or MEKi (trametinib; 10 nM) for 6-8 hours. Afterwards, PBS or T-VEC (MOI 1) was added to cells and cells were cultured for 24 h, stained for Annexin V (apoptosis), and analyzed by flow cytometry. **(B)** Quantitative analysis of A. **(C)** Annexin-V staining of cells SKMEL-28 cells treated with T-VEC and MEKi or T-VEC, MEKi and Z-VAD (pan caspase inhibitor). Annexin-V staining could be blocked by treatment with Z-VAD FMK (20 μM)**. (D)** Western blot analysis of cleaved PARP. SK-MEL-28 Cells (3.5 x 10^5) were seeded in 6-well plate, treated with vehicle or MEKi (trametinib; 10 nM) as in A. six to eight hours later cells were inoculated with T-VEC (MOI 1). 24 h post-viral infection, total cell lysates were harvested and cleaved PARP level is detected by immunoblotting. This experiment was conducted twice, similar results were obtained. Data are presented as mean ± SEM and statistical differences between groups were measured by one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Only significant differences are shown.

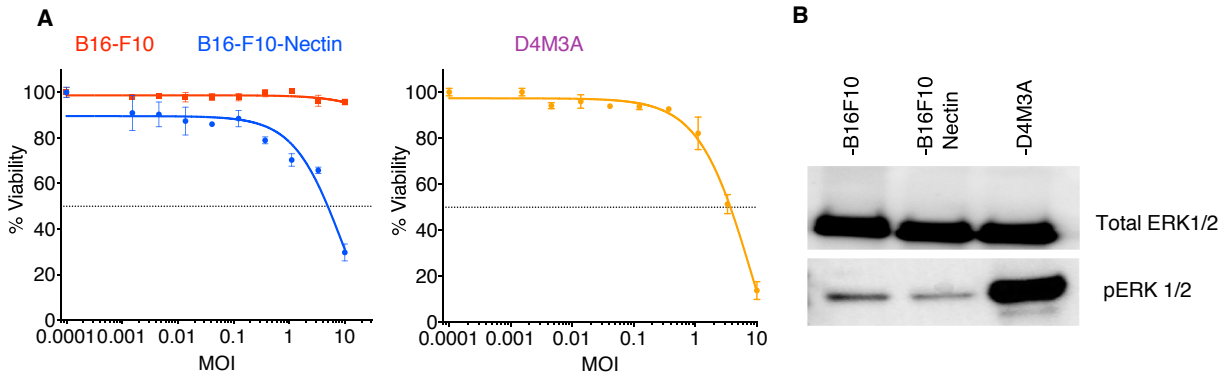

Figure S4. Characterization of murine D4M3A cells

(**A**) MTS assay measuring cell viability of B16-F10 (red) B16-F10-Nectin-1 (blue) and D4M3A (yellow) at 3 days post T-VEC treatment. (**B**) Immunoblot measuring the levels of phosphorylated ERK1/2.

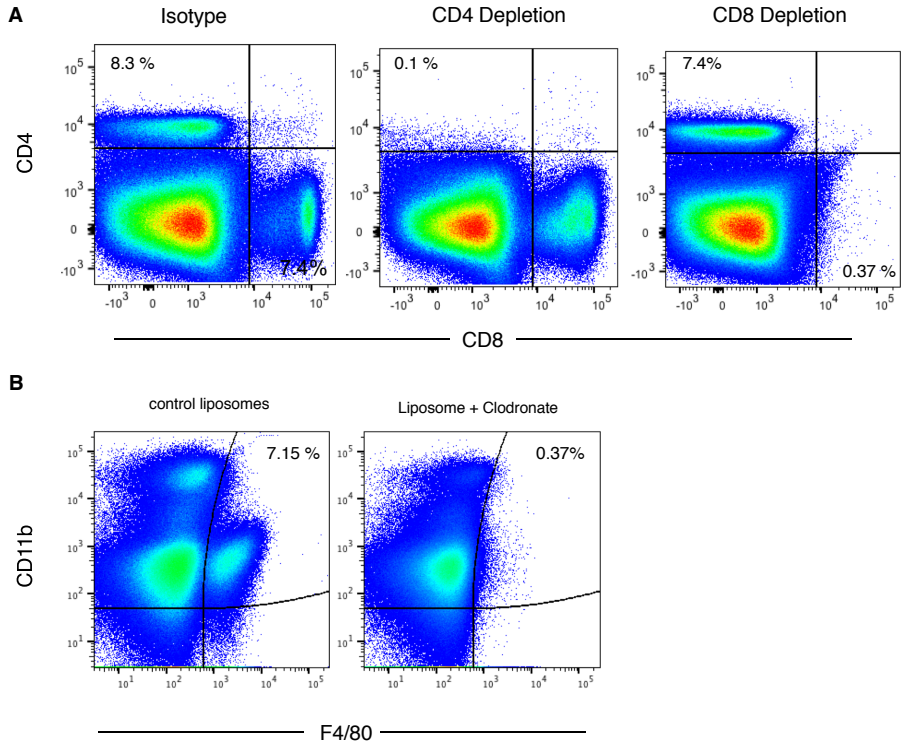

Figure S5. Validation of immune cell depletion

In vivo effects of depletion antibodies (αCD4 and αCD8) on $CD4^+$ and $CD8^+$ cell populations (**A**), and clodronate liposomes on macrophage (CD11b+F4/80+) populations (**B**) in splenocytes from treated mice. B6 mice were injected with either anti-mouse CD8α (clone 2.43; 10 mg/kg), or anti-mouse CD4 (clone GK1.5; 10 mg/kg), control liposomes or clodronate liposomes (first dose 50 mg/kg followed by 25 mg/kg) were given by intra-peritoneal (i.p.) injection every 72 hours for 3 doses. Mice were sacrificed 24 h. after the last dose, splenocytes isolated, and stained with or without anti-mouse CD4 (clone 129.19) and CD8a (clone 53-6.7) antibodies, or anti-mouse CD11b and F4/80 antibodies, and analyzed by flow cytometry.

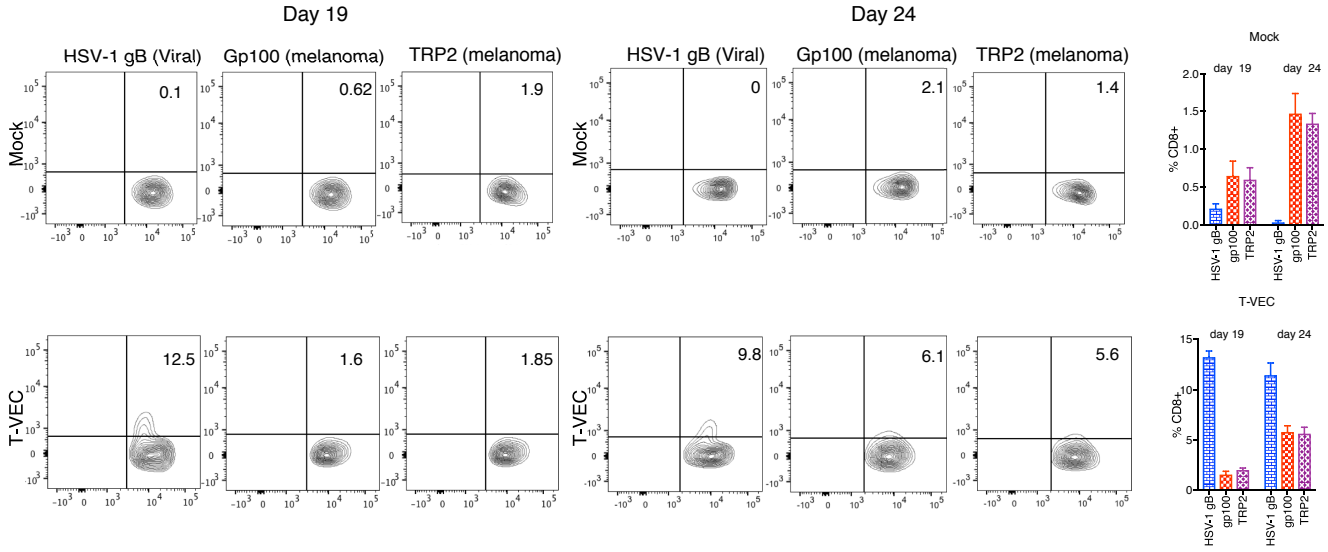

Figure S6. Time course analysis of tumor infiltrating CD8⁺ T cells during mT-VEC treatment

Day 19: Mice bearing D4M3A tumors were treated with 1 x 10^6 pfu of mT-VEC on days 15 and 18 and tumors collected on day 19. Bar graph indicating the % $CD8^+$ antigen specific T cells as indicated.

Day 24: Mice bearing D4M3A tumors were treated with 1 x 10^6 pfu of mT-VEC on days 15, 18, 21 and 23 and tumors harvested on day 24. Bar graph indicating the % $CD8^+$ antigen specific T cells as indicated.

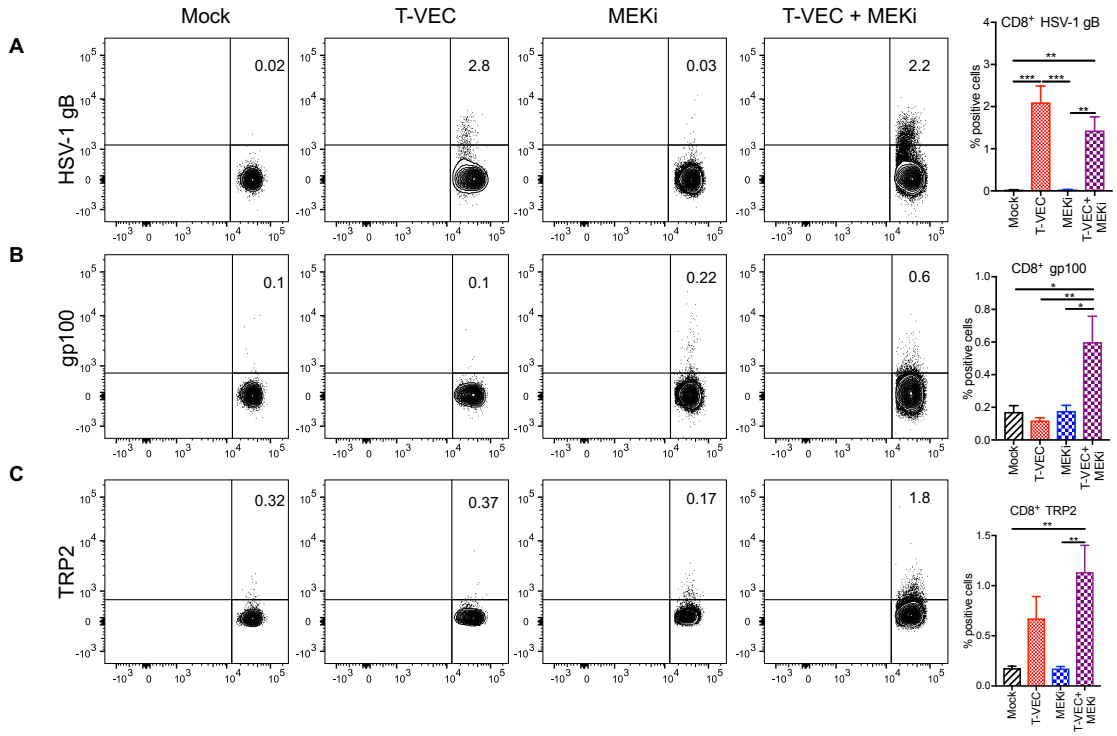

Figure S7. Analysis of CD8⁺ T cells from spleen during mT-VEC + MEKi treatment

C57BL/6J mice implanted s.c. in the right flank with 3 x 10^5 D4M3A cells and treated with mT-VEC (1 x 10^6 pfu) or sterile water i.t. for 3 doses on days 15, 19 and 22 and or trametinib (0.5 mg/kg) or vehicle (0.2% Tween 80 and 0.5% Hydroxypropyl methyl cellulose) orally once daily on days 15-19. Spleens were harvested on day 24 and flow cytometry was performed. Cells were gates on live, CD45⁺, CD3⁺, CD8⁺ and further analyzed for antigen specificity.

(A) Representative plots and bar graph showing quantification of HSV-1-specific H-2Kb-HSV-1gB dextramer positive CD8 T cells from spleen.

(B) Representative plots and bar graph showing quantification of melanoma antigen specific H-2Db-gp100 dextramer positive CD8 T cells from spleen.

(C) Representative plots and bar graph showing quantification of melanoma antigen specific H-2Kb-TRP2 dextramer positive CD8 T cells from spleen.

Data presented as mean ± SEM and the statistical differences between groups was measured by one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Only significant values are indicated.

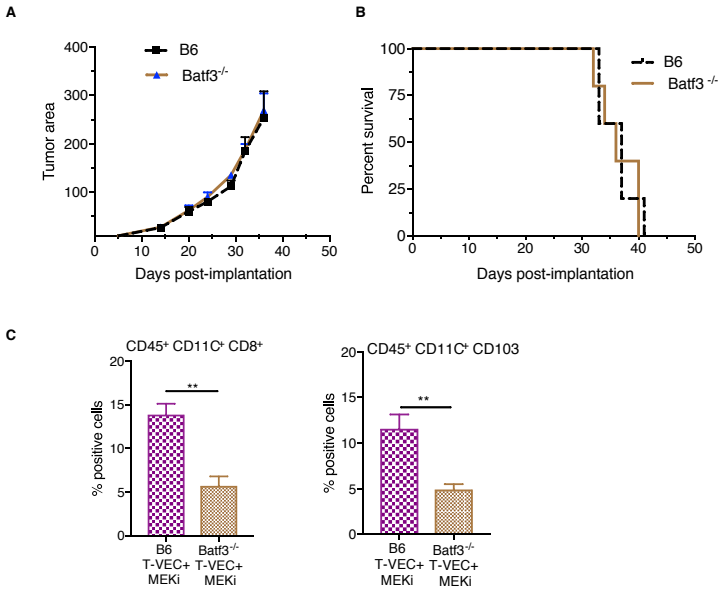

Figure S8. Characterization of D4M3A tumor cells in Batf3 knockout mice

In order to test growth kinetics of D4M3A cells in Batf3$^{-/-}$ mouse mode compared to C57BL/6J mice, age-matched C57BL/6J and Batf3$^{-/-}$ mice were implanted subcutaneously in the right flank with 3 x 10^5 D4M3A murine melanoma cells on day 0. **(A)** Tumor growth and **(B)** Survival were monitored **(C)** C57BL/6J (n = 5) mice and Batf3$^{-/-}$ mice (n = 5) were treated as in Fig. 6C and mice were euthanized on day 24. Tumors were harvested, dissociated cells stained with fluorochrome-conjugated anti-mouse antibodies, and multicolor flow cytometry performed. **(C)** Bar graph indicating frequency of live CD45$^+$ MHC II$^+$ CD11b$^-$ CD11c$^+$ CD8 cells (left panel) and CD45$^+$ MHC II$^+$ CD11b$^-$ CD11c$^+$ CD103 (right panel) cell subsets from B6 and Batf3$^{-/-}$ mice. Data are presented as mean ± SEM and the statistical differences between groups was measured by two-tailed student *t* test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Only significant differences are indicated

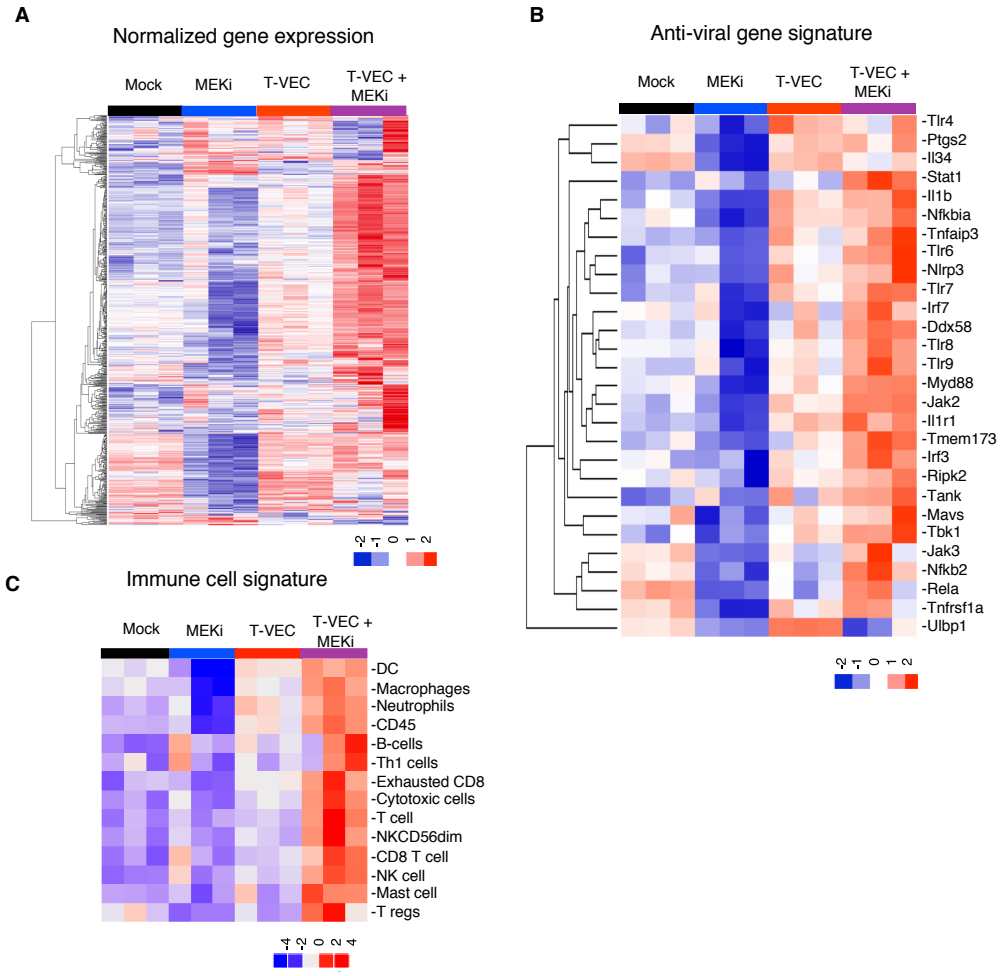

Figure S9. NanoString gene expression heat maps for all genes profiled and by gene function.

C57BL/6J (n=3) mice implanted with 3 x 10^3 D4M3A cells on day 0, treated with sterile water or mT-VEC (10^6 pfu) on days 15, 19 and 22 and vehicle or MEKi (trametinib; 0.5 mg/kg) gavaged orally once daily from days 15-19 (n = 5/group), and euthanized on day 24. Tumors were harvested, total RNA was isolated using Qiagen RNAeasy kit and gene expression analysis was done using the PanCancer Immune panel as described in the Materials and Methods. N=(3). This experiment was conducted twice with similar results. **(A)** Heat map representing normalized gene expression of all genes included in the Nano String PanCancer Immune panel. **(B)** Heat map representing the normalized gene expression signature of genes associated with innate anti-viral immune responses**. (C)** Heat map representing the normalized gene expression signature of genes associated with specific immune cell function.

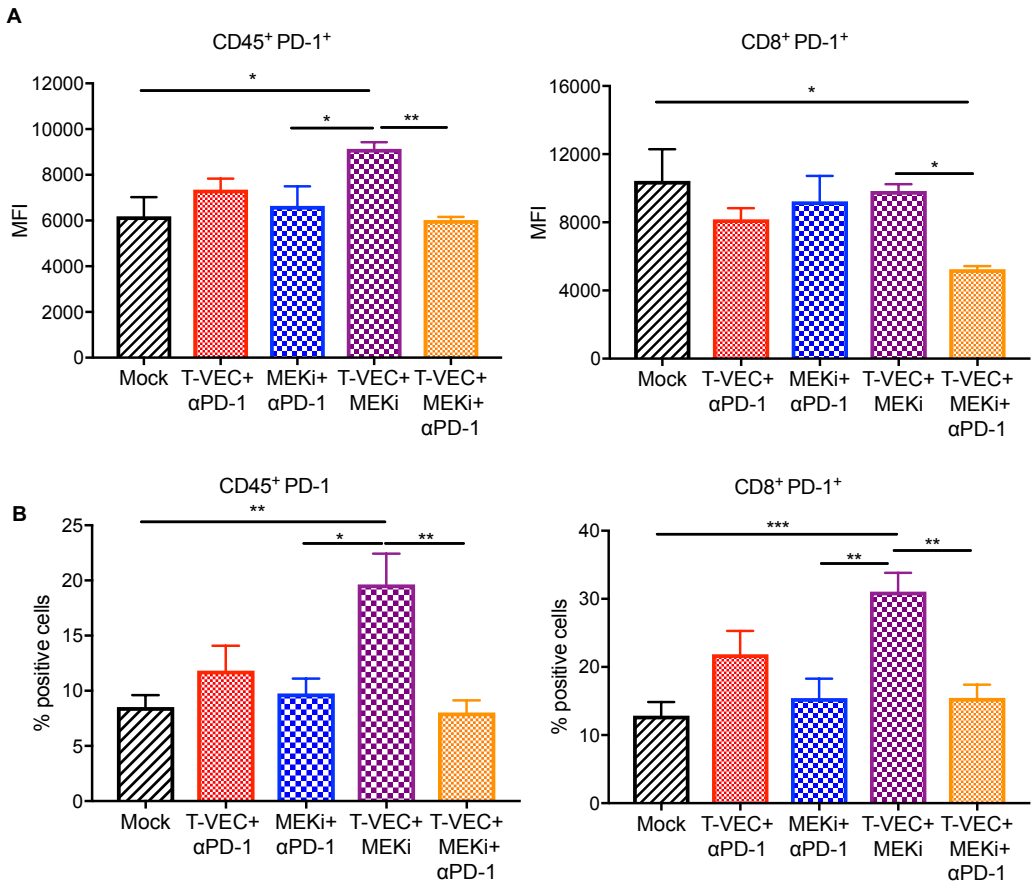

Figure S10. Mean fluorescence intensity (MFI) expression of PD-1 expression and frequency of PD-1⁺ cells

C57BL/6J mice (n=5 per group) were implanted subcutaneously in the right flank with D4M3A cells (3 x 10^5) on day 0 and treated with T-VEC (1x10^6 pfu) or sterile water via intra-tumoral injection on days 15, 19 and 22, trametinib (0.5 mg/kg) or vehicle control on days 15-19 via oral gavage and αPD-1 antibody (clone: RMP1-14, 10 mg/kg) via i.p. injection on days 15, 19 and 22. In addition, groups received double combination treatment with T-VEC and trametinib (and rat IgG isotype control) or triple therapy with m-TVEC, trametinib, and αPD-1 antibody. **(A)** Bar graph indicating the mean fluorescence intensity (MFI) of $CD45^+PD\text{-}1^+$ (right panel) and $CD45^+CD8^+PD\text{-}1^+$ (left panel). **(B)** Bar graph indicating the percent positive $CD45^+PD\text{-}1^+$ (right panel) and percent positive $CD45^+CD8^+PD\text{-}1^+$ (left panel). Each experiment was repeated at least two times with similar results. Data are presented as mean ± SEM and statistical differences between groups were measured by using one-way ANOVA *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Only significant differences are indicated.

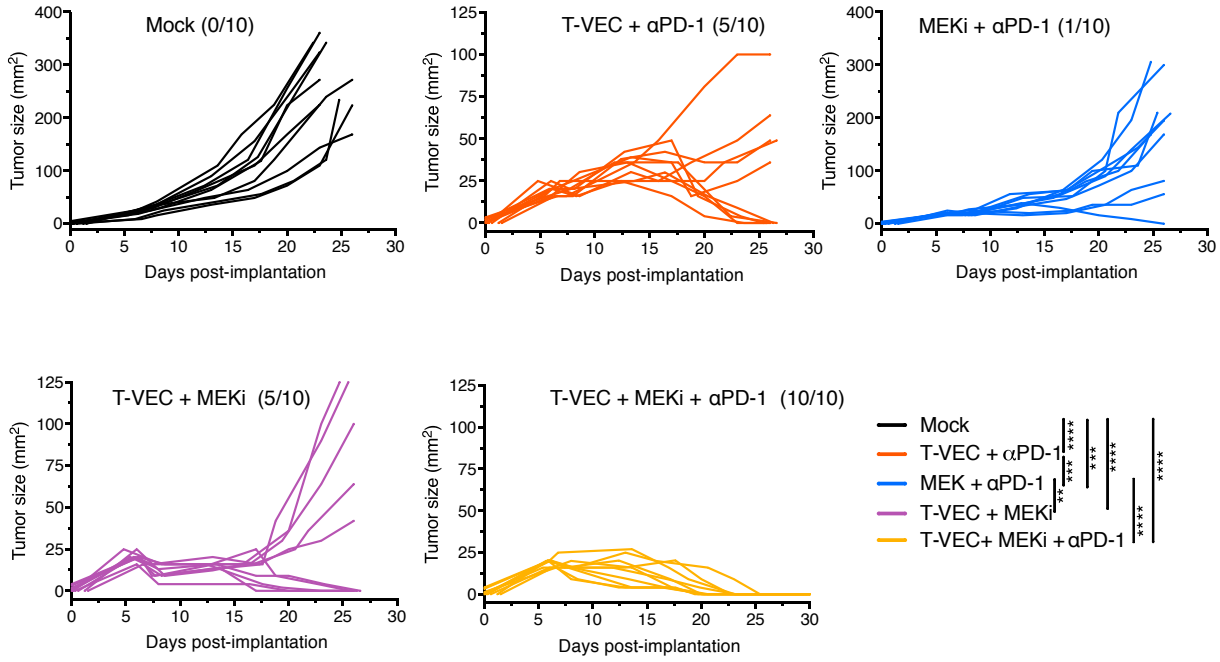

Figure S11. Individual tumor growth curves of BALB/c mice bearing CT26 tumors.

Growth curves from two individual experiments. BALB/c mice (n=10 per group) were implanted subcutaneously in the right flank with CT26 cells (2 x 10^5) on day 0 and treated with T-VEC (5x10^5 pfu) or sterile water via intratumoral injection on days 8, 12, 15, 19 and 22, MEKi (0.5 mg/kg) or vehicle control on days 8-20 via oral gavage and αPD-1 antibody (clone: RMP1-14, 7.5 mg/kg) via i.p. injection on days 8, 12, 15 and 19. In addition, groups received equivalent doses of double combination treatment with mT-VEC and MEKi (and rat IgG isotype control) or triple therapy with TVEC, MEKi and αPD-1 antibody. Mean tumor area across groups was calculated on day 26 and statistical differences between groups were measured by one-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Only significant differences are shown.

Supplemental Tables (Table S1. – Table S6.)

Table S1. Antibodies

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC anti-mouse CD4	BioLegend	Cat#100406, RRID:AB_312713
PE anti-mouse CD3	BioLegend	Cat#100206, RRID:AB_312663
Pacific Blue™ anti-human/mouse Granzyme B	BioLegend	Cat#515408, RRID:AB_2562195
Brilliant Violet™ 605 anti-mouse PD-L1	BioLegend	Cat#124321, RRID:AB_2563635
Brilliant Violet 650™ anti-mouse CD45	BioLegend	Cat#103151, RRID:AB_2565884
Brilliant Violet 711™ anti-mouse IFN-γ	BioLegend	Cat#505836, RRID:AB_11219588
Brilliant Violet 785™ anti-mouse CD8a	BioLegend	Cat#100750, RRID:AB_2562610
APC anti-mouse FOXP3	Thermo Fischer Scientific	Cat#17-5773-82, RRID:AB_469457
Alexa Fluor® 700 anti-mouse Ki-67	BioLegend	Cat# 652420, RRID:AB_2564285
APC/Cy7 anti-mouse I-A/I-E (MHCII)	BioLegend	Cat#107628, RRID:AB_2069377
APC anti-mouse F4/80	BioLegend	Cat#123115; RRID: AB_893493
PerCP/Cy5.5 anti-mouse CD3	BioLegend	Cat#100218, RRID:AB_893318
PE/Cy7 anti-mouse CD11c	BioLegend	Cat#117318, RRID:AB_493568
Brilliant Violet 421™ anti-mouse Ly-6G/Ly-6C (Gr-1)	BioLegend	Cat#108434, RRID:AB_2562219
Brilliant Violet™ 605 anti-mouse PD-1	BioLegend	Cat#135220, RRID:AB_2562616
Brilliant Violet 650™ anti-mouse CD44	BioLegend	Cat#103049, RRID:AB_2562600
Brilliant Violet 711™ anti-mouse CD11b	BioLegend	Cat#101242, RRID:AB_2563310
Brilliant Violet 785™ anti-mouse CD8a	BioLegend	Cat#100750, RRID:AB_2562610
Alexa Fluor® 700 anti-mouse CD45	BioLegend	Cat#103128, RRID:AB_493715
APC/Cy7 anti-mouse CD4	BioLegend	Cat#100414, RRID:AB_312699
FITC anti-mouse CD103	BioLegend	Cat#121420, RRID:AB_10714791
APC/Cy7 anti-mouse PD-L1	Thermo Fischer Scientific	Cat#46-5982-82,RRID:AB_2573819
PerCP/Cy5.5 anti-mouse CD4	BioLegend	Cat#100434, RRID:AB_893324
Brilliant Violet 605™ anti-mouse F4/80	BioLegend	Cat#123133, RRID:AB_2562305
Anti-mouse PD-1	BioXCell	Cat#BE0146; RRID: AB_10949053
Anti-mouse CD4	BioXCell	Cat#BE0003-1; RRID: AB_1107636
Anti-mouse CD8	BioXCell	Cat#BE0061; RRID: AB_1125541
InVivoMAb Rat IgG2b	BioXCell	Cat#BE0090; RRID: AB_1107780
InVivoMAb Syrian Hamster IgG	BioXCell	Cat#BE0087; RRID: AB_1107782
Anti-mouse CD8a	Thermo Fischer Scientific	Cat#14-0808-80; RRID: AB_2572860
Cleaved caspase-3 (Asp175) antibody	Cell Signaling	Cat#9661; RRID: AB_2341188
Phospho-p44/42 MAPK (Erk1/2)	Cell Signaling	Cat#4376; RRID: AB_331772
Anti-Ki67	Abcam	Cat#ab16667; RRID: AB_302459
HRP anti-rat IG	Vector Lab	Cat#MP-7444-15; RRID: AB_2336530

Table S2. Chemicals

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
RPMI 1640 Medium	Thermo Fischer Scientific	Cat#11875093
DMEM (Dulbecco's Modified Eagle Medium)	Thermo Fischer Scientific	Cat#11965118
DMEM/F-12	Thermo Fischer Scientific	Cat#10565018
Trypsin-EDTA (0.25%)	Thermo Fischer Scientific	Cat#25200056
Penicillin-Streptomycin-Glutamine	Thermo Fischer Scientific	Cat#10378016
TRIzol	Thermo Fischer Scientific	Cat#15596018
Fetal Bovine Serum	Sigma Aldrich	Cat#12306C
TWEEN 80	Sigma Aldrich	Cat#SKU-P4780
(Hydroxypropyl)methyl cellulose	Sigma Aldrich	Cat#SKU-H7509
Phenazine methosulfate (PMS)	Sigma Aldrich	Cat#SKU-P9625
CellTiter 96® AQueous MTS Reagent Powder	Promega	Cat#G1111
Standard macrophage depletion kit	Encapsula nanoscience	Cat#SKU-8901
DAB+ substrate chromogen system	Dako	Cat#K3468
Hematoxylin 2	Thermo Fischer Scientific	Cat#7231
Eosin Y 1% alcoholic solution	Fisher Scientific	Cat#245-658
10% Formalin W/V	Fisher Chemical	Cat#SF98-4
Cytoseal XYL	Thermo Fischer Scientific	Cat#8312-16E
Trametinib (GSK1120212)	Selleck Chemicals	Cat#S2673
Vemurafenib (PLX4032, RG7204)	Selleck Chemicals	Cat#S1267
Z-VAD-FMK	Selleck Chemicals	Cat#S7023
FITC TRP2 Dextramer	Immudex	Cat# JD2199
PE GP-100 Dextramer	Immudex	Cat# JA3570
APC HSV-1 gB Dextramer	Immudex	Cat#JD2670

Table S3. Commercial Assays

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Commercial Assays		
LookOut Mycoplasma PCR kit	Sigma	Cat#MP0035
Mouse Pan-cancer immune gene panel	NanoString Technologies	XT_PGX_MmV1_CancerImm
Apoptosis Detection Kit	BioLegend	Cat#640914
RNeasy plus mini kit	Qiagen	Cat#74134

Table S4. Experimental cell lines.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental cell lines		
Human: SK-MEL-28	ATCC	HTB-72
Human: SK-MEL-2	ATCC	HTB-68
Human: SK-MEL-5	ATCC	HTB-70
Mouse: B16-F10	ATCC	CRL-6475
Mouse: B16-F10-Nectin	Amgen	N/A
Mouse: D4M3A	(Jenkins et al., 2014) (Ref:13)	N/A
Mouse: CT26	ATCC	CRL-2639

ATCC - American Type Culture Collection.

Table S5. Experimental Models

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/strains		
Mouse: C57BL/6J	Jackson Labs	Stock#000664
Mouse: NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ	Jackson Labs	Stock#005557
Mouse: B6.129S(C)-*Batf3*^{tm1Kmm}/J	Jackson Labs	Stock#013755
Mouse: BALB/c	Jackson Labs	Stock#000651

Table S6. Tumor area from mouse studies

Included as a separate excel document.