SUPPLEMENTAL MATERIALS

Histoculture conditions

The B16F10, CT26, and EL4 murine cell line were maintained in complete RPMI-1640 media consisting of RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1X penicillin-streptomycin. The MB49 murine urothelial carcinoma cell line was maintained in DMEM supplemented with 10% (v/v) heat inactivated FBS and 1X penicillin-streptomycin.

Recombinant bacterial minicells

VAX014 rBMCs were manufactured and characterized by Vaxiion Therapeutics and met all applicable and current product specifications utilized for clinical release. The control rBMC VAX-I (Inv^{+},PFO^{-}) were produced and characterized by Vaxiion Therapeutics as described previously.[16]

Determination of integrin expression by flow cytometry

B16F10 integrin expression confirmation was performed as previously described.[14 15] Briefly, B16F10 cells were washed once, dissociated from the flask using TrypLE Express (Thermo Fisher), and centrifuged to collect cell pellet. Cells were concentrated and non-specific protein interaction blocked using secondary antibody host serum prior to being stained with primary integrin antibodies for 30 minutes at 4°C as shown in **Supplemental Table S1**. Cells were washed twice and stained with secondary Alexa Fluor® 488 conjugated antibodies for 30 minutes at 4°C. Cells were washed three times prior to data acquisition using Stratedigm flow

cytometer. Data was analyzed using CellCapture. Hamster isotype and secondary antibody only stains were used as negative controls.

Determination of cellular internalization of rBMCs

B16F10 cells were seeded at $2.0x10^5$ cells/well in a 6-well tissue culture plate and allowed to adhere for 4 hours prior to the addition of carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher) stained VAX-I rBMCs at 1,000:1 rBMCs to mammalian cell ratio (referred to as "MOI" for all *in vitro* work presented). Cells/rBMCs were centrifuged, co-incubated for 2 additional hours, and subsequently washed prior to flow cytometry. Live cell populations were analyzed for CFSE signal as a measure of rBMC internalization. B16F10 cells incubated with $CFSE^-$ VAX-I rBMCs were used to set negative gates. CFSE labelled VAX-I rBMCs were generated by incubating 12 μM CFSE with $1.5x10^{10}$ VAX-I rBMCs/mL in 1X PBS supplemented with 2mM magnesium sulfate ($MgSO_4$) overnight at 37°C. CFSE labeled VAX-I rBMCs were washed twice with 1X PBS containing 2mM $MgSO_4$ and fluorescent labelling verified using flow cytometry (data not shown).

***In vitro* cell potency, lactate dehydrogenase assay, and propidium iodide staining of cells**

In vitro potency and oncolytic activity of VAX014 was measured in B16F10 cells as previously described.[14 15] Briefly, target cells were seeded in a 96-well tissue culture plate at 5,000 cells/well and were allowed to adhere for 4 hours prior to the addition of titrated VAX014 or VAX-I rBMCs at a MOI ratio ranging from 2,000:1 to 4:1. Cells/rBMCs were centrifuged for 10 minutes, co-incubated for an additional 20 hours, and assayed for viability using 1/10 volume

Prestoblue™ (Thermo Fisher). After cells incubated for 5 hours with viability dye, colorimetric signal was read at 560 ex/600 em using SpectraMax M3 per manufacturer's instructions. Oncolytic activity of VAX014 was measured using the identical setup as described above except 10,000 cells/well were plated and lactate dehydrogenase (LDH) release was used to measure oncolysis. After cells/rBMCS co-incubated for 2 hours, 25 µL of supernatant was removed from each well and assayed for LDH content using a standardized LDH assay kit (Sigma, Cat# TOX7) per manufacturer's instructions. Potency and oncolytic activity assays were performed in triplicate.

For propidium iodide (PI) staining, B16F10 cells were plated at $1.0x10^6$ cells/well in a 12-well tissue culture plate and allowed to adhere for 4 hours. Cells were treated with VAX014 at MOI of 2,000:1, centrifuged, and co-incubated for 2 hours. Cell culture media was then removed, and cells mechanically dissociated from the plate, washed once, and stained with 2 µg/mL PI (Sigma Aldrich) for 5 minutes prior to flow cytometry. Heat treated (65°C for 20 minutes) cells were stained with PI and served as a positive control.

Mitochondrial membrane potential assay

To determine loss of mitochondrial potential, cells were plated in a 12-well tissue culture plate and treated with rBMCs as described above with the exception of a 5-hour co-incubation of cells/rBMCs prior to staining with 10 nM Mitotracker RedCMXRos (Thermo Fisher) for 1 hour. Cells were mechanically dissociated from plates and washed once prior to flow cytometry.

Visual morphologic assessment of cells following VAX014 treatment *in vitro*

Pyknotic assays were performed using the same 96-well plate set-up and VAX014 rBMC treatment as described above except 0.15 µg of 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher) was added per well. Images were captured at 60X magnification using EVOS microscope system.

Intradermal tumor installation

Unless indicated otherwise, female C57BL/6 or Balb/C mice were anesthetized by isoflurane inhalation, right hind flanks were shaved, and $1.25x10^5$ B16F10, MB49 cells, or CT26 cells were implanted into the dermis of the right hind flank in a 30 µL volume on Day 0 using an insulin syringe. For all tumor models, tumors were measured every other day using a digital caliper in mm and tumor volume (mm^3) calculated using the formula: π/6 x length x width x width. Mean tumor growth rates were plotted up to the median day of survival for saline treated control or tumor-naïve mice and any exceptions are noted. Mice were observed for clinical symptoms and termination criteria were met when tumor volume was >1500 mm^3 or morbidity was observed.

Single i.d. efficacy experiments

A single i.d. tumor was installed as described above. When tumors reached 2-5 mm in length, weekly i.t. treatments were initiated. An i.t. dose of $1.5x10^8$ VAX014 rBMCs was used for both B16F10 and MB49 models and $5x10^6$ VAX014 rBMCs/dose was used for the CT26 model. Each i.t. treatment was administered in a 30 µL dose volume once weekly for a total of up to 6 treatments or until injected tumors exhibited CR. Treatment response groups in mice treated with repeat weekly i.t. administration of VAX014 were defined as complete response (CR),

responding (Rs) tumors (≤ 70% of mean saline treated tumor volume), or rebounding (Rb) tumors (> 70% of mean saline treated tumor volume).

Immune cell depletion experiments

Depletion of CD8α$^+$ T cells, CD4$^+$ T cells, or NK cells in the single i.d. B16F10 tumor model was accomplished by intraperitoneal (i.p.) administration of depletion antibodies specific for CD8α (250 µg/dose), CD4 (250 µg/dose), or NK1.1 (200 µg/dose) in 100 µL volume 1X PBS. T cell depletion antibodies were given once weekly prior to i.t. treatment and NK depletion antibody given on Day 3 and 5 prior to tumor implantation and continued twice weekly throughout the course of treatment. In the single i.d. CT26 tumor model, CD8α$^+$ T cell depletion was achieved by i.p. administration of CD8α (250 µg/dose) depletion antibodies on 2 consecutive days prior to treatment initiation and given twice weekly thereafter. Depletion antibodies or placebo controls were administered until injected tumors achieved CR or termination criteria were met.

In the bilateral i.d. MB49 tumor model, CD8α$^+$ T cell and CD4$^+$ depletion was achieved by i.p. administration of either CD8α (100 µg/dose) or CD4 (100 µg/dose) depletion antibodies on 3 consecutive days prior to tumor implantation, day of implantation, and three days prior to treatment initiation. Depletion antibodies or placebo controls were administered once weekly thereafter to at least Day 47 or until termination criteria were met. In all cases, depletion of each cell type was confirmed by flow cytometry. Refer to **Supplemental Table S1** for list of depletion antibodies used in this study.

Evaluation of TILs in injected tumors

C57BL/6 mice were implanted with a single i.d. B16F10 tumor as described above. When tumors reached 2-5 mm in length, a single reduced i.t. dose of 1×10^8 VAX014 rBMCs was administered and tumor growth measured for each subject as described above. Mice were categorized into treatment groups defined as having achieved CR (no measurable tumor beyond Day 20), as actively Rs tumors (≤ 5 mm in length), or as Rb tumors (≥ 7 mm in length).

To evaluate TIL populations, tumors were excised from euthanized mice on Days 12-28, minced using forceps, and enzymatically dissociated in digestion buffer (3 mg/mL collagenase D, 200 U/mL DNaseI in RPMI media, 10% heat inactivated FBS) for 20 minutes at 37°C. Crude dissociated tumor cell suspensions were passed through a 70 µm nylon mesh to generate single cell suspensions, which were then concentrated by centrifugation/resuspension in wash buffer (1X PBS, 10% heat inactivated FBS, 5 mM EDTA). Single cell suspensions from injected tumors with extensive necrosis were further enriched for leukocytes via 80%/40% Percoll density gradient. Following concentration and/or enrichment, cell suspensions were washed at least once and filtered through a 35 µm nylon mesh to remove residual cell debris prior to downstream analysis by flow cytometry (described below).

CTL assays

For B16F10 stimulator cell preparation in the single i.d. B16F10 tumor model, B16F10 cells were first incubated 24 hours in 50 ng/mL recombinant murine interferon gamma (mIFN-γ) (Sigma Aldrich) to ensure adequate MHC-I expression (**supplemental figure S6**). Upregulation of MHC-I was confirmed by flow cytometry prior to conversion to stimulator cells via a 1-hour incubation in a cytostatic concentration of 50 µg/mL mitomycin C (MMC). B16F10 stimulator

cells were washed three times to remove residual MMC, mechanically dissociated from the tissue culture flask, centrifuged, and resuspended in expansion media consisting of complete RPMI media supplemented with 50 μM β-mercaptoethanol, 1% MEM non-essential amino acids, and 40 U/mL murine IL-2 (Sigma Aldrich) and plated at $1.5x10^5$ cells/well in a 24-well tissue culture plate for 4 hours prior to splenocyte addition. On Day 14, animals were euthanized, spleens were dissociated and passed through a 70 µm mesh, centrifuged, and red blood cell(s) (RBC) lysed twice using RBC lysis buffer (0.135M ammonium chloride, 0.015M tris hydrochloride) for 10 minutes. Splenocytes were washed in wash buffer, filtered through a 35 μM mesh, and splenocytes added at a 15:1 splenocyte to B16F10 stimulator cell ratio or pulsed with either TRP-$2^{180-188}$ or $GP100^{25-33}$ peptides (10 µg/mL) in expansion media. After 5 days of clonal expansion, B16F10 target cells were pretreated with 50 ng/mL mIFN-γ then seeded at 10,000 cells/well in a 96-well tissue culture plate for 4 hours. Syngeneic haplotype matched murine EL4 thymoma cells were also pulsed with either TRP-2, GP100, or no peptide and seeded at the same density in a U-shaped plate for 1 hour prior to splenocyte addition. EL4 cells pulsed without peptide served as a non-specific target cell control. Stimulated splenocytes were washed twice with 1X PBS and added to duplicate test wells at E:T ratios of 100:1, 50:1 and 10:1 indicated in the figure. Splenocytes/target cells co-incubated for 4 hours prior to assaying LDH release as a measure of CTL activity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) per manufacturer's instructions.

Bilateral i.d. tumor models for determining abscopal effects

Bilateral i.d. B16F10 tumors were established as described above with the exception that i.d. tumors were implanted on opposing flanks using $1.25x10^5$ B16F10 cells for the right flank

(designated as injected tumor) and a reduced inoculum of 3.13×10^4 B16F10 cells for the left flank (designated as distal noninjected tumor) on Day 0. Bilateral i.d. MB49 tumors were also established as described with the exception that each i.d. tumor was implanted using 1.25×10^5 MB49 cells.

Weekly i.t. treatment with either saline or VAX014 was given as described above when tumors (designated as injected tumor) reached 2-5 mm in length (Day 5 for B16F10, and Day 6-8 for MB49). In both models, treatment continued weekly for up to 6 total doses or until injected tumors exhibited CR. Both injected and distal noninjected tumor volumes were measured by digital caliper as described.

Determining specificity of antitumor response for the injected tumor type *in vivo*

To determine specificity of antitumor responses for the injected tumor type following i.t. treatment with VAX014, i.d. tumors were implanted on opposing flanks using 1.25×10^5 B16F10 cells into the right flank (designated as injected tumor) and 1.25×10^5 MB49 cells into the left flank (designated as noninjected tumor) on Day 0. When B16F10 tumors reached 2-5 mm in length (Day 5), weekly i.t. treatment of saline or VAX014 was administered as described above into the B16F10 tumor only. Treatment continued weekly for up to 6 total doses or until injected B16F10 tumors exhibited CR.

The same experiment was then repeated except MB49 was designated as the injected tumor and the contralateral B16F10 tumor served as the noninjected tumor. Both the injected and noninjected tumor growth rates were measured and splenic CTL activity against the injected tumor type (MB49 as target cells) in comparison to the noninjected tumor type (B16F10 as target

cells) was performed as described above on Day 21 with the exception that lymphocyte stimulation was performed using MMC-treated MB49 stimulator cells.

Systemic ICB combination therapy experiments

Combination with systemic ICB against either CTLA-4 (αCTLA-4) or PD-1 (αPD-1) was conducted using the bilateral i.d. B16F10 model and treatment schedule as described above. Treatment continued weekly for up to 6 total doses or until injected tumors exhibited CR. Systemic administration of αCTLA-4 (100 µg/dose) and/or αPD-1 (200 µg/dose) was given in a 100 µL volume i.p. bolus on the same day as i.t. treatment and continued thereafter every 3-4 days for a maximum of 5 doses (Refer to **Supplemental Table S1** for list of ICB therapeutic antibodies used in this study). Saline vehicle controls for i.t. and i.p. injections were kept on the same schedule, respectively. Both injected and distal noninjected tumor volumes were measured by caliper as described.

Immunotranscriptome analysis

Intradermal B16F10 tumors were implanted and i.t. treated as described with either saline, VAX014 monotherapy, or VAX014 in combination with systemic ICB, above. Mice were euthanized, tumors surgically excised on days indicated, and total RNA extracted using RNeasy protect kit and DNase I (Qiagen) according to manufacturer's instructions. Each RNA sample (20 ng) was prepared for analysis using the NanoString Mouse PanCancer Immune Profiling panel chip (NanoString Technologies, Inc., Seattle, WA). The assay was performed on nCounter MAX Analysis System (Sanford Consortium for Regenerative Medicine Stem Cell Genomics

Core, La Jolla, CA) according to the manufacturer's instructions. Data was analyzed by ROSALIND® (https://rosalind.bio/) with a Hyperscale architecture developed by ROSALIND, Inc. (San Diego, CA). Read Distribution percentages, violin plots, identity heatmaps, and sample multidimensional scaling plots were generated as part of the quality control step. Normalization, fold changes, and *p*-values were calculated using criteria provided by NanoString. Abundance of various cell populations was calculated on ROSALIND® using the Nanostring Cell Type Profiling Module. Fold changes and *p*-values were calculated using the fast method as described in the nCounter® Advanced Analysis 2.0 User Manual. *P*-value adjustment was performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR). Clustering of genes for heatmaps of transcripts was generated using the PAM (Partitioning Around Medoids) method. Threshold for comparative heatmaps and clustered gene signatures was set at $\leq$ -1.5 and $\geq$ 1.5 and *p*-value $\leq$ 0.05.

Evaluation of TILs in noninjected tumors

Bilateral i.d. B16F10 tumors were implanted in mice and i.t. treatment ± systemic ICB given as described. Distal noninjected tumors were excised from euthanized mice on Days 13-14 and tumors processed for downstream analysis by flow cytometry as described for injected tumors, above.

Flow cytometry

For flow cytometry from histocultures, cells were grown to confluency in a T25 cm^2 flask and were then washed, enzymatically dissociated, and were washed again by

centrifugation/resuspension prior to staining. Single cell suspensions from spleens and tumors were generated as described above. Tumor draining lymph node(s) (DLN) were dissociated and passed through a 70 µm nylon mesh to generate single cell suspensions, centrifuged/washed in wash buffer, and filtered through a 35 µm nylon mesh. Blood samples were extracted via cardiac puncture and placed in 0.5M EDTA when tumors reached sizes indicated in the figures. Blood samples were centrifuged/washed twice with complete media and subjected to two rounds of RBC lysis using RBC lysis buffer (described above) for 10 minutes followed by the addition of 1/10 volume 10X PBS. Blood samples were centrifuged/washed twice with wash buffer and filtered through a 35 µm nylon mesh.

Fc receptors were blocked for all samples using anti-CD16/32 antibody (Biolegend). Cells were then labeled with either eFluor™ 780 Fixable Viability Dye (Thermo Fisher) or PI for 10 minutes at 4°C. Staining with surface antibodies was performed for a minimum of 30 minutes at 4°C for all samples. TRP-2 dextramer (Immudex) staining was performed per manufacturer's instructions. Where needed, intracellular staining was performed after cell surface staining. Fixation/permeabilization was achieved using either the BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences) or the FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher) per manufacturer's instructions. Intracellular markers were stained for a minimum of 30 minutes at 4°C. Data were acquired using Stratedigm, BD FACSCanto (BD Biosciences) or CytoFlex-S (Beckman Coulter) and analyzed withBD FACSDiva 6.1.3 or CytExpert 2.4 software platforms, respectively.

The gating strategy for TIL analysis, MDSC analysis, and TRP-2 dextramer staining in the single i.d. tumor model was to first gate on live cell populations (PI negative). For TIL analysis, gates were further established using the leukocyte compartment ($CD45^{+}$) and all $CD4^{+}$,

$CD8^+$, $CD4^+FoxP3^+$ (Tregs) expressing cells. The gates for TIL analysis in the bilateral i.d. B16F10 model were established using fluorescence minus one (FMO) controls followed by gating on live cell populations (viability dye negative), the leukocyte compartment ($CD45^+$), lymphocyte compartment ($CD3\varepsilon^+$), respective lineage markers ($CD8\alpha^+/CD4^-$ or $CD4^+/CD8\alpha^-$), and then downstream phenotype/effector molecule targets as indicated. Antibodies and reagents utilized for flow cytometry are listed in **Supplemental Table S1**.

Tumor rechallenge

As indicated in each model, mice that had achieved CR following i.t. treatment of VAX014 monotherapy or VAX014 in combination with systemic ICB were rechallenged with an i.d. B16F10 tumor ($1.0x10^5$ cells), MB49 tumor ($1.25x10^5$ cells), or CT26 tumor ($1.25x10^5$ cells) on Days 25 – 45 post CR. No further treatment was provided, and mice were evaluated for tumor growth (where present) and survival.