

Intravenously deliverable oncolytic virus VET3-TGI favorably alters the tumor microenvironment

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ABSTRACT

Background Oncolytic viruses have pledged to combat cancer by causing immunogenic cell death of cancerous cells while priming cancer-specific immune responses. These outcomes have proven elusive in the clinic. Current-generation vectors rarely reach effective concentrations inside tumors when given intravenously, and consequently are limited to intratumoral administration. Even after direct tumor infusion, these therapies frequently fail to overcome local immunosuppression caused by inappropriate activation of transforming growth factor beta (TGF- β) signaling throughout the tumor microenvironment.

Methods VET3-TGI is a vaccinia virus that expresses the chemokine receptor C-X-C chemokine receptor 3 (CXCR3) in infected cells within the first hours after infection. Circulating cells infected in situ on intravenous infusion are driven to migrate to tumors that express cognate chemokines, thus delivering the virus to its target. Infected tumor cells subsequently secrete interleukin-12 (IL-12) and a soluble TGF- β inhibitor.

Results Expression of CXCR3 from vaccinia virus boosted delivery of virus to tumors containing C-X-C motif chemokine 9 and C-X-C motif chemokine 10, which are known to be upregulated in many human cancers. VET3-TGI effectively combated tumor growth when administered intravenously as a single agent in multiple mouse tumor models. In these models, IL-12 provoked inflammation-driven antitumor activity from immune cells. This activity was strengthened by local TGF- β blockade, which crippled the ability of tumors to suppress the immune response.

Conclusions VET3-TGI demonstrates the efficacy of three different transgenes with distinct and complementary activities: CXCR3 for tumor targeting to enable intravenous delivery, a TGF- β inhibitor to disrupt immunosuppression, and IL-12 to promote antitumor immunity.

BACKGROUND

Cancer remains a prominent threat to health and well-being worldwide in the 21st century, ranking highly among the leading causes of death in many nations. On average, an estimated one in five people will develop this disease within their lifetime, virtually assuring

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Interleukin-12 (IL-12) stimulates anticancer immunity, but this process is blocked in many tumors by transforming growth factor beta (TGF- β) signaling.
- ⇒ Non-localized blockade of TGF- β signaling can restore the efficacy of antitumor treatments but produces intolerable side effects.
- ⇒ Oncolytic viruses (OVs) can allow localized transgene production, but failure to get enough virus to tumors by intravenous infusion has limited the OV field to intratumoral treatments.

WHAT THIS STUDY ADDS

- ⇒ We enhanced tumor-targeted delivery of intravenously infused virus by expressing the chemokine receptor C-X-C chemokine receptor 3 early after infection.
- ⇒ Cells infected in circulation migrated toward tumors expressing cognate chemokines.
- ⇒ Such effective tumor-targeted delivery of an IL-12 and TGF- β inhibitor-armed virus was well-tolerated and slowed or reversed tumor progression in multiple mouse models.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Chemokine receptor-aided delivery could provide a broad and generally applicable strategy to target different cancer types using intravenously delivered oncolytic virus therapies.
- ⇒ The ability to achieve tumor-targeted delivery after systemic administration opens treatment options for patients that do not present tumors suitable for injection.
- ⇒ The specificity afforded by this approach potentially allows deployment of anticancer payloads that are too aggressive to deliver using non-targeted methods.



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that the direct or indirect consequences are felt by all.¹ Medical interventions against cancer are often painful, lengthy, and expensive. Annual care nationwide is estimated to

cost hundreds of billions of dollars in the USA alone.² Yet, because of its diverse causes and presentations, cancer remains difficult to effectively treat.

Established interventions such as surgery, radiation, chemotherapy, and immunotherapy, singly or in combination, often fail to target cancer at a single-cell level, meaning both that healthy cells are killed and that some cancer cells may escape and regrow.³ Long-term control of cancer typically requires induction of the patient's immune system to recognize residual malignant cells as foreign. Successful induction triggers eradication of cancerous cells by immune cells and, critically, creates an immunological memory that prevents relapse.

Recently deepened understanding of the mechanisms of immune activation and suppression in tumors has guided the development of immunotherapies, which are treatments that persuade a patient's immune cells to attack their cancer. Among these recent developments are chimeric antigen receptor (CAR) T cells for hematological malignancies and immune checkpoint inhibitors (ICIs), which override self-versus-foreign recognition checkpoints that are misused by tumors to block immune attack.^{4,5} While such treatments have achieved commercialization, non-specific delivery, toxicity from imperfect tumor targeting, and failure to overcome immunosuppression within complex tumor microenvironments lead to estimated inefficacy in up to 80% of solid tumors.⁶ Additionally, cell-based treatments such as CAR T cells are costly, in part due to their complicated manufacturing pipeline and personalized nature.⁷ Exemplified by the herpes simplex virus (HSV) vector IMLYGIC and the adenovirus cretostimogene grenadenorepvec (reviewed elsewhere^{8,9}), oncolytic viruses (OVs) induce immunogenic cell death preferentially in tumors. Engineered OVs enable in situ expression of therapeutic transgenes from within tumors so that a cancer-targeted immune response is mounted.

The large, enveloped virus vaccinia (*Orthopoxvirus vaccinia*) possesses many properties favorable for OV backbone engineering. Its use clinically as a smallpox vaccine has cemented its relative safety and efficacy in creating strong and enduring immunity.¹⁰ The vaccinia genome is a single double-stranded DNA chromosome that can stably host thousands of bases of foreign DNA.¹¹ Vaccinia does not modify the genome of infected cells. Its replication is cytoplasm-bound and inherently non-mutagenic, producing both cell surface-associated virions to infect neighboring cells and free-floating virions adapted for dispersal via circulation (unlike HSV and adenovirus).¹² Vaccinia can be infused intravenously, and its replication is enhanced by cancer hallmarks including aberrant nutrient acquisition, metabolism, immune suppression, and uncontrolled cell cycle progression.¹³ Deletion of the gene *J2R*, encoding the J2 protein, a viral thymidine kinase (TK), has been demonstrated clinically to confine replicating virus to tumors by impairing DNA synthesis in healthy cells.¹⁴ Vaccinia can additionally be modified to increase adaptive T-cell responses and decrease virulence

by deleting virulence factor gene *A52R*.¹⁵ Although these properties position vaccinia as a naturally suitable vehicle for anticancer therapy, wild-type vaccinia lacks independent motility. Previous approaches employing vaccinia have sought to hone its cancer-destroying abilities while dismissing its inability to actively seek out and pursue targets.

Chemokines are a class of small secreted signaling proteins that direct cellular movement and localization after binding to specific compatible chemokine receptors. Abnormal expression of chemokines marks cancerous tissue from normal tissue. While some chemokine signatures support cancer by coordinating tolerance and formation of 'cold' immunosuppressed microenvironments,¹⁶ they also comprise a homing signal to target tumors and therefore an opportunity to undermine disease progression.¹⁷ Here, we show that an OV encoding the chemokine receptor CXCR3 can compel infected cells in situ to act as 'virus buses,' migrating toward and actively delivering intravenously infused virus into tumors that overexpress cognate chemokines. This cell-enhanced delivery provides a powerful new method to increase the potency of cancer-fighting viruses. Our approach differs from small-molecule drugs, such as ICIs, biologics, such as antibody-drug conjugates, and OVs, since these current embodiments of the state of the art are passively delivered, meaning that their initial distribution is at the mercy of diffusion and circulation from the injection site. Only after contacting their target can target-specific binding or replication confer specificity. The highly tumor-specific delivery achievable by our strategy enabled us to arm our OVs with therapeutic transgenes that instigate aggressive antitumor immunity.

Interleukin-12 (IL-12), a heterodimeric cytokine, is a potent stimulator of tumoricidal activities of natural killer (NK) and both CD4+ and CD8+ T cells. IL-12 signaling promotes antitumor inflammation and development of long-term adaptive immunity by directing clonal expansion of effector and memory cells.¹⁸ Clinical translation of IL-12-based therapeutics is impeded by its toxicity and limited ability to overcome immune suppression.¹⁹ Transforming growth factor beta (TGF- β) comprises humans and mice of the highly conserved and closely related TGFB1, TGFB2, and TGFB3 proteins. Ligand-induced hetero-tetramerization of TGF- β type II receptor (T β RII) with TGF- β type I receptor (T β RI) is a key initiator of immune regulation, self-tolerance, wound-healing, and tissue development and remodeling.²⁰ Clinical translation of TGF- β inhibitors also remains stymied by toxicity due to this target's pleiotropic functions, which include halting runaway inflammation in healthy tissue.²¹ Within tumors, the TGF- β signaling pathway is a pivotal mediator of cancer progression. TGF- β signaling orchestrates formation of malignant niches that exclude immune cells, and instructs surrounding fibroblasts, macrophages, and regulatory T cells to adopt tumor-supportive roles.^{22–24} Pro-tumor TGF- β signaling further promotes angiogenesis and epithelial-to-mesenchymal transition

(EMT), suppresses antitumor immunity by upregulating immune checkpoint molecules,²⁵ blocks IL-12-stimulated immune activation, and sensitizes activated cytotoxic T cells to exhaustion,²⁶ causing tumors to immunologically 'go cold.'

Genetically encoded soluble TGF- β -targeting 'mini monomer' inhibitors engineered to specifically block interaction between T β RII and T β RI have been developed.²⁷ This technology promises to synergize with IL-12 while avoiding off-target toxicity if combined with effective delivery and tumor-targeted expression in an OV.

Elaborating on this approach, we present preclinical development of VET3-TGI, a vaccinia virus-based oncolytic immunotherapy that incorporates CXCR3-motivated tumor homing and tumor-restricted expression of therapeutic transgenes IL-12 and the TGF- β inhibitor 'dnTGF β mm,' designed for intravenous delivery.

METHODS

Vaccinia and cell origins

The Western Reserve strain of vaccinia virus was obtained unencumbered from an academic laboratory (Thorne Lab, University of Pittsburgh). Next-generation sequencing (NGS, data collection by Azenta Life Sciences using the Illumina MiSeq platform) of the whole viral genome was used to confirm strain identity. Cell strains B16-F10 (CRL-6475; RRID:CVCL_0159), Renca (CRL-2947; RRID:CVCL_2174), EMT-6 (CRL-2755; RRID:CVCL_1923), CT-26 (CRL-2638; RRID:CVCL_7256), A20 (TIB-208; RRID:CVCL_1940), murine embryonic fibroblasts (MEF) (SCRC-1040; RRID:CVCL_5251), human foreskin fibroblasts (HFF-1) (SCRC-1041; RRID:CVCL_3285), human lung carcinoma (A549) (CCL-185; RRID:CVCL_0023), human cervical cancer (HeLa) (CCL-2; RRID:CVCL_0030), 143B (CRL-8303; RRID:CVCL_2270), and U-2OS (HTB-96; RRID:CVCL_0042) were purchased from American Type Culture Collections (ATCC). Cell strain LL/2 (Lewis lung carcinoma (LLC), REF 90020104; RRID:CVCL_4358) was purchased from Sigma-Aldrich. Murine colon adenocarcinoma (MC-38) cells (RRID:CVCL_B288) were obtained via Kerafast (ENH204-FP). Unless specified otherwise, cells were cultured using vendor-recommended medium composition at 37°C with 5% CO₂ in humidified incubators, and all fetal bovine serum (FBS) was heat-inactivated by the vendor. Mycoplasma surveillance was performed on cultured cells and virus preparations using the Universal Mycoplasma Detection Kit (ATCC 30-1012K) in accordance with manufacturer-supplied protocols. Surveillance for microbiological contamination was carried out by direct inoculation of test articles in thio-glycolate or tryptic soy broths, and monitoring growth for 14 days; growth-positive materials were not used in any experiments.

Analysis of CXCR3-homing signals from tumor expression databases

The GEPIA²⁸ web server was queried to compare RNA sequencing-measured expression of C-X-C motif chemokine 9 (CXCL9), C-X-C motif chemokine 10 (CXCL10), and C-X-C motif chemokine 11 (CXCL11) between cancer and normal tissues using data sets from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. Median transcripts per million values and corresponding disease states were visualized to establish the scope of targetable indications.

Construction and selection of recombinant vaccinia virus

Vaccinia transfer vectors were constructed in *Escherichia coli* by classical restriction enzyme/ligase-mediated cloning. Transfer vectors were constructed in the pUC57-amp vector backbone obtained from Genewiz with the desired promoter and transgene encoding sequence, accompanied as needed by a visual selection-enabling reporter gene, flanked on the 5' and 3' termini by recombination-directing sequences of 250–1000 bp in length to enable substitution of native viral genes encoding either A52 or J2. Single-transgene viruses carrying IL-12 or TGF- β inhibitor were constructed by recombination across the locus encoding TK. For early exploratory studies, viral gene *J2R* was replaced in wild-type Western Reserve with a gene consisting of strong constitutive promoter P7.5 followed by a single-chain murine IL-12 encoding the p40 subunit joined to the p35 subunit via a flexible linker, or with a late promoter-driven, secreted TGF- β inhibitor. The single-chain design was selected for IL-12 to drive stoichiometric p70 assembly using one promoter. J2-deletion and A52-deletion viruses and partial component viruses were constructed to serve as controls. Design parameters of each virus are provided in figure 1A.

Plasmids comprising transfer vectors were introduced into 143B human osteosarcoma cells by transfection and recombination with parental virus and were initiated by infection of cells. Recombinant viruses were obtained by plaque selection from infected 143B cell monolayers using the presence (or absence) of reporter signal to distinguish recombinant from parental virus plaques. Absence of wild-type virus on completion of plaque selection was confirmed by PCR using primers against parent-specific genes. Recombinant virus identity was confirmed by Sanger sequencing across modified loci or NGS (Azenta Life Sciences).

VET3-TGI was made by combining efficacious components found in earlier studies into a single virus, replacing viral TK (gene *J2R*, *VACWR094*) in wild-type Western Reserve with DNA encoding a single-chain human IL-12 under the weak late viral promoter 135, and secreted soluble dnTGF β mm under strong constitutive viral promoter P7.5. The open reading frame of A52R (*VACWR178*), a nuclear factor kappa B (NF- κ B)-inhibiting virulence factor, was then replaced with DNA encoding human CXCR3 (isoform 1), placing CXCR3 expression under the natural weak early promoter for

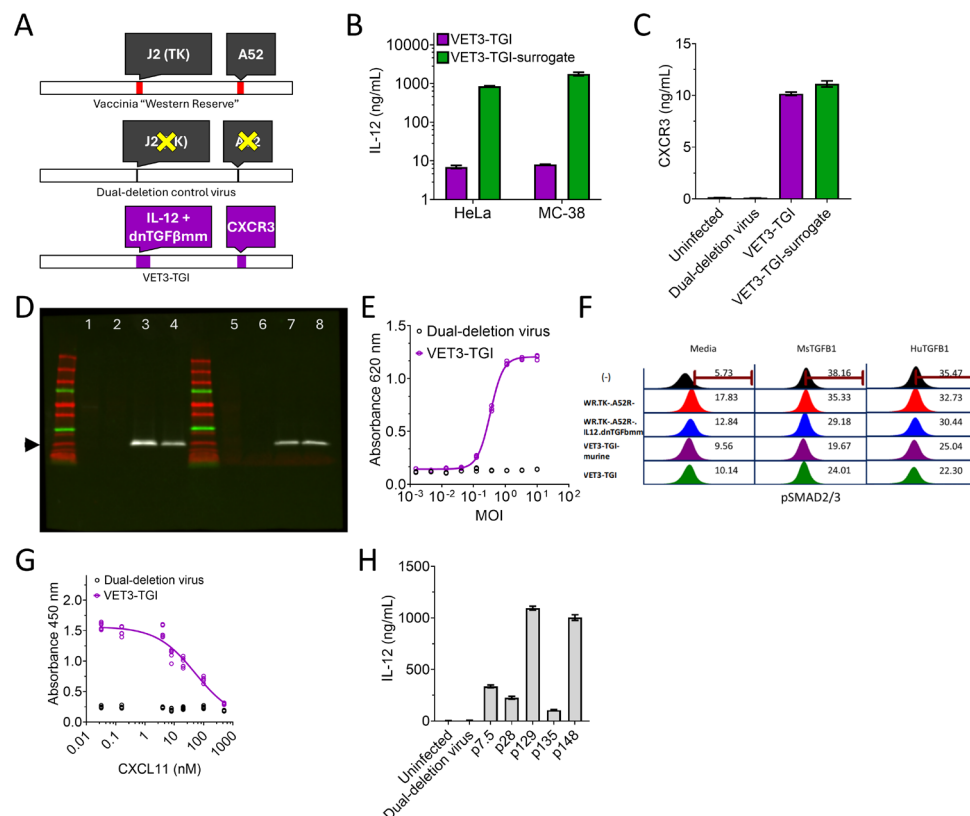


Figure 1 VET3-TGI is a recombinant vaccinia virus that produces biologically active IL-12, dnTGFβmm, and CXCR3 in place of virulence factors J2 (thymidine kinase) and A52. (A) The genome of vaccinia is depicted showing approximate locations of virulence factor genes *J2R* and *A52R* and modifications implemented to make the ‘dual-deletion’ control virus and VET3-TGI. (B) Presence and biological activity of VET3-TGI components: IL-12 in conditioned medium was quantified using species-specific ELISA kits targeting the p70 heterodimer to confirm expression (bars mean with SD, each $n=4$). Control virus IL-12 production was below limit of quantitation (not plotted). (C) CXCR3 protein from VET3-TGI and surrogate-infected cells was quantified by ELISA (mean with SD, $n=2$). (D) Expression of dnTGFβmm protein from VET3-TGI and VET3-TGI-surrogate infected human (lanes 1–4) and murine (lanes 5–8) cell lines was confirmed by western blot analysis on SDS-denatured samples; loading order: uninfected, dual-deletion control-infected, VET3-TGI-infected, surrogate-infected. Arrow: 15 kDa marker; predicted mature MW 14 kDa. (E) IL-12 signaling induced by conditioned medium from control virus-infected cells was compared with VET3-TGI-infected cells across a range of infection MOIs using a reporter cell line. Increased absorbance corresponds to more IL-12 reporter activation (points: all measurements, $n=3$ per MOI, solid line: 4PL fit). (F) Phospho-SMAD2/3 was used to measure TGF-β pathway activation by either human or murine TGF-β1 recombinant protein. Conditioned medium from uninfected or various virus-infected cells was used to block receptor signaling complex assembly on reporter cells, enabling detection of inhibitory activity. (G) Cell surface CXCR3 on infected cells was measured after treatment with various concentrations of CXCL11, with loss of signal corresponding to ligand-induced endocytosis (points: all measurements, $n=3$ per concentration, solid line: 4PL fit). (H) IL-12 expression from viruses under different promoters (p7.5, p28, p129, p135, p148) measured by ELISA (bars: mean with SD, $n=2$ per promoter). 4PL, four-parameter logistic; CXCL11, C-X-C motif chemokine 11; CXCR3, C-X-C chemokine receptor 3; IL-12, interleukin-12; MOI, multiplicity of infection; MW, molecular weight; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor beta.

the *A52R* locus. Since human IL-12 is non-functional in mice,²⁹ a functional surrogate ‘VET3-TGI-surrogate,’ was constructed for in vivo mouse studies by replacing the human single-chain IL-12 in VET3-TGI with a sequence encoding the murine equivalent. A third virus, ‘VET3-TGI-murine,’ was constructed in the same manner as VET3-TGI to carry murine CXCR3 and murine single-chain IL-12 in addition to dnTGFβmm.

Ultracentrifuge purification of recombinant vaccinia virus

Recombinant viruses were expanded in HeLa human cervical carcinoma cells grown in five-layer flasks. For each typical lot, six confluent five-layer flasks were infected at a

multiplicity of infection (MOI) of 0.02 for 2 days. Infected cells were washed, dissociated, and then lysed using 10 mL of hypotonic buffer (10 mM Tris base, pH 9.0), assisted by three freeze-thaw cycles from -80°C to room temperature. Infectious lysate was treated sequentially with Benzonase (Millipore, 1.01697.0010; 180 μL ; ≥ 45 kU, 60 min) and 10X TrypLE (Thermo Scientific, A1217702), diluted to 1X in the lysate for 60 min at room temperature before mechanical Dounce homogenization. The homogenate was clarified by low-speed centrifugation (4°C , $500 \times g$, 10 min) in a swinging-bucket rotor. Virus from the supernatant was purified by density using sucrose-gradient ultracentrifugation.

Bioreactor purification of VET3-TGI-surrogate

HeLa cells from six freshly confluent T-175 flasks were used to seed a preconditioned iCellis Nanopilot-scale bioreactor equipped with mPATH controller. Cells were cultured under 5% CO₂ with gentle circulation in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS for 6 days. Cells were quantified by nuclei counting after carrier lysis. Virus was introduced to achieve a target MOI of 0.002 in fresh, prewarmed medium. Infection proceeded at 36°C and 5% CO₂ with gentle circulation for 3 days. The bioreactor bed contents were washed after medium draining using Tris buffer (pH 7.3) to remove residual medium, then infected cells were lysed by circulating incubation in hypotonic buffer (Tris, pH 9.6 with magnesium chloride and Benzonase). The resulting infectious lysate was clarified, treated with Benzonase for at least 48 hours, and then purified by tangential flow filtration (TFF) using a preprimed KrosFlo KR2i (Repligen) system fitted with 0.1 µm-rated hollow-fiber polysulfone membrane. The sample was concentrated, then soluble contaminants were removed by diafiltration for a minimum of 20 sample volumes before final concentration in 20 mM Tris, pH 7.8, with 8.5% w/v sucrose. After passage through a 1.2 µm depth filter, the virus preparation was supplemented with polysorbate-80 (PS-80) to achieve a final concentration of 0.01% w/v PS-80.

TFF-purified virus was tested for comparability with ultracentrifuge-purified virus and no differences in biological activity were observed. Virus prepared with this method was used in one experiment and so noted in the corresponding figure legend.

Quantitation of virus infectivity by plaque assay

Virus titers were measured using a plaque assay. Monolayers of U-2OS human osteosarcoma cells in 6-well CellBIND plates (Corning, 3335) were infected with virus serially diluted in serum-free McCoy's 5A base medium (Gibco, 16600-082) for 2 hours. Infectious medium was then removed and replaced with overlay medium (complete medium containing 1.5% carboxymethylcellulose). Plaques were allowed to form undisturbed for 3 days. Infected monolayers were gently rinsed with phosphate-buffered saline (PBS) and then stained for 1 hour at room temperature with crystal violet (CV) staining solution (0.1% CV in 20% aqueous ethanol). Unbound stain was removed by rinsing with distilled water. Plaques on dried plates were counted by a minimum of two operators per well. Wells from dilutions yielding fewer than 30 or more than 300 plaques were excluded from analysis. All multiplicity of infection calculations were performed using values measured in U-2 OS cells.

Comparison of viral replication in cancerous versus non-cancerous cultured cells

Cultured cell lines (HeLa, A549, MC-38, LLC, HFF-1, and MEF) were infected at an MOI of 1.0 by VET3-TGI or VET3-TGI-surrogate for 1 hour, with three biological

replicates prepared concurrently per condition. Infectious medium in each well was then replaced with complete medium. Infected cells were grown for 24 hours. Cells and medium were collected and frozen/thawed (−80°C/37°C) to release virus particles. Crude lysates were sonicated. Titers of lysates were measured by plaque assay.

Detection, quantitation, and in vitro biological activity of VET3-TGI transgenes

CXCR3: HeLa cells infected (MOI: 1.0, 1 day) with VET3-TGI, VET3-TGI-surrogate, or controls (dual-deletion virus and uninfected HeLa cells) were lysed (50 mM Tris buffer, 150 mM NaCl, 1% Triton X-100) with protease inhibitors, followed by freeze-thaw cycles (−80°C/37°C). Supernatants were collected after sonication and centrifugation. CXCR3 ELISA was performed using a commercial reagent kit (R&D Systems, DY008B), capture antibody (BioLegend, 353702 RRID:AB_10983073), detection antibody (Abcam, ab288446; RRID:AB_3675773), and horseradish peroxidase (HRP) conjugate (Abcam, ab205718; RRID:AB_2819160). Quantitation was performed using diluted reference standards (R&D Systems, H00002833-P01-2ug). Activation-induced CXCR3 internalization was measured using cell-based ELISA on cells infected (MOI: 1.0, 6 hours) with either dual-deletion control virus or VET3-TGI. Different concentrations of human CXCL11 (PeproTech, 300-46-100UG) were added (30 min, 37°C). Samples were chilled on ice and fixed with 2% paraformaldehyde in PBS. External CXCR3 was labeled using clone 1C6 (BD Biosciences, 557183; RRID:AB_396594), followed by HRP conjugate (Promega, W402B; RRID:AB_430834). Absorbance from tetramethylbenzidine substrate (Thermo Scientific, 34028) was measured using a Spark 10M plate reader (Tecan). For surface-specific labeling of A549 and HeLa cells, cells were infected (MOI: 20). After the indicated durations, cells were dissociated and fixed with paraformaldehyde. Cells were washed and CXCR3 was labeled with a fluorescent conjugate (BD Biosciences, 561730; RRID:AB_397008) at 4.0 µg/mL. MC-38 cells were infected (MOI: 5, overnight). Dissociated cells were washed with PBS and stained with Zombie Yellow (BioLegend, 423104), followed by phycoerythrin (PE)-conjugated anti-CXCR3 (BioLegend, 126506; RRID:AB_1027650). Signal from washed samples was collected via Attune NxT flow cytometer.

IL-12: HeLa cells were infected (MOI: 5.0, 18 hours) with viruses containing murine single-chain IL-12 under various vaccinia promoters. Conditioned medium was clarified by centrifugation and IL-12 was quantified by ELISA targeting the p70 heterodimer (RayBiotech, ELM-IL12P40P70-1). VET3-TGI and surrogate were assayed similarly using both HeLa and MC-38 cell lines paired with species-appropriate ELISA kits (Thermo Scientific, BMS238 and BMS6004). Production of biologically active IL-12 from HeLa cells infected at different MOIs for 1 day with VET3-TGI was measured by treating reporter cells (InvivoGen, hkb-il12; RRID:CVCL_UF31) with 0.1

µm-filtered conditioned medium. Reporter activity was measured using a Tecan Spark 10M plate reader.

TGF-β inhibitor (dnTGFβmm): HeLa cells were infected (MOI: 5.0, 1 day) with VET3-TGI or VET3-TGI surrogate alongside controls: dual-deletion virus and uninfected HeLa. Medium was removed, and cells were treated with 2X Laemmli buffer (Bio-Rad, 1610737) containing 5% β-mercaptoethanol, subjected to freeze-thaw cycles (−80°C/37°C), and centrifuged. The supernatants were heated (50°C, 10 min) before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, 4561094) and subsequent transfer to polyvinylidene difluoride membrane (Bio-Rad, 1704156). The membrane was rinsed (Tris-buffered saline plus 0.05% v/v Tween-20; Acros Organics, 23336-0010), blocked with 5% non-fat cow's milk (Research Products International, M17200-1000.0), rinsed again, and probed (Cell Signaling Technology, 3709S; RRID:AB_2063357) for 24 hours before HRP attachment (Abcam, ab205718). Enhanced chemiluminescence signal (Bio-Rad, 1705061) was detected using an Azure 600 (Azure Biosystems). **Phospho-SMAD2/3 cytometry:** HeLa cells were infected (MOI: 10, 1 day) with VET3-TGI or VET3-TGI-murine, dual-deletion virus, and non-infected controls to make conditioned medium. A20 cells were treated (30 min) with filtered (0.1 µm) conditioned medium and then stimulated (30 min) with recombinant TGF-β1 (murine, R&D Systems, 7666-MB-005/CF or human, PeproTech, AF-100-21C, 50 ng/mL). Treated cells were washed, fixed with 4% formaldehyde, rinsed (BD Biosciences, 554656), and resuspended with chilled methanol. After rinsing, cells were stained using a phospho-SMAD2/3 antibody at a 1:100 dilution (BD Biosciences, 562696; RRID:AB_2716578). Unbound stain was rinsed away. Stained cells were suspended in 4% formaldehyde, and fluorescence was captured using an Attune NxT flow cytometer (Invitrogen). For reporter assays, conditioned medium was prepared similarly (except MOI: 5) and applied to the reporter cell line (Signosis, SL-0016) for 2 hours. Recombinant TGF-β1 (PeproTech) was added to the final concentrations shown. Signal was developed using a Bright-Glo luciferase assay kit (Promega, E2620) and measured using a Spark 10M plate reader.

Detection of CXCR3 expression on infected peripheral blood mononuclear cells

Frozen peripheral blood mononuclear cells (PBMCs) from three different donors were thawed, washed with complete medium (RPMI-1640 with 10% FBS), counted, and spinoculated in a swinging-bucket rotor (23°C, 935 × g, 60 min) at a target MOI of 5.0 with either dual-deletion control virus or VET3-TGI. Cells were collected after infection and placed in a cell culture incubator overnight alongside the uninfected control. Cells were collected, washed, stained with Zombie Yellow (BioLegend, 423103), washed again, and resuspended in a marker staining mixture containing anti-CD14-BV711 (BioLegend, 301838; RRID:AB_2562909),

anti-CD20-Alexa Fluor 647 (BioLegend, 302318; RRID:AB_493229), anti-CD34-BV421 (BioLegend, 119321; RRID:AB_10900980), and anti-CXCR3-PE (BioLegend, 353706; RRID:AB_10962912), each at a 1:100 dilution in FBS staining buffer (BD, 554656). Stained cells were washed and fixed with 4% paraformaldehyde. Data were collected with compensation and unstained controls using an Attune NxT flow cytometer (Invitrogen). Population percentages were measured after excluding dead cells.

In vitro migration of VET3-TGI-infected PBMCs

Human PBMCs from leukapheresis cones (STEMCELL Technologies, 200-0093) were purified by Ficoll gradient centrifugation, quantified, and infected with VET3-TGI or the dual-deletion control virus at an MOI of 5. PBMCs were spinoculated in a swinging-bucket rotor (935 × g, 60 min) and loaded onto the apical side of a migration chamber insert. Transwell inserts (Corning, 3422) were loaded into reservoirs in which CXCL9-expressing MC-38 were seeded, and cell migration occurred overnight. Cell types that migrated across the porous membrane into the lower chamber were labeled using antibodies against cell type-specific markers (1:100 dilution): anti-CD20-Alexa Fluor 647 (B cells, BioLegend, 302318); anti-CD33-PE (myeloid cells, BioLegend, 303404; RRID:AB_314348); and anti-CD3-BV421 (T cells, BioLegend, 300434; RRID:AB_10962690). Vaccinia virus-infected MC-38 cells were labeled with antivaccinia virus L1 Fab-Atto 488 conjugate at 1:200 dilution (Absolute Antibody, AB00774-23.0; custom fluorophore conjugation). Samples were analyzed using an Attune NxT flow cytometer (Invitrogen). MC-38 tumor cells were collected, and cells infected with vaccinia virus were stained with fluorescently labeled anti-L1 Fab and quantified. Uninfected MC-38 cells and control virus were used to compare VET3-TGI-induced specific migration and increased delivery of virus to MC-38 cells. Identical tests were performed using four other viruses that express CXCR3 alongside different transgene payloads to demonstrate general applicability. Data analysis was performed using FCS Express 7 analysis software.

Murine ligand-induced migration of human CXCR3-expressing T cells

Human buffy coat (Vitalant, Leukopak, 2 mL) was washed once with cell culture medium (RPMI-1640 with 10% FBS) and centrifuged in a swinging-bucket rotor (23°C, 336 × g, 5 min). The recovered cells were resuspended in 20 mL of cell culture medium (RPMI-1640 with 10% FBS) supplemented with human IL-2 to 1 U/mL and incubated at 37°C overnight. The stimulated cells were passed through a strainer and enumerated, then diluted to 5×10⁶ cells/mL. One million cells were seeded per Transwell insert (Corning, 3422) and loaded into reservoirs containing either human or murine CXCL9 (PeproTech, human: 031087-1; murine: 314166), CXCL10 (PeproTech, human: 081539A; murine: 1213153), or CXCL11 (PeproTech, human: 911151; murine: 0202232)

at 10, 100, or 1000 ng/mL, with no-chemokine controls. Migration proceeded at 37°C for 90 min. Inserts were discarded, and migrated cells were collected from the reservoirs after centrifugation. Pellets were resuspended in labeling solution containing a 1:200 dilution of each of CD3-BV421, CD4-APC/Fire750, and CD8-Alexa Fluor 488 (BioLegend, 300434, 357426; RRID:AB_2716183; and 301021; RRID:AB_2561281, respectively) in buffer (BD Bioscience, 1308379). Cells were washed twice and fixed with 4% paraformaldehyde for analysis of the CD3+ population (T cells).

Animal studies

All preclinical studies involving live animals were conducted in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities at either Hilltop Lab Animals (Scottsdale, Pennsylvania) or Translation Drug Development laboratories (Scottsdale, Arizona). All studies complied with and were performed in accordance with Institutional Animal Care and Use Committee-approved protocols. All C57BL/6 mice received closed-loop isoflurane anesthesia during tumor implantation procedures and intratumoral injections. For all other procedures, animals were manually restrained. Animals were closely monitored for signs of morbidity, at which time they were promptly euthanized by carbon dioxide inhalation, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Death was not used as an endpoint in any experiment.

For syngeneic tumor model studies, Renca cells were cultured in RPMI-1640 supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin/streptomycin. Female BALB/c mice aged 8–10 weeks were implanted with 4×10^5 cells/mouse (suspended in Hanks' Balanced Salt Solution (HBSS)) subcutaneously in the lateral dorsolumbar region. EMT-6 cells were cultured in Waymouth's medium supplemented with 15% FBS and 1% penicillin/streptomycin. Female BALB/c mice aged 8–10 weeks were implanted with 8×10^5 cells/mouse (suspended in HBSS) subcutaneously in the lateral dorsolumbar region. MC-38 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Female C57BL/6 mice aged 8–10 weeks were implanted with 5×10^5 cells/mouse (suspended in HBSS) subcutaneously in the lateral dorsolumbar region. All implantation calculations used viable cell quantification from an Invitrogen Countess II cell counter with trypan blue exclusion.

Tumors were allowed to grow to an average of about 50 mm³. Tumor volumes were calculated by measuring the length and width of each tumor in millimeters and applying the modified ellipsoid approximation: volume (in mm³) = $0.5 \times \text{length} \times \text{width}^2$. Mice were sorted so that each treatment group had the same average tumor volume. HLA staff, blinded to treatment conditions, performed tumor measurements twice weekly and weight measurements weekly. Mice were sacrificed in cases of

extreme weight loss (>20%), paralysis, cachectic appearance, or excessive tumor burden (1000 mm³ for Renca, and 1400 mm³ for EMT-6 and MC-38). In all studies of therapeutic efficacy, a complete response (CR) due to treatment was defined as a tumor volume of <100 mm³ that had previously measured at least 50 mm³.

Virus preparation for in vivo mouse studies

Virus was sonicated in closed tubes immersed in chilled water at 75% amplitude for a total of 120 s in 20 s on/off intervals (Qsonica Q125 equipped with cup horn), then diluted in a solution of 10% sucrose in 10 mM Tris base, pH 8.0 (VFB), to the required working concentrations. For intratumoral treatments, viruses were diluted to 1×10^7 plaque-forming units (PFU) in 60 µL and injected via a 28 G insulin syringe directly into the tumor. For intravenous infusion treatments, viruses were diluted to either 1×10^7 PFU in 100 µL, 1×10^6 PFU in 100 µL, or 1×10^5 PFU in 100 µL and given via tail vein using a 27 G insulin syringe. All preparations were performed immediately before treatment.

Animal studies of virus delivery to tumors

Subcutaneous Renca implantations were performed as stated elsewhere in the 'Methods' section. Engrafted BALB/c mice were treated 14 days postimplantation by tail-vein injection of 1×10^7 PFU of '+CXCR3' virus (vaccinia virus encoding a leptin-IL-2 fusion in the TK locus and CXCR3 in the A52R locus) or with '-CXCR3' virus (vaccinia virus encoding a leptin-IL-2 fusion in the TK locus, with A52R deleted). Each treatment group contained 10 animals. Mean tumor volume was 59 mm³ at time of treatment. BALB/c mice were implanted with EMT-6 (8×10^5 cells/mouse) and subcutaneous tumors grew for 13 days, averaging a volume of 217.2 mm³ on treatment day. Mice (n=5/group) were treated by tail vein with 1×10^7 PFU of either VET3-TGI-surrogate or an otherwise identical virus lacking CXCR3. C57BL/6 mice bearing MC-38 tumors (mean volume, 104.6 mm³) implanted as described in the 'Methods' section (n=5/group) were treated by tail-vein injection 13 days postimplantation with 1×10^7 PFU of either VET3-TGI-surrogate or an otherwise identical virus lacking CXCR3. For all virus delivery studies, at 18–24 hours post-treatment, subjects were sacrificed. Tumors were excised and frozen in liquid nitrogen for viral genome quantitation by quantitative PCR (qPCR).

Carcinogen-induced tumors

The carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (N-nitrosamine ketone (NNK); CAS 64091-91-4; Toronto Research Chemicals, TRC-M325750) was administered to FVB mice (n=48; Jackson Laboratory, 001800; RRID:IMSR_JAX:001800) by intraperitoneal injection. Individual doses contained 3.0 mg of NNK in 200 µL of sterile warmed PBS. Induction spanned 4 weeks, with three doses given per week. Mice were allocated to treatment groups (vehicle: n=18; dual-deletion control: n=15;

and VET3-TGI surrogate: $n=15$ /per group) and monitored for 13 weeks after the last NNK treatment. Virus solutions were prepared for intravenous dosing as stated elsewhere in the 'Methods' section and given by tail vein at 1×10^7 PFU/mouse, establishing treatment day 0, with final group sizes of vehicle: $n=17$; dual-deletion control: $n=15$; and VET3-TGI-Surrogate: $n=13$). Treatments were repeated on day 2. Subjects were rendered unresponsive by isoflurane and sacrificed 2 weeks after initial treatment. Lungs were inflated by gravity-driven perfusion with 10% neutral buffered formalin, dissected, submerged in buffered formalin for 1 day, and then transferred into ethanol before nodules were counted.

Quantitation of virus genomes from infected tissue by qPCR

Tumor weights were measured to the nearest one-tenth of a milligram (Mettler, MS104TS), then homogenized with 1.0 mL of Qiagen Buffer ATL by ceramic bead beating in four 30 s agitation intervals (4°C , 6 m/s, 50% duty cycle; Fisherbrand Bead Mill 24). The homogenate was incubated overnight with proteinase K at 56°C , then held at 65°C for 4 hours to eliminate infectivity. DNA was recovered after heat treatment using the DNeasy Blood and Tissue Kit (Qiagen) in accordance with the supplied protocol and quantified by absorbance using a Tecan Spark 10M plate reader equipped with a NanoQuant plate. Purified DNA was used as the template for qPCR at either 1:10, 1:100, 1:1000, or 1:10000 dilution, as appropriate, using assay diluent (Qiagen Buffer AE with 1.0 $\mu\text{g}/\text{mL}$ of sheared salmon sperm DNA). Primers (900 nM) and probe (250 nM) targeting the viral gene *E9L* were combined with PrimeTime 2X Master Mix containing ROX (Integrated DNA Technologies, Coralville, Iowa, USA) to achieve the indicated final concentrations. Each final reaction mixture, prepared in MicroAmp optical 96-well reaction plates (Applied Biosystems, N8010560), consisted of 15.0 μL of working master mix, to which 5.0 μL of control, reference standard, or sample was added. The reference standard was prepared at defined concentrations from a linearized plasmid (3058 bp, synthesized by Genewiz) hosting a copy of the target sequence. Amplification (95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 62°C for 1 min) was performed using a QuantStudio 6 Flex instrument (Life Technologies, 4485689). Genome copies were calculated by comparison with reference wells using the instrument software. Values were converted to copies per unit of sample mass using the initial sample weights.

Screening of untreated murine tumor models for CXCL9, CXCL10, and CXCL11 expression

Mice (aged 8–10 weeks) were implanted to produce subcutaneous tumors for chemokine screening, with five mice allocated per cell line. Renca cells were implanted into BALB/c mice (4×10^5 cells/mouse), and tumors were allowed to grow for 13 days before harvest. B16-F10 cells, mixed at a 1:1 ratio in chilled Matrigel Matrix HC (Corning, 354248), were implanted into C57BL/6 mice

(2×10^4 cells/mouse) and allowed to grow for 11 days before harvest. EMT-6 cells were implanted into BALB/c mice (1×10^5 /mouse) and allowed to grow for 10 days before harvest. MC-38 cells were implanted into C57BL/6 mice (5×10^5 cells/mouse) for 11 days before harvest. LLC cells were implanted into C57BL/6 mice (4×10^5 cells/mouse) for 8 days before harvest. Tumors, excised immediately after sacrifice, were plunged into liquid nitrogen and stored at -80°C before analysis. Tumors were thawed, weighed, and homogenized using a Bead Mill (same settings as for qPCR) in PBS containing protease inhibitors (Thermo Scientific, A32965, 20012-027). Quantification of CXCL9 and CXCL10 in homogenates was carried out by ELISA (RayBiotech, ELM-MIG and R&D Systems, DY466-05, respectively) in accordance with the kit-supplied protocols. Signal was measured using a Tecan Spark 10M plate reader; 4PL fitting and interpolation of measured values to standard curves were performed using GraphPad Prism V.9.0.1.

Serum cytokine and inflammation marker detection via LEGENDplex

Female C57BL/6 mice aged 8–10 weeks were implanted with 5×10^5 MC-38 cells. Mean tumor volume was approximately 100 mm^3 on initiation of intravenous treatment on days 0, 3, and 7 with 1×10^7 per dose of either VET3-TGI-murine or a CXCR3-lacking control virus with IL-12 and dnTGF β mm transgenes. Approximately 6 hours after the final treatment, animals were sacrificed and whole blood was collected in heparinized tubes. Cytokines were quantified using LEGENDplex (BioLegend, 740150, 741023). Sera from treated animals were diluted 1:3 in supplied Assay Buffer before applying to beads for 2 hours at room temperature with agitation. Washes, application of the detection antibody, and streptavidin-PE were performed in accordance with the kit manual. Data acquisition was performed using an Attune NxT flow cytometer (Invitrogen). Population median fluorescence intensities (MFIs) were converted to cytokine concentrations using the manufacturer's analysis suite. Each quantified cytokine was compared between treatments (VET3-TGI-murine vs control) using Student's t-test (GraphPad Prism).

Immunofluorescence assessment of T-cell infiltration of virus-treated tumors

Tumors were grown to an average volume of 62 mm^3 in BALB/c mice after subcutaneous implantation of 4×10^5 Renca cells per animal before treatment with either vehicle buffer, dual-deletion control virus, or VET3-TGI-murine (viruses administered at 1×10^7 PFU by tail-vein injection). MC-38 tumor-bearing C57BL/6 mice were treated with the same viruses and doses at a mean group tumor volume of 55 mm^3 . Subjects were sacrificed, and tumors were collected 9 days post-treatment, frozen in optimal cutting temperature compound, and sliced into 10 μm sections. Slices were immersed in 4% paraformaldehyde solution, then rinsed with PBS and blocked by sequential treatment with BLOXALL (Vector Laboratories, SP-6000),

avidin-biotin (Vector Laboratories, SP-2001), and PBS containing 1% bovine serum albumin (BSA) and Tween-20 (Millipore, 655204-100ML). Biotin-antibody conjugates were applied in sequence with streptavidin-HRP (Thermo Scientific, N100) to achieve tyramide-amplified, antigen-specific labeling with anti-CD3-biotin×CF532 (BioLegend, 100244; RRID:AB_2563947×Biotium, 9066), followed by anti-CD8a-biotin×CF488 (BioLegend, 100704; RRID:AB_312743×Biotium, 92171), with each antibody prepared in labeling buffer (Biotium, 22029). Sections were then labeled for granzyme B using antigranzyme B-Alexa Fluor 647 conjugate (BioLegend, 515406; RRID:AB_2566333). The remainder were stained for cytokeratin (BioLegend, 914204; RRID:AB_2616960), CD45 (TONBO Biosciences, 30-0451 U100×Biotium, 96006), and CD31 (BioLegend, 102404; RRID:AB_312899×Biotium, 96022). Cellular nuclei were labeled with Hoechst 33342 (Biotium, 40046). Rinsed slices were mounted using EverBrite medium (Biotium, 23001). Labeled tumor slices were imaged using a CellInsight CX7 LZR spinning-disk confocal microscope (Thermo Scientific) equipped with a 20× air objective. Cells were typed using fluorescent markers and enumerated in ImageJ (National Institutes of Health) to present cell counts per field alongside representative images. Each enumeration was performed manually using 15–25 fields acquired randomly from five tumor sections.

Tumor CXCL9, CXCL10, and CXCL11 quantitation by LEGENDplex

Female C57BL/6 mice were implanted with cultured LLC cells (4×10^5 cells/mouse). Tumors attained a mean volume of 253 mm³ before intratumoral treatment with either TK-lacking control virus or the IL-12-equipped virus (single dose, 1×10^7 PFU). Female BALB/c mice were implanted with cultured Renca cells (4×10^5 cells/mouse). Tumors attained a mean volume of 192 mm³ before intratumoral treatment with either the TK-lacking control virus or IL-12-equipped virus (single dose, 1×10^7 PFU). Animals were sacrificed 24 hours post-treatment. Tumors were frozen in liquid nitrogen. After thawing, sample weights were measured to the nearest one-tenth of a milligram (Mettler, MS104TS), then samples were homogenized with 1.0 mL of PBS supplemented with protease inhibitors (1% final concentration, Life Technologies, A32965) by ceramic bead beating in four 30 s agitation intervals (4°C, 6 m/s, 50% duty cycle, Fisherbrand Bead Mill 24), followed by a 5-minute rest period. Total protein was quantified by bicinchoninic acid assay (Pierce Rapid Gold BCA Protein Assay Kit, Life Technologies, A53225) using included BSA standards. Lysates were diluted 1:5 using LEGENDplex assay buffer to create assay samples. Cytokines were quantified using LEGENDplex kits (BioLegend, 741067 and 740007). Briefly, all reagents and standards were prepared in accordance with kit manual, and the filter plate was prewetted and then emptied. Standards or samples were added to the assay plate, with each well mixing 25.0 µL of assay buffer and

25.0 µL of standard or sample. Beads were then added (25.0 µL per well) to the assay plate. The assay plate was shaken at 500 rpm at room temperature for 2 hours and washed twice with the kit wash buffer. Detection antibodies were added (25.0 µL per well) prior to a 60 min shake (500 rpm) at room temperature. Washed as previously stated, the sample plate was then incubated with streptavidin-PE conjugate (25.0 µL per well) and shaken at 500 rpm at room temperature for 30 min. The plate was washed twice, the beads were suspended, and briefly shaken with 300 µL per well of wash buffer, and then data were collected using an Attune NxT flow cytometer. Population MFIs were converted to cytokine concentrations using the manufacturer's analysis suite and presented as pg analyte/µg of total protein. Analyte abundance was compared between treatment groups using GraphPad Prism V.10.6.1.

Detection of antivaccinia neutralizing antibodies

Female C57BL/6 mice were implanted subcutaneously with MC-38 (5×10^5 cells each) and assorted into treatment groups (n=20/group) with a mean tumor volume of 117 mm³. Intravenous treatments were performed by tail-vein injection of 1×10^7 PFU of virus (one of the following per group: (1) dual-deletion control, (2) CXCR3+ virus, (3) IL12+ virus, (4) IL12+/dnTGFβmm+ virus, or (5) VET3-TGI-murine). From each treatment group, five mice were sacrificed for blood collection on post-treatment days 7, 10, 15, and 21. Brachial bleeds were performed on sedated unresponsive animals, with blood collected into EDTA tubes (Kent Scientific, 3072122) for centrifugation to obtain plasma, which was diluted 1:100 in PBS with 0.05% Tween-20, 'wash buffer,' prior to use. ELISA plates (Corning EIA, 3590), coated overnight at 4°C with dual-deletion virus in PBS, were emptied, then incubated at room temperature for 60 min with 2% Fraction V BSA (Research Products International, 9048-46-86) in PBS (blocking buffer), and then washed five times with wash buffer. Standards were prepared as a 12-step, twofold dilution series of a rabbit antivaccinia polyclonal antibody (Abcam, ab35219-1001; RRID:AB_778768) in blocking buffer. Standards (in triplicate) or samples (biological replicates) were placed into coated plates at 50 µL per well, rested at room temperature for 90 min, and washed five times with wash buffer. Wells were loaded with HRP-conjugate: goat antirabbit IgG HRP (Thermo Scientific, 31466; RRID:AB_10960844), diluted 1:5000 in wash buffer, was added to reference standard wells, while goat antimouse IgG HRP (Thermo Scientific, A16066; RRID:AB_2534739), diluted similarly, was added to wells containing samples. After resting at room temperature for 30 min, the plate was washed five times and loaded with complete substrate (equal parts of Thermo Scientific, 1854060, 1854050). Substrate conversion was halted by addition of 2 M sulfuric acid and absorbance (450 nm) was read immediately using a Tecan Spark 10M plate reader.

Gene set enrichment analysis of virus-treated MC-38 and Renca tumors

Female BALB/c mice were implanted subcutaneously with 4×10^5 Renca cells and tumors were allowed to grow to a mean volume of 95 mm^3 . C57BL/6 mice were implanted subcutaneously with either 5×10^5 of MC-38 cells or 4×10^5 of LLC cells, which attained mean tumor volumes of 101 mm^3 and 166 mm^3 , respectively. All mice were then treated by tail-vein injection at a dose of 1×10^7 PFU with either TK-deleted control virus, virus expressing dnTGF β mm, virus expressing IL-12, or virus expressing both dnTGF β mm and IL-12. On each of post-treatment days 3 and 8, five mice were sacrificed and tumors harvested, two of which were sectioned, embedded in OCT, and stored overnight at -80°C under isopentane. A Leica CM UV cryostat was used to remove and discard the outer surface (approximately $25 \mu\text{m}$) of each frozen section from day 8 harvests. Then $10 \mu\text{m}$ slices were cut from the freshly exposed surface and mounted onto slides for GeoMX Digital Spatial Profiling analysis. Immune marker labeling, transcript recovery, data acquisition, mapping (murine whole-transcriptome atlas), and quality filtering were performed by Azenta Life Sciences. One row assigned to symbol 'NegProbe-WTX,' containing non-integer count values, was removed from the raw read count.

In MC-38 samples, regions with immune infiltrate (CD45) were preferentially selected from slices for analysis: four regions from one tumor (TK-lacking virus), six regions from one tumor (dnTGF β mm-only virus), three regions from one tumor (IL-12-only virus), and two regions from one tumor (IL-12 and dnTGF β mm combo virus) were selected. Gene expression from these within-tumor spatial subsets were analyzed using a limma-voom workflow (V.3.66.0) in RStudio (Posit Software V.2026.01.0): QC-filtered non-normalized read counts from each region were assigned to groups by virus treatment, with contrasts assigned to compare expression associated with each treatment virus (IL-12 only, dnTGF β mm only, or the combo) against the TK-lacking control virus. Each gene from each sample was scored by multiplying $-\log_{10}(\text{p value})$ by the computed logFC. The gene list was then sorted (descending values) and analyzed using the gene set enrichment analysis (GSEA) function of clusterProfiler³⁰ (V.4.18.4) by comparing samples against all curated gene sets (50) within the mouse-ortholog hallmark collection.³¹ The normalized enrichment scores of enriched gene sets (adjusted $p \leq 0.05$, Benjamini-Hochberg post hoc) from the IL-12 vs TK-minus contrast were compared with sets enriched from the IL-12+dnTGF β mm combo vs TK-minus contrast.

Renca samples were processed using the same method with no changes: two regions (TK-lacking virus), five regions (dnTGF β mm-only virus), four regions (IL-12-only virus), and two regions (IL-12 and dnTGF β mm combo virus) comprised the analyzed data set. As with MC-38 samples, for each treatment condition, all regions of interest were drawn within the same tumor slice.

Biological pathway analysis of VET3-TGI-treated MC-38 and Renca tumors

Female C57BL/6 mice were implanted subcutaneously with 5×10^5 MC-38 cells and treated by tail-vein injection with either vehicle, 1×10^5 PFU of dual-deletion control virus, or 1×10^5 PFU of VET3-TGI-murine virus after attaining an average tumor volume of 58 mm^3 . Additionally, female BALB/c mice were implanted subcutaneously with 4×10^5 Renca cells and treated by tail-vein injection with either vehicle, 1×10^7 PFU of dual-deletion control virus, or 1×10^7 PFU of VET3-TGI-murine after reaching an average tumor volume of 62 mm^3 . Treatment groups for both tumor types were sacrificed on days 3 and 9 post-treatment. Tumors were homogenized in Lysing Matrix D tubes (MPBio, 6913100) containing Qiazol (Qiagen, 79306). RNA was purified for RNA sequencing using a miRNeasy Micro kit (Qiagen, 74106) according to manufacturer's instructions. One sample per virus treatment was sent to Azenta Life Sciences for library preparation and NGS. Per Azenta's service description, library preparation was completed in accordance with the manufacturer's protocol using the NEBNext Ultra II RNA Library Prep Kit (New England Biolabs), which consisted of enrichment of polyadenylated transcripts by oligo-dT capture, followed by fragmentation, first-strand and second-strand complementary DNA synthesis, and terminal adapter ligation. Data acquisition was performed using an Illumina HiSeq instrument with a 2×150 bp paired-end configuration. Image analysis and base calling were conducted using the HiSeq Control software from Illumina. Illumina's bcl2fastq 2.17 software was employed to de-multiplex and convert the raw .bcl sequencing data into .fastq file format.

The .fastq paired-end Illumina data were uploaded into a local instance of Galaxy V.25.0.³² Read quality for each sample was verified using FastQC (Galaxy V.0.74+galaxy1).³³ Each sample's paired reads were aligned to the *Mus musculus* GRCm39 reference genome (RefSeq ID: GCF_000001635.27) using HISAT2 (Galaxy V.2.2.1+galaxy1).³⁴ Mapped transcripts for each sample were converted to raw read count matrices using featureCounts (Galaxy V.2.1.1+galaxy0).³⁵ Differential gene expression was analyzed by limma-voom (Galaxy V.3.18.0+galaxy0).³⁶ Virus treatment and harvest days were defined as factors. Contrast was established for virus treatment levels by subtracting the dual-deletion virus group from the VET3-TGI-murine virus group. Reads were filtered at a threshold of 1.0 counts per million and normalized by Trimmed Mean of M-values (TMM). Gene symbols and corresponding t-statistics were sorted to make a preranked gene data file. Gene set enrichment was detected in RStudio using fast gene set enrichment analysis (fgsea³⁷ for each tumor type against 'allograft rejection,' 'inflammatory response,' 'interferon-gamma signaling,' and 'TGF- β signaling' murine gene sets from Molecular Signatures Database (MSigDB). Coordinated expression trends were plotted based on the gene rank and its corresponding enrichment score for all genes present in the preranked

data file. P values were adjusted to control false discovery rate per the Benjamini-Hochberg method.

Statistical analyses

Except RNA sequencing data analyses, tests for significant differences were performed using GraphPad Prism (V.10). The comparison and tests applied are stated alongside each instance where a comparison outcome is reported as 'significant' or 'not significant' in 'Results' section and display item captions.

RESULTS

VET3-TGI expresses functional transgenes in infected cells

VET3-TGI hosts genes enabling expression of IL-12 and a TGF- β inhibitor 'dnTGF β mm' in place of viral gene *J2R*, and CXCR3 in place of viral gene *A52R* (figure 1A). Secretion of IL-12 from VET3-TGI and a surrogate (with murine IL-12) was confirmed using commercial ELISA kits (figure 1B); expression of CXCR3 by infected cells was similarly verified by ELISA, and placement on the surface of human and murine cells was confirmed by flow cytometry (figure 1C, see online supplemental material). Production of the TGF- β inhibitor was confirmed by western blot analysis (figure 1D) since commercial ELISA kits are not available for this protein. The virus dose-dependent secretion of biologically active IL-12 from VET3-TGI-infected cells into medium was confirmed using a reporter cell line (figure 1E). Suppression of TGF- β 1-induced SMAD2/3 phosphorylation and activity in a TGF- β signaling reporter cell line were used to verify the activity of the dnTGF β mm protein (figure 1F, see online supplemental material). Dose-dependent agonist-induced internalization of CXCR3 was verified by treating infected cells with the natural agonist CXCL11 and monitoring surface CXCR3 by cell-based ELISA (figure 1G).

Intratumoral delivery of vaccinia virus expressing IL-12 inhibits tumor growth

To qualitatively explore whether IL-12 or TGF- β inhibition exerts antitumor activity, the corresponding viruses were used to treat Renca tumor-bearing BALB/c mice at 1×10^7 PFU single dose via intratumoral injection, in comparison with TK-lacking (disrupted) control virus and vehicle-only treatments. Treatment with the IL-12-expressing virus caused profound tumor growth inhibition, with eight subjects lacking detectable tumors at 56 days post-treatment. All treatments proved well tolerated. Unlike what we have reported previously in other models,³⁸ TGF- β inhibition alone did not provide a therapeutic advantage over the TK-lacking (disrupted) control in the Renca model (see online supplemental material).

Early viruses designed to test intravenous delivery of IL-12 or IL-12 and TGF- β inhibiting transgenes displayed toxicity on intravenous delivery in B16-F10-bearing or Renca-bearing mouse models. These viruses used the early and late promoter p7.5 to drive strong constitutive IL-12 expression after infection. Weak late-only

promoters, to restrict IL-12 expression to cells that are permissive to virus replication, were explored. Yields from viruses encoding IL-12 under the core promoter sequences controlling open reading frames VACWR129, VACWR135, and VACWR148, and p28 and p7.5, were compared by ELISA (figure 1H). The core promoter sequence from VACWR135 was adopted for subsequent virus designs as 'p135.'

Gene expression profiling of tumors shows elevated CXCL9, CXCL10, and CXCL11

Human gene expression profiling revealed many diseases in which tumor expression of CXCL9, CXCL10, CXCL11, or a combination of these chemokines was greater than expression from normal tissues—a prerequisite for enhanced tumor targeting with CXCR3 (see online supplemental material).

Murine CXCL9, CXCL10, and CXCL11 induce migration through human CXCR3

Migration assays using different concentrations of the murine chemokines CXCL9, CXCL10, and CXCL11 were performed to test whether human CXCR3 responds to these murine orthologs of its natural ligands. Human PBMC-derived T cells, known to naturally contain a CXCR3⁺ subset, migrated in a dose-dependent fashion toward all three cognate murine homologs (see online supplemental material). Murine chemokines displayed similar or clear-but-weaker induction of migration via human CXCR3 compared with human chemokines.

VET3-TGI confers CXCR3-mediated migration of infected cells in vivo

To investigate whether VET3-TGI-driven expression of CXCR3 compels infected hematopoietic cells to migrate toward cognate chemokines CXCL9, CXCL10, and CXCL11, human PBMCs were purified from blood and infected with dual-deletion control virus or VET3-TGI. Infection with VET3-TGI increased the percentage of PBMCs positive for CXCR3 labeling compared with both the control virus and uninfected cells, confirming receptor expression (figure 2A). Migration of infected PBMCs toward CXCL9/10/11-expressing MC-38 cells was used to compare the chemotactic responsivity conferred by control virus versus VET3-TGI. More migrating cells were obtained from VET3-TGI-infected PBMCs compared with those infected with the control virus. The CD20⁺ population (B cells) is the principal migrating component of the population (figure 2B). The delivery of virus by PBMCs to the tumor itself was also measured by counting virus-infected (vaccinia L1-positive) MC-38 cells in each migration chamber: chambers that hosted VET3-TGI-infected PBMCs contained more L1-positive MC-38 cells than chambers that drew control virus-infected PBMCs (figure 2C).

CXCL9 and CXCL10 in subcutaneous tumors grown from B16-F10, EMT-6, Renca, LLC, or MC-38 cell lines were quantified to inform model selection for in vivo

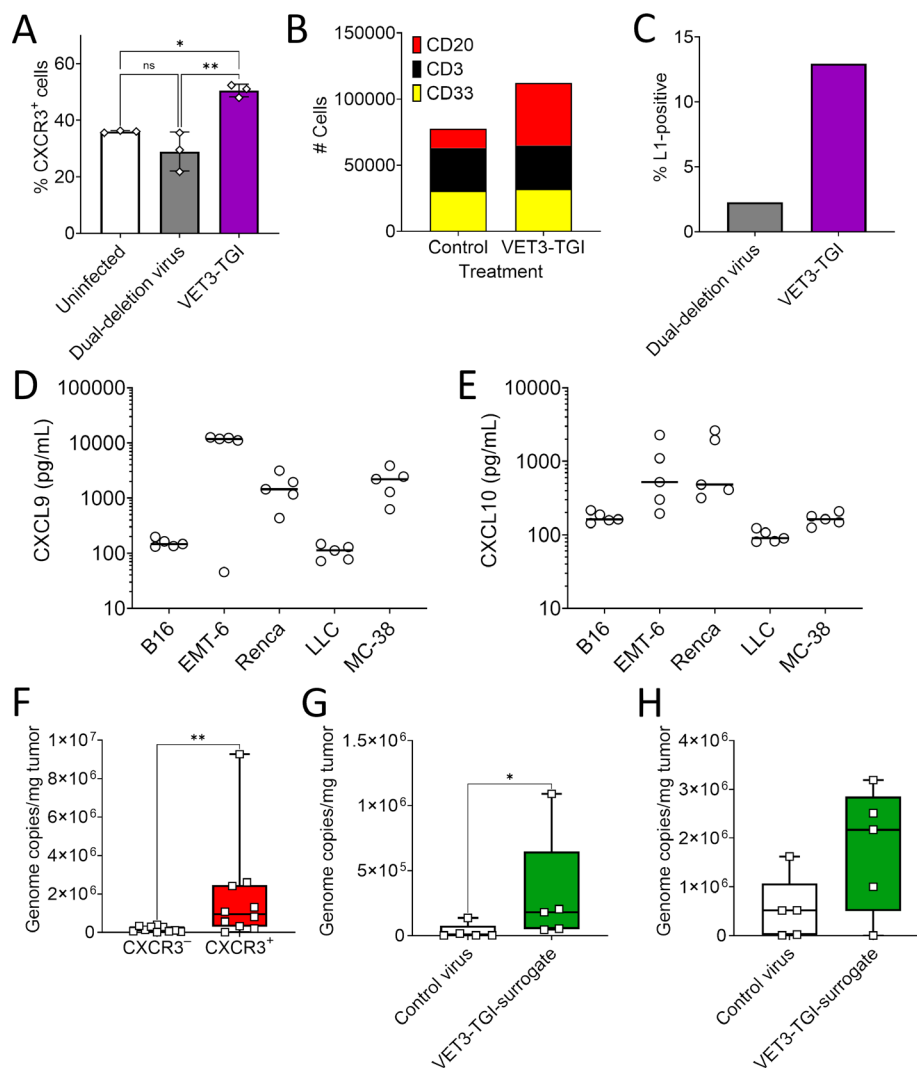


Figure 2 VET3-TGI uses CXCR3 to enable migration of B cells toward CXCL9 and CXCL10, causing increased virus delivery in in vitro and in vivo systems. (A) Extracellular CXCR3 was labeled on primary human PBMCs after infection with VET3-TGI or controls; gates were drawn on CXCR3 signal after compensation and exclusion of dead cells. Data from each of three donors per condition are plotted as points; bars indicate the mean with SD, * $p \leq 0.05$, ** $p \leq 0.01$, one-way ANOVA, Tukey post hoc test. (B) Migrating cells from human PBMCs infected with VET3-TGI or dual-deletion control virus were quantified by flow cytometry after cell type-specific labeling. (C) Delivery of VET3-TGI was compared with dual-deletion control virus by detecting infected (viral L1) MC-38 cells resulting from CXCR3-directed migration of infected PBMCs across Transwell membranes. Panels B and C display data from one assay outcome representative of results from five different CXCR3-equipped viruses as bar plots; see the 'Methods' section. (D) CXCL9 and (E) CXCL10, chemokines that activate CXCR3, were quantified from tumor lysates prepared from the indicated mouse models by ELISA. Symbols indicate measurements from individual tumors constituting biological replicates; lines indicate medians ($n=5$). Concentrations are plotted on a logarithmic scale. (F) Intravenous delivery efficacy between otherwise identical CXCR3-equipped and CXCR3-lacking viruses in mice hosting subcutaneous Renca tumors was compared by qPCR, with results visualized by box plot (whiskers: extrema; points: biological replicates, $n=10$ /group, $p=0.0089$, Mann-Whitney U test). (G) Delivery efficacy of VET3-TGI-surrogate was compared against an otherwise identical CXCR3-lacking analog in a subcutaneous EMT-6 tumor model ($n=5$ /group, $p=0.0317$, Mann-Whitney U test). (H) Delivery of VET3-TGI-surrogate was assessed similarly in animals hosting MC-38 tumors ($n=5$ /group, $p=0.2222$, Mann-Whitney U test). ANOVA, analysis of variance; CXCL9, C-X-C motif chemokine ligand 9; CXCL10, C-X-C motif chemokine ligand 10; CXCR3, C-X-C motif chemokine receptor 3; LLC, Lewis lung carcinoma; ns, not significant; PBMCs, peripheral blood mononuclear cells; qPCR, quantitative PCR.

studies of CXCR3-directed delivery. EMT-6 lysates contained the most CXCL9, while Renca and MC-38 tumor lysates yielded intermediate amounts. B16 and LLC tumors yielded negligible CXCL9. All tumors produced CXCL10: EMT-6 and Renca exhibited relatively high concentrations, while B16 and MC-38 tumors yielded

lower levels. LLC tumors contained the lowest amount of CXCL10 (figure 2D,E).

Intravenous delivery of CXCR3-expressing virus versus delivery of the receptor-lacking control virus was therefore compared using the identified tumorigenic cell line models (Renca, MC-38, and EMT-6) by quantifying viral

genomes in tumors shortly (18–24 hours) after tail-vein injection. Renca tumors (figure 2F) showed a clear and significant ($n=10$, $p\leq 0.01$, Mann-Whitney U test) increase in viral genomes detected within tumors of mice treated with CXCR3-equipped virus versus those treated with the receptor-lacking control. Similar trends with other pairs of CXCR3-equipped versus lacking viruses were observed in both MC-38 (figure 2G) and EMT-6 (figure 2H) subcutaneous tumor models, establishing a correlation between CXCR3 expression and enhancement of virus delivery to the tumor by intravenous route.

VET3-TGI inhibits or reverses tumor growth in immunocompetent animals

To test if TGF- β inhibition enhances the antitumor activity of IL-12, a virus hosting both transgenes in the TK locus was compared against the IL-12-equipped virus and against TK-deleted control virus or vehicle treatment. Zero CRs were observed in vehicle-treated mice ($n=20$, all groups), while intratumoral treatment of subcutaneous Renca tumors with a single dose (1×10^7 PFU) of control virus produced CR in 20% of treated animals (figure 3A). Same-dose treatment using virus equipped with IL-12 alone resulted in 35% CR. Addition of the TGF- β inhibiting transgene to the IL-12 virus doubled the CR to 70%. Groups treated with viruses hosting IL-12 or the IL-12 and TGF- β inhibitor combination achieved greater overall survival over the 67-day study period following initial treatment. To answer whether tumor eradication requires persistence of the recombinant virus and corresponding expression of its transgenes, or whether bona fide antitumor adaptive immunity had arisen, surviving CR mice were re-challenged by implantation with Renca cells. All mice previously treated with IL-12-equipped or IL-12 plus dnTGF β mm-equipped viruses failed to grow tumors during the 36-day observation period following rechallenge.

To assess the antitumor performance of a vaccinia virus combining delivery-enhancing CXCR3 with IL-12 and dnTGF β mm, tumor-bearing (EMT-6: figure 3B or MC-38: figure 3C) mice were treated by tail-vein injection with vehicle control, dual-deletion control virus at 1×10^7 PFU, or VET3-TGI-murine at varying doses (1×10^5 to 1×10^7 PFU). No CR occurred in groups treated with vehicle or with the dual-deletion control virus. Half of the EMT-6 implanted mice treated at 1×10^5 PFU achieved CR, and 90% of those treated at 1×10^6 were tumor-free at the end of the study. In groups hosting faster-growing MC-38 tumors, no CR occurred in mice given vehicle, dual-deletion control virus, or dosed with 1×10^5 PFU of VET3-TGI-murine. However, survival analyses from these studies (figure 3D–F) revealed that this lowest dose in MC-38 conferred a benefit in median survival (group $n=10$, $p=0.0003$, Mantel-Cox) when compared with treatment at 1×10^7 with control virus. CRs prevailed at higher doses of VET3-TGI-murine, with 70% CR observed among the 1×10^6 -treated group and 90% at the highest treatment dose; median survival at these higher doses could not be

confidently extrapolated due to insufficient mortality within these groups.

To establish whether VET3-TGI is therapeutically effective in non-subcutaneous models of cancer, FVB mice hosting NNK carcinogen-induced tumors were treated with vehicle, dual-deletion control virus, or VET3-TGI-surrogate. Lung tumors were counted 2 weeks post-treatment. Control virus treatment was equally ineffective as vehicle treatment ($p=0.3669$, one-way analysis of variance (ANOVA), Dunnett's post hoc test). Mice treated with VET3-TGI-surrogate formed significantly fewer nodules than vehicle-treated mice ($p=0.0158$), with the majority of this treatment group remaining free of visible cancer (figure 3G).

VET3-TGI and surrogate replicate better in cancerous versus non-cancerous cells

The ability of VET3-TGI and VET3-TGI-surrogate to replicate in cancerous cell lines (HeLa, A549, MC-38, LLC) was compared with non-cancer cells (HFF-1 and MEF). Viral titers from infected cell lysates were measured by plaque assay. A549 and HeLa supported significantly greater production of progeny virus (figure 4A and $p\leq 0.05$ and $p<0.0001$, respectively via two-way ANOVA, Sidák's post hoc test). Similarly in murine cells, infection of both MC-38 and LLC resulted in higher titers than those from MEF cells (figure 4B, $p\leq 0.0001$ and $p<0.05$, respectively).

Inclusion of CXCR3 in VET3-TGI reduces markers of systemic toxicity

To examine the effects of CXCR3-assisted tumor delivery on systemic toxicity, cytokine release syndrome factors, and inflammation-associated markers in sera of virus-treated tumor-bearing mice were quantified using LEGENDplex. VET3-TGI-murine was compared with an analog without CXCR3 and with A52R intact (figure 4C–K). Serum concentrations of inflammation-associated cytokines interferon (INF)- α , IL-6, C-C motif chemokine ligand 2, and tumor necrosis factor (TNF)- α were significantly higher ($p\leq 0.05$, t-test) in mice treated with CXCR3-lacking virus compared with mice treated with VET3-TGI-murine. IL-10, a component of compensatory signaling to regulate inflammation, was significantly elevated in control virus-treated mice ($p=0.0041$, t-test). Differentiation of circulating levels of IFN- γ was hampered since samples from control virus-treated mice exceeded the maximum quantifiable limit of the assay. IFN- γ signaling promotes expression of CXCL9, which was measured significantly higher in serum from control virus-treated mice ($p=0.0366$). Circulating IL-12 and CXCL10 also decreased in animals treated with VET3-TGI-murine, but differences were not significant ($p=0.1690$ and $p=0.1998$, respectively).

VET3-TGI treatment specifically increases cell-mediated immunity signatures

To characterize immune infiltrates linked to tumor regression, tumors from animals treated either with

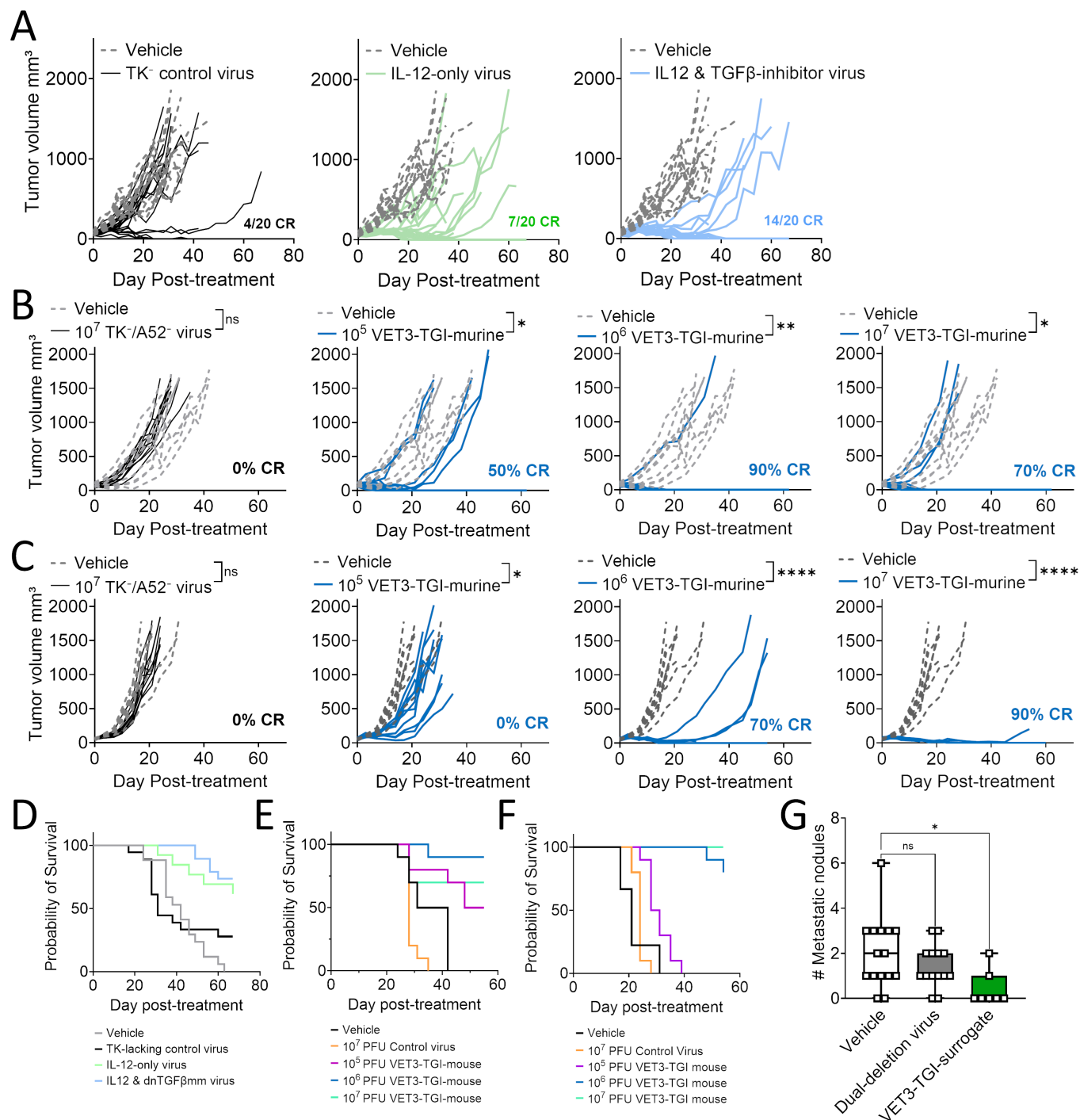


Figure 3 Vaccinia virus equipped with both IL-12 and dnTGF β mm demonstrates superior antitumor activity and inclusion of CXCR3 enables systemic delivery by intravenous treatment. (A) Tumor growth inhibition by volume was assessed between groups of Renca tumor-bearing mice treated by intratumoral injection with either vehicle buffer or the indicated recombinant vaccinia virus (each $n=20$): left: vaccinia with inactivated TK gene; middle: vaccinia virus with IL-12, right: vaccinia virus with both IL-12 and TGF- β inhibitor transgenes; CR (where tumor volume shrank to $<100\text{mm}^3$) count/total group count. (B) Tumor volume was monitored over time after intravenous treatment of BALB/c mice bearing EMT-6 tumors with either 1×10^7 PFU of TK-/A52 $^-$ control virus or indicated escalating doses of VET3-TGI-murine, versus vehicle control ($n=10/\text{group}$). (C) Same as B, but with C57BL/6 mice bearing MC-38 tumors. Groupwise comparisons were performed on median survivals from corresponding Kaplan-Meier analyses; ns, $p>0.05$, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$; Mantel-Cox test with Holm-Šidák's post hoc correction for multiple comparisons. (D), (E), (F) Kaplan-Meier survival analyses of subjects from A, B, and C, respectively, which were used to calculate and compare the effect of treatment on median survival times. (G) Postmortem count of lung metastases in an NNK-induced cancer model is displayed as box plot; * $p<0.05$, one-way ANOVA on counts (vehicle $n=15$; dual-deletion virus $n=14$; VET3-TGI-surrogate $n=7$), with treatments compared with vehicle using Dunnett's post hoc test. ANOVA, analysis of variance; CR, complete response; CXCR3, C-X-C motif chemokine receptor 3; IL-12, interleukin-12; NNK, N-nitrosamine ketone; ns, not significant; PFU, plaque-forming units; TGF- β , transforming growth factor beta; TK, thymidine kinase.

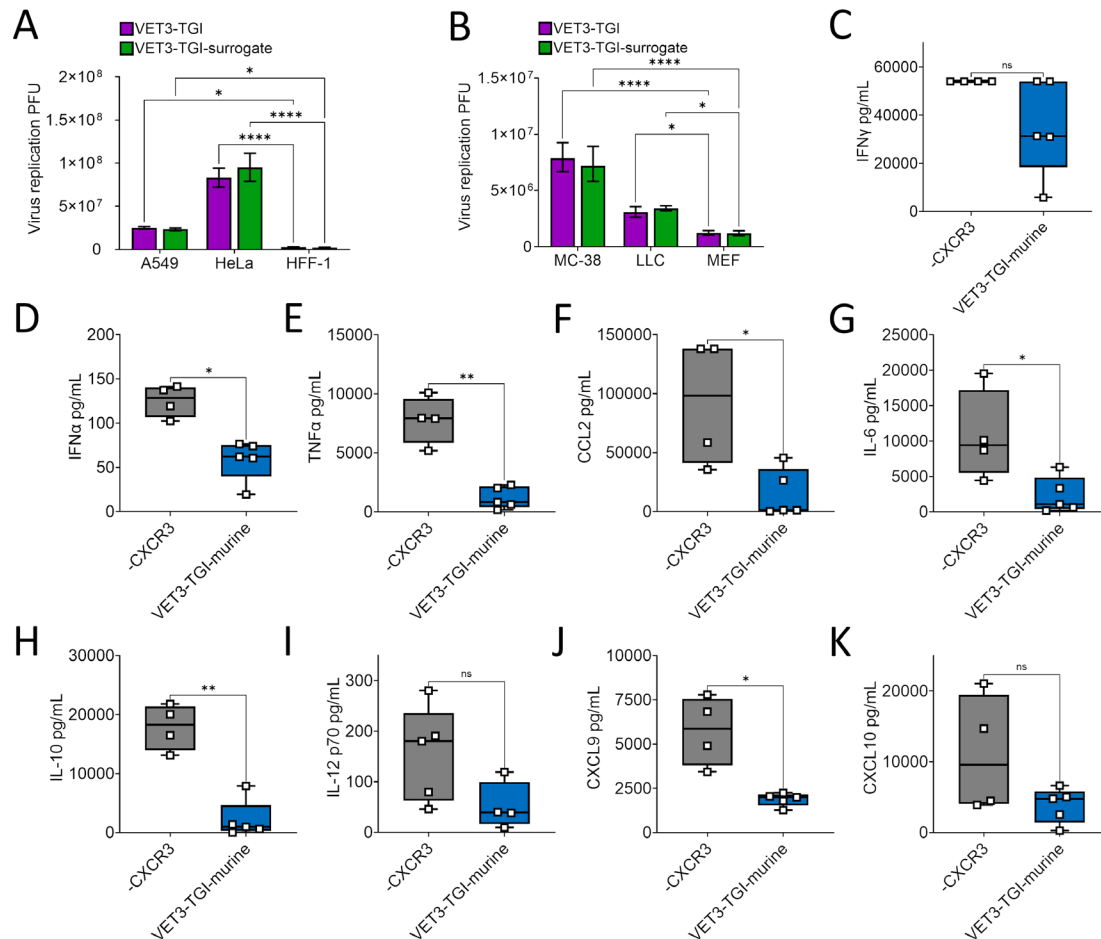


Figure 4 VET3-TGI preferentially replicates in cancerous over non-cancerous cells, and systemic toxicity is subdued by inclusion of CXCR3. (A) Indicated human cancerous (A549, HeLa) and non-cancerous (HFF-1) cells were infected for 1 day and lysate titers quantified by plaque assay. Bars indicate mean with SD, $n=3$; comparisons via two-way ANOVA, Šidák's post hoc test, $*p\leq 0.05$, $****p\leq 0.0001$. (B) Same as A to compare replication in murine cancerous (MC-38, LLC) versus non-cancerous (MEF) cell lines. Panels A and B used TFF-purified VET3-TGI-surrogate. (C–K) Mice treated three times with 1×10^7 PFU per dose ($n=5$ /group) of VET3-TGI-murine or a CXCR3-lacking analog ('-CXCR3' with intact A52R) were sacrificed, and circulating indicators of systemic toxicity from serum were quantified for display as box plots (whiskers: extrema; points: individual biological replicates). Differences between treatment outcomes were assessed by t-test, $*p\leq 0.05$, $**p\leq 0.01$. A549, human lung carcinoma; ANOVA, analysis of variance; CXCR3, C-X-C motif chemokine receptor 3; HFF-1, human foreskin fibroblasts; HeLa, human cervical cancer; LLC, Lewis lung carcinoma; MC-38, murine colon adenocarcinoma; MEF, murine embryonic fibroblasts; ns, not significant; PFU, plaque-forming units; TFF, tangential flow filtration.

vehicle control, dual-deletion virus, or with VET3-TGI-murine were collected and stained for CD3, CD8, and granzyme B, or cytokeratin, CD45, and CD31 markers. Renca tumors treated with control virus trended toward modestly elevated counts of CD45+, CD3+, and CD8+ cells, differing significantly in number with CD3 and CD8 markers ($p\leq 0.0001$, one-way ANOVA, Tukey post hoc test) from those of vehicle-treated mice (figure 5A,B). Tumors from animals treated with VET3-TGI-murine displayed significantly greater amounts of infiltrate positive for these markers, corresponding to increased recruitment of B-cell, T-cell, and NK-cell subsets when compared with either the control virus or vehicle ($p\leq 0.0001$, one-way ANOVA, Tukey post hoc test). These outcomes were consistent in MC-38 tumors (figure 5C).

To explore how IL-12 changes the chemokine profile of tumors, the IL-12-associated chemokines CXCL9,

CXCL10, and CXCL11 were quantified in LLC and Renca tumors collected 24 hours postintratumoral treatment with controls or an IL-12-equipped virus. LLC tumors demonstrated significant elevation ($p\leq 0.005$, one-way ANOVA with Tukey post hoc test) of CXCL9 (mean $38.7\text{ pg}/\mu\text{g}$ total protein, SD 16.3) after treatment with IL-12-equipped virus compared with both TK-lacking control virus and vehicle (figure 6A). CXCL10 increased modestly when treated with IL-12-equipped virus versus the TK-lacking control ($p=0.0210$) but remained comparable with vehicle treatment. Different treatments caused no significant changes in CXCL11 produced in LLC tumors. Renca tumors displayed striking changes when treated with IL-12-equipped virus: CXCL9 increased 10-fold (mean $352.6\text{ pg}/\mu\text{g}$ total protein, SD 251.8). Similarly, we observed significantly ($p\leq 0.05$) elevated CXCL10 production linked to IL-12 (figure 6B). Treatment with

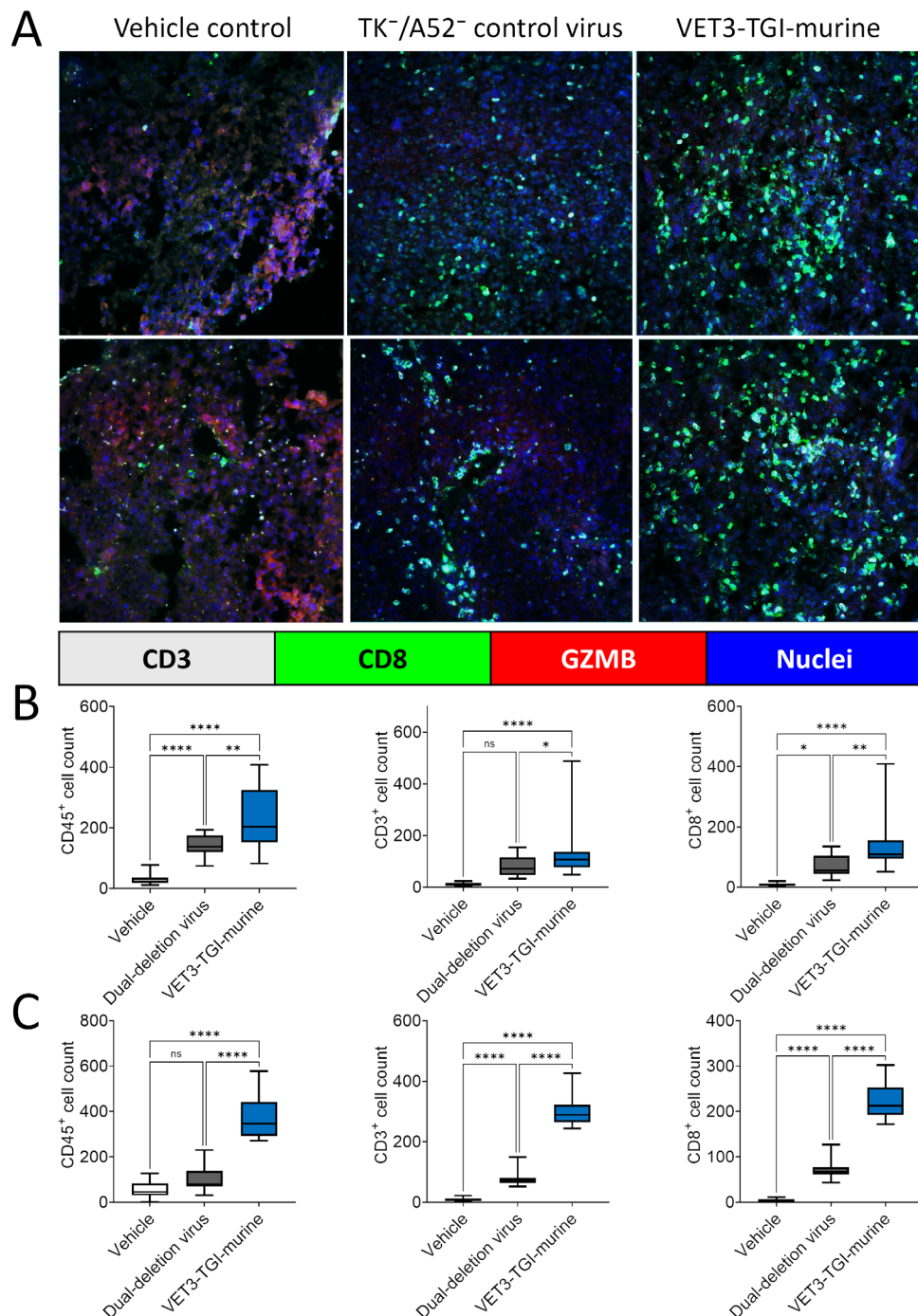


Figure 5 VET3-TGI treatment increases Th1-associated tumor immune infiltrates. (A) Images were acquired using a CellInsight CX7 LZR confocal microscope. Renca tumors treated intravenously with either vehicle control, dual-deletion control virus, or VET3-TGI-murine were sectioned and stained for CD3, CD8, and GZMB; two representative fields are presented. A separate stain set was used to enumerate CD45⁺ cells (not shown). (B) Samples treated as in panel A were counted manually from 15 fields per treatment condition (acquired from sections from five different tumors) and displayed as box plots (whiskers: extrema). Group counts were compared by one-way ANOVA with Tukey post hoc test; ns, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. (C) The IF imaging experiment and analysis described in panels A and B was repeated using an MC-38 tumor model. ANOVA, analysis of variance; GZMB, granzyme B; IF, immunofluorescence; MC-38, murine colon adenocarcinoma; ns, not significant; Th1, T helper 1.

the IL-12-equipped virus elevated CXCL11 expression when compared with vehicle but not when compared against treatment with the TK-lacking control ($p = 0.0943$). Together, these results link IL-12 to increased tumor reservoirs of the inflammation-associated and

CXCR3-recruiting chemokines CXCL9, CXCL10, and CXCL11.

To investigate the effect of virus treatment on the T helper (Th)2-directing activity of Th cells, induction of vaccinia-reactive antibodies, an indicator of humoral

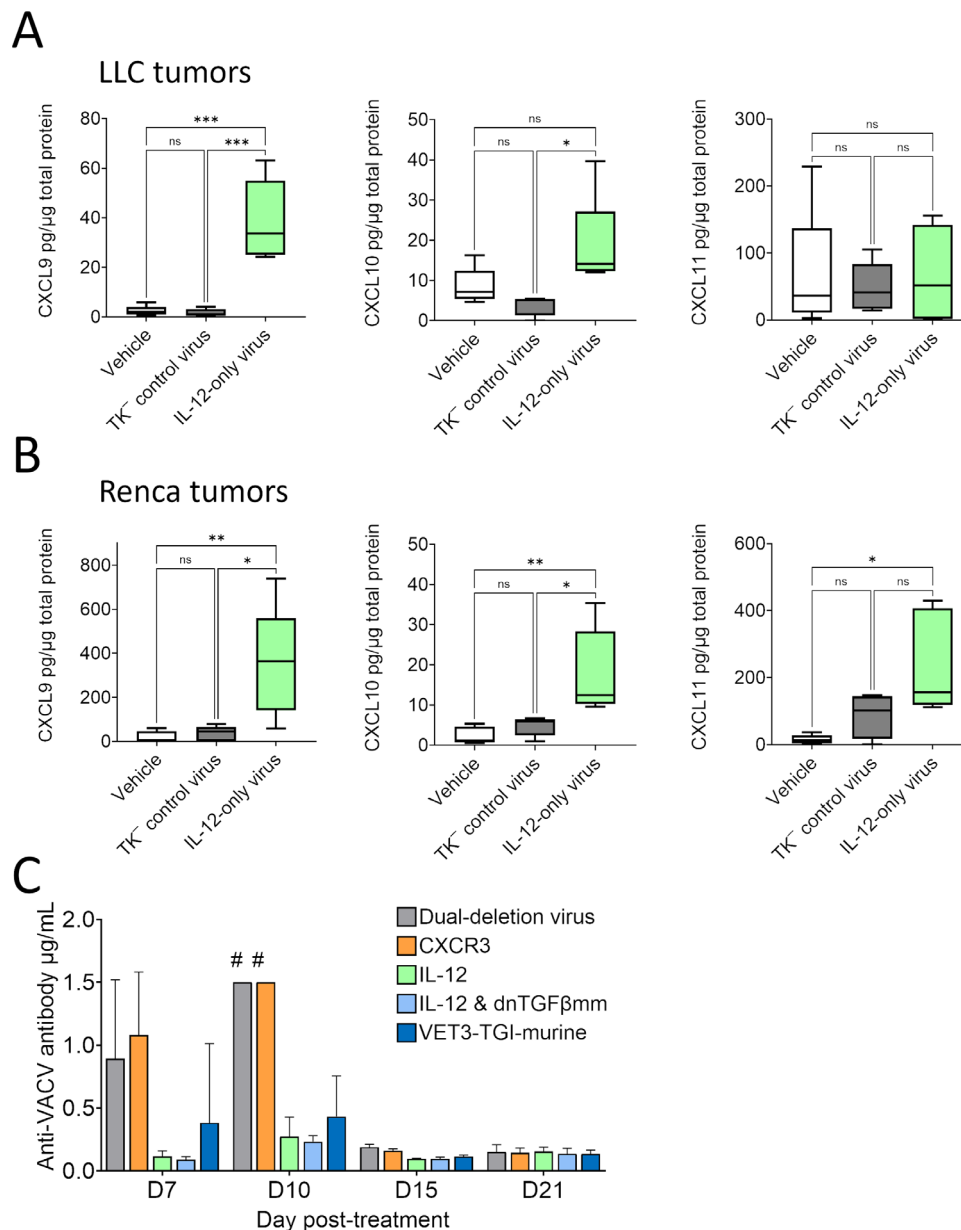


Figure 6 IL-12 invokes markers of Th1-promoting type II IFN-driven immunity. (A) Cytokines linked to CXCR3 delivery and IFN- γ signaling were quantified in tumor lysates obtained from LLC tumor-bearing mice after indicated treatment or control. Left, CXCL9. Middle, CXCL10. Right, CXCL11 measurements displayed as box plots (whiskers: extrema). Differences between each treatment group (CXCL11 treatment group IL-12 virus: n=4; all others n=5) were assessed by one-way ANOVA with Tukey post hoc test, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.005. (B) Repeat of A using Renca-bearing mice (CXCL11 treatment group TK-deleted control virus: n=4; all others n=5). (C) Vaccinia-reactive antibodies from MC-38 tumor-bearing mice treated with indicated viruses were quantified at multiple times post-treatment. Symbols (#) indicate samples that exceeded the quantifiable range of the assay. Bars: mean with SD, n=5/group. ANOVA, analysis of variance; CXCL9, C-X-C motif chemokine ligand 9; CXCL10, C-X-C motif chemokine ligand 10; CXCL11, C-X-C motif chemokine ligand 11; CXCR3, C-X-C motif chemokine receptor 3; IFN- γ , interferon-gamma; IL-12, interleukin-12; LLC, Lewis lung carcinoma; MC-38, murine colon adenocarcinoma; Th1, T helper 1; TK, thymidine kinase.

response, was measured after treatment of mice with viruses containing different components of VET3-TGI. Virus-reactive antibody titers were elevated on days 7 and 10 post-treatment in mice dosed with dual-deletion virus or with CXCR3-only virus (figure 6C). Lower titers were seen following treatment with all viruses carrying IL-12. Titers decreased to low levels across all treatment groups by day 21. Differences between IL-12

containing viruses versus IL-12-lacking viruses were significant (p $\leq$ 0.005, two-way ANOVA with Dunnett's post hoc test) in samples collected on day 15. Confidences were not calculated for differences observed on day 10 due to oversaturation of the assay by samples treated with IL-12-lacking viruses. Overall, IL-12-equipped viruses provoked weaker humoral responses than those without.

TGF- β inhibition strengthens antitumor gene signatures invoked by IL-12

Transcriptome changes in MC-38 tumors were explored to contrast gene expression repertoires induced after treatment with IL-12-only virus, or the combination of IL-12 and dnTGF β mm transgenes. Differential expression invoked by each treatment (IL-12 or 'combination') was calculated relative to MC-38 tumors treated with a TK-lacking control virus. GSEA³⁹ was applied to identify coordinated changes. All 50 sets in the murine hallmark collections (Molecular Signatures Database) were screened for enrichment. Combo-treated tumors showed positive enrichment in gene sets associated with Th1-directed antitumor activity: IFN- γ response, allograft rejection, and inflammatory response. Treatment with IL-12 only and with the combination caused depletion in gene sets associated with cell cycle progression and drivers of EMT. Importantly, depletion of WNT/ β -catenin and TGF- β signaling sets was detected only with combo-treatment (figure 7A). Renca tumors were treated and analyzed identically to MC-38. Increased enrichment of antitumor gene sets reflected those observed in MC-38, although IL-12 alone also produced significant positive enrichment scores. Positive enrichment of cholesterol homeostasis and unfolded protein response gene sets were observed from IL-12-treated samples, while IL-2/STAT5 signaling, TNF- α signaling via NF- κ B, and EMT signatures increased only with combination treatment (figure 7B).

In a separate study, transcriptome remodeling caused by intravenous infusion of VET3-TGI murine was compared with those similarly treated with dual-deletion control virus in MC-38 and Renca tumors harvested days 3 and 9 post-treatment. Treatment-dependent transcript changes, controlling for harvest day as a factor, were scored and analyzed by fgsea against hallmark gene sets specific to tumor rejection (allograft rejection, inflammatory response, and IFN- γ) and TGF- β signaling (figure 7C). Enrichment probability values were adjusted for false discovery rate per Benjamini-Hochberg. Corroborating our previous study and GSEA analysis, all tumor rejection-related gene sets showed positive enrichment scores, while depletion was indicated across the TGF- β signaling gene set. Increased transcription linked to allograft rejection and IFN- γ response meets a stringent adjusted $p \leq 0.05$ threshold, whereas changes to the inflammatory response and TGF- β signaling had slightly weaker confidence (adjusted $p = 0.05927$ and $p = 0.10810$, respectively). Renca tumors were similarly analyzed to MC-38 tumors. All tumor rejection-associated gene sets had increased enrichment, while the TGF- β signaling gene set was suppressed (all adjusted $p \leq 0.05$) (figure 7D).

DISCUSSION

After a surge in approvals of novel cancer immunotherapeutics, many recent and initially promising therapies have stumbled in the clinic, re-emphasizing the need for

new targets and approaches. Oncolytic viral immunotherapies remain a promising approach with a high potential for inducing a potent immune response against the tumor because they enable coordinated expression of multiple immune activators from within the cancer itself. However, the complexity of this approach and the limited ability to deliver these therapies intravenously has reduced the historical excitement in this field.

To date, intravenous dosing of vaccinia virus has depended on non-targeted dissemination of virions in vivo, since this vector has no intrinsic homing ability. Most of any intravenous inoculum initially infects non-tumor cells in circulation. Ordinarily, infection of these cells reduces therapeutic potential because virus particles meant to infect tumor cells are wasted, and although viral replication is attenuated in these non-tumor cells, off-target effects are induced. This approach therefore produces narrow therapeutic windows between efficacy and toxicity. OV's have also historically been inadequately equipped to overcome immunosuppression once they reach tumors. To overcome these limitations, we equipped VET3-TGI with CXCR3, IL-12, and a TGF- β inhibitor. These transgenes enable intravenously infused virus to get to tumors, locally lift immune-suppressive signaling, and precipitate the development of antitumor immunity.

VET3-TGI uses a novel and unique mechanism to boost the systemic delivery potential of the vaccinia backbone and allow delivery to occur at levels resulting in therapeutic activity. VET3-TGI causes cells to express CXCR3 immediately after infection, and in turn, we saw through increased virus deposition in vivo that at least some of the non-tumor cells infected early after intravenous delivery migrated into CXCL9-expressing and/or CXCL10-expressing tumors. Furthermore, we saw that VET3-TGI treatment incurs less serum markers of systemic toxicity than an analogous virus with wild-type A52R, lacking CXCR3. Removal of A52R in VET3-TGI relieves NF- κ B inhibition, increasing IL-12 production, and intensifying inflammatory signaling, and would be expected to increase toxicity outside of the tumor. Instead, reduced toxicity likely reflects CXCR3-associated virus delivery to the tumor and away from other organs and tissues. While not unique to tumors, data from TCGA and GTEx reveal that these CXCR3-cognate chemokines along with CXCL11 are elevated in many cancers, including breast, bladder, esophageal, renal, ovarian, and uterine carcinomas, head and neck, and cervical squamous cell carcinomas, and colorectal cancer, with little homeostatic expression in normal tissue, rendering them targetable. Consistent with prior work,^{40 41} treatment with IL-12-expressing virus in our study further increased expression of these chemokines in tumors, including those that initially expressed very low levels. This has the potential to create a positive feedback loop to facilitate repeat dosing in the clinic.

TGF- β inhibition has the potential to overcome many immune suppressive and related mechanisms employed

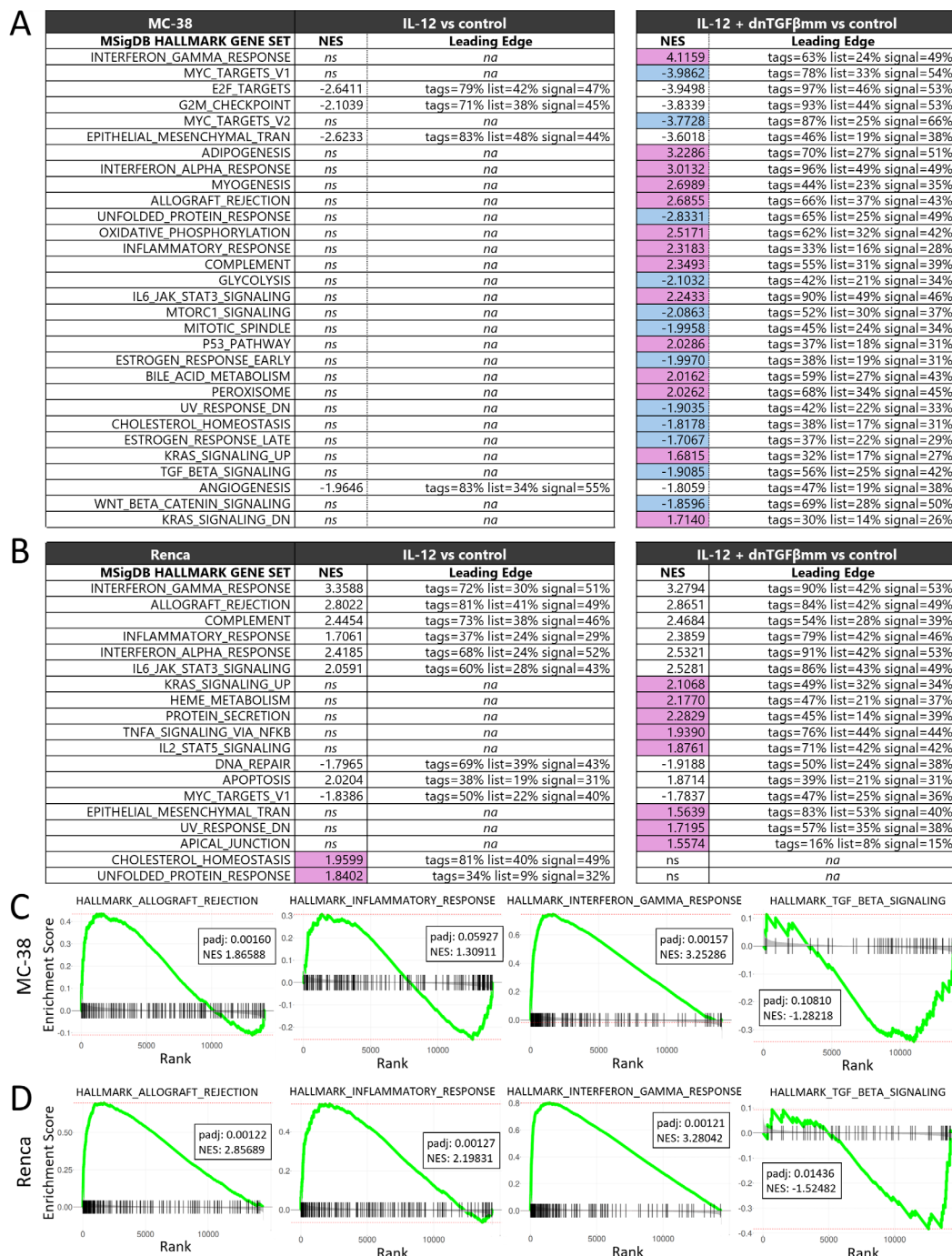


Figure 7 VET3-TGI transgenes cooperate to produce gene expression patterns indicative of antitumor inflammation in both intratumoral and intravenous infusion treatment modalities. (A) GSEA was used to investigate coordinated transcriptional differences arising from intratumoral treatment of MC-38 tumors with IL-12-equipped virus with TGF-β inhibitor ('IL-12+dnTGFβmm') or without ('IL-12'). NES for every set in the mouse-ortholog hallmark collection (MSigDB) were calculated from differentially expressed genes identified by contrasting the specified treatment against treatment with a TK-lacking control virus. The top 30 results were filtered by p value after adjustment for false discovery rate (adjusted $p \leq 0.05$, BH post hoc) for multiple tests; ns: no gene set enrichment detected. (B) GSEA analysis was performed as described in A on analogously treated Renca tumors. All hallmark sets significantly enriched (adjusted $p \leq 0.05$, BH post hoc) in at least one treatment are shown. Highlights indicate gene sets where treatment-based differences in enrichment were detected (pink: positive enrichment, blue: depletion). (C) fgsea was used to interrogate tumor-bound pathway-level gene expression patterns produced after intravenous delivery of VET3-TGI-murine versus the dual-deletion control virus. Enrichment scores, NES, and adjusted p values were calculated to assign pathway expression trends. The difference in transcription between the virus treatment groups in MC-38 tumors was compared with four mouse gene sets of interest related to antitumor immunity. (D) fgsea analysis was performed identical to C on analogously treated Renca tumors. BH, Benjamini-Hochberg; fgsea, fast gene set enrichment analysis; GSEA, gene set enrichment analysis; IL-12, interleukin-12; MC-38, murine colon adenocarcinoma; NES, normalized enrichment score; ns, no gene set enrichment detected; TGF-β, transforming growth factor beta; TK, thymidine kinase.

by solid tumors and we have previously demonstrated the benefits of suppressing this pathway from an OV. However, unlike the more sensitive mouse tumor models used in our previous publication,³⁸ TGF- β inhibition alone bestowed negligible benefit in the heavily immune-infiltrated Renca model.⁴² Interestingly, Renca cells lack expression of T β RII, needed for dnTGF β mm binding.⁴³ Nonetheless, co-expression of TGF- β inhibitor and IL-12 transgenes doubled remission over IL-12 treatment alone, with similar remission outcomes between intratumoral and intravenous delivery seen across multiple models. Adaptive immunity-mediated tumor rejection is implicated since subjects with CR failed to support tumor take on rechallenge. Efficacy in these models coincides with increased B-cell, T-cell, and NK-cell infiltration compared with control virus treatment, revealing potential participants in a cell-based immunity response. Elevated tumor CXCL9, CXCL10, and CXCL11 after IL-12 exposure is consistent with Th1-biased immune differentiation,^{40 44 45} further supported by our observation that IL-12-equipped viruses dampen the induction of systemic vaccinia-reactive antibodies. Elaboration of immunity along the Th1 axis instead of Th2⁴⁶ should further aid repeat dosing by reducing or delaying the development of neutralizing antibodies.

MC-38 tumors in this study showed quintessential transcriptional signatures of TGF- β pathway inhibition and IL-12 response after treatment with correspondingly equipped viruses and provided many benefits over expression of IL-12 alone including depletion across TGF- β signaling, angiogenesis, WNT/ β -catenin signaling, glycolysis, and EMT gene sets. Inflammatory and type II IFN response signatures intensified. A colon adenocarcinoma-derived cell line with a high mutation burden, MC-38 tumors grow rapidly. Untreated established tumors feature extensive extracellular matrix with embedded cancer-associated fibroblasts, immune-depleted cores, and disorganized vasculature,⁴⁷ phenotypes supported by TGF- β activity. MC-38 cells express TGF- β 1 but its inhibition alone lacks therapeutic benefit.⁴⁸ Altogether, the transcriptome remodeling induced uniquely by dnTGF β mm combined with IL-12 from VET3-TGI emphasize the importance of simultaneously inducing immune activation while combating immune suppression.

Similarly, in Renca tumors, treatment with VET3-TGI enriched IFN- γ , inflammatory response, and allograft rejection-associated gene sets. Significant depletion of the TGF- β signaling gene set in the Renca model was also observed despite loss of T β RII and associated autocrine signaling in these cells.

VET3-TGI contains multiple active components: an oncolytic vaccinia virus backbone combined with the expression of CXCR3, IL-12, and a TGF- β inhibitor. For such a complex therapeutic agent to provide significant benefit, it is critical that these components function in a coordinated fashion. Here, we show how the components of VET3-TGI cooperate to safely and specifically attack tumors: (i) early expression of CXCR3 from initially

infected non-tumor cells induces their chemotaxis toward ligand-expressing tumors, an effect that is reinforced by IL-12-induced production of CXCL9, CXCL10, and CXCL11 from within the tumor and by reduction in induction of antiviral neutralizing antibody after initial treatments; (ii) this CXCR3-mediated active targeting of the tumor reduces viral delivery to other organs, thereby reducing the potential for IL-12 and TGF- β inhibitor toxicity. This safety is further enhanced by restricting virus replication to tumors through deletion of TK-encoding and A52-encoding genes, and by late-only expression of the potent immunostimulatory cytokine IL-12 from a weak promoter; (iii) VET3-TGI marks the debut of genetically encoded TGF- β inhibitors delivered via vaccinia virus as a method to fully and safely unleash IL-12-driven antitumor immune activity in tumors and not within healthy tissue. Since IL-12 expression increases TGF- β pathway signaling, the benefits of this cytokine are reduced within the tumor, an effect that is overcome by co-expression of the TGF- β inhibitor.

While we have introduced VET3-TGI in the context of an intravenously deliverable monotherapy, we anticipate future exploration of its therapeutic potential when combined with checkpoint inhibitors to provide further therapeutic benefits.

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