

The oncolytic viral immunotherapy ASP1012 optimizes memory T cell responses after systemic delivery

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Clinical use of oncolytic viral immunotherapies (OVIs) has been hindered by the requirement for intratumoral delivery and inconsistent activity. Here, we describe a novel, engineered strain of vaccinia virus (ASP1012) designed to overcome both limitations. ASP1012 displays improved systemic delivery even in the face of pre-existing anti-viral immunity. This is achieved through expression of the chemokine receptor CCR2, which, in turn, allows the virus to take advantage of inherent infection of lymphocytes in circulation and redirect these infected lymphocytes toward tumors expressing the chemokine CCL2, which is expressed by most solid human tumors. We have previously described potent T cell activation mediated by expression of the metabolic modulator leptin from an OVI. Here, we demonstrate significantly further increased therapeutic activity through expression of a Leptin-IL2 fusion protein and describe how this mediates changes in the tumor microenvironment leading to therapeutic activity in multiple mouse tumor models. ASP1012 expresses both CCR2 and Leptin-IL2, resulting in a therapy capable of producing complete responses in multiple mouse tumor models after intravenous delivery.

INTRODUCTION

Cancer immunotherapy is often limited by the small numbers of pre-existing T cells in many tumors. Vaccinia-based oncolytic viral immunotherapies (OVIs) have demonstrated a remarkable ability to attract new T cells into even cold tumors, but these T cells often rapidly become exhausted or subverted within the tumor microenvironment, especially after the OVI has been cleared.¹

The *LEP* gene, which expresses the metabolic modulating adipokine leptin, has previously been expressed from oncolytic vaccinia resulting in the ability to maintain the T cell immune response subsequent to its attraction into the tumor.² This effect is mediated through binding to the leptin receptor expressed on effector T cells. However, because many other cell types within the tumor microenvironment also express leptin receptors, these effects can be limited. Here we

show that leptin fused to interleukin-2 (IL2) *Leptin-IL2* could more effectively and specifically target leptin receptor on T cells, while the IL2 provides additional therapeutic benefits.

IL-2 is a key cytokine that has been identified as a T cell growth/expansion factor.³ It was the first cytokine approved by the US Food and Drug Administration (FDA) for use in cancer immunotherapy and has demonstrated clinical promise^{4,5} despite several limitations,^{6,7} including poor half-life,⁸ systemic toxicities,⁹ promotion of regulatory T cells,^{10,11} and its ability to induce T cell exhaustion.^{12,13} Fusion of IL-2 to leptin helps overcome many of these limitations by extending its half-life, increasing retention of the cytokine in the tumor to reduce toxicities, and increasing its selectivity for and maintenance of effector T cells.

Furthermore, in the current study, the Vaccinia Enhanced Template (VET) is described for the first time. VET is a platform approach to allow improved systemic delivery of OVI and selective expression of therapeutic transgenes, such as *Leptin-IL2*, within the tumor microenvironment after intravenous delivery. The ASP1012 virus contains several modifications that combine to create a prototype VET backbone. These include the combination of a viral thymidine kinase (TK/J2R) gene deletion allowing for selective replication of virus in tumor cells,¹⁴ with the A52R gene deletion, a negative regulator of NF-κB,¹⁵ and a master controller of inflammation. Together, these virulence gene deletions enhance safety and increase activity of the vector. Furthermore, the human *CCR2* transgene was expressed resulting in targeted migration of lymphocytes and other cells infected *in situ* toward CCL2-expressing tumors. ASP1012 combines this VET backbone with expression of Leptin-IL2. The goal of this study was to test ASP1012 in pre-clinical models after intravenous delivery.

Received 13 February 2026; accepted 2 July 2026;
<https://doi.org/10.1016/j.ymthe.2026.07.005>

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RESULTS

Leptin-IL2 fusion demonstrates increased therapeutic activity relative to Leptin or IL-2

Initially, we looked to compare the therapeutic activity and effects on the tumor microenvironment of expression of either leptin, IL-2, or a Leptin-IL2 fusion from a Western Reserve strain of vaccinia virus with a single thymidine kinase deletion (WR.TK-).

To create mouse tumor models that recapitulated T cell-rich (“hot”) or myeloid cell rich (“cold”) tumors in a similar genetic background, tumor cell lines were cultured from spontaneous pancreatic ductal adenocarcinoma (PDAC) tumors formed in *Kras*^{G12D/+}; *Trp53*^{R172H/+}; *p48-Cre* (KPC) mice. Upon re-implantation into wild-type (WT) C57BL/6 mice, these KPC derived tumor cell lines formed tumors either rich in CD3+ cells (hot phenotype) or CD11b (cold phenotype) (Figure S1A). Two of these, KPC1 (CD3+ lo.CD11b+ high; cold) and KPC7 (CD3+hi.CD11b+ lo; hot) were selected for evaluation.

Mice with subcutaneously implanted tumors >50 mm³ were treated by intratumoral injection with viral formulation buffer (vehicle), or 1×10^7 plaque-forming units (PFU) of different virus treatments: thymidine kinase deletion alone (WR.TK-) or this viral backbone expressing individual transgenes.

KPC1-implanted tumors were anticipated to present an aggressive and treatment-resistant model because they are enriched with cells expressing CD11b, a marker linked to immunologically cold phenotypes. Treatment with WR.TK- or WR.TK-.Leptin viruses each improved survival probability over treatment with vehicle. However, these treatments failed to achieve complete responses (CRs), in this work defined as a tumor that grew to exceed 50 mm³ after implantation and subsequently shrank and maintained a volume below 100 mm³ after treatment until the end of the study. IL-2 expression caused significant tumor growth inhibition starting at 24 days post treatment (one-way ANOVA on per-treatment tumor volumes, with Tukey’s *post hoc*, $p = 0.0166$) and resulted in 47% (seven of 15) CRs in this model. The Leptin-IL2-expressing virus displayed the greatest activity with tumor growth inhibition established by day 24 post treatment (one-way ANOVA, Tukey’s *post hoc*, $p = 0.0034$), and an overall CR rate of 70% (seven of 10) in addition to significant ($p < 0.01$) survival benefit over vehicle treatment (Figure 1A). This greater therapeutic efficacy was accompanied by tumor microenvironment changes, seen most strongly at late (day 21) times after initial treatment, which included increased effector memory T cell populations, and reduced exhaustion (TIM-3) and immune checkpoint (PD1) expression on the T cells within the tumor after treatment with leptin-IL2-expressing viruses (Figures 1B and S1B), though this observation was taken with caution due to this group’s relatively small ($n = 2$) sample size.

KPC7 hot tumors were less aggressive, with all viruses demonstrating some activity (Figure 1C). All virus treatments conferred survival benefits in this model, and all virus treatments caused significant tu-

mor growth inhibition by day 14 post treatment (one-way ANOVA with Tukey’s *post hoc*, $p \leq 0.0001$). Furthermore, treatment with WR.TK- yielded a single CR out of 15 evaluable subjects, while 33% (five of 15) CRs were observed with WR.TK-.Leptin treatment, and 40% (six of 15) CRs with WR.TK-.IL2. Leptin-IL2 expression was highly beneficial with 60% (nine of 15) CRs. These outcomes were associated with changes in the tumor T cell population, with Leptin-IL2-expressing viruses showing reduced regulatory T cell (Treg) populations and decreases in LAG3 and PD1 expression on CD8+ T cells (Figures 1D and S1B).

Leptin-IL2 therefore provided significant therapeutic advantage over either Leptin or IL2 expression alone in both models, and this correlated with alterations in the T cell repertoire within the tumor, including increased numbers of effector memory T cells, and reduced levels of Tregs and of inhibitory markers (LAG-3, TIM-3, and PD1) on CD4+ or CD8+ T cells. These effects were most evident at later times after treatment, when much of the OVI would have been expected to have been cleared by the immune response.

Construction and *in vitro* testing of ASP1012

To enhance systemic delivery of the virus and consequently increase tumor-selective expression of the *Leptin-IL2* therapeutic transgene, we designed an OVI from our VET virus platform, ASP1012, which consisted of a Western Reserve vaccinia strain with deletions of the viral *TK/J2R/VACWR094* and the *A52R/VACWR178* virulence genes and expressing human *CCR2* (from the *A52R* gene deletion site and under control of the native, early *A52R* promoter). The *Leptin-IL2* fusion transgene was expressed from the site of the *TK* gene, driven by the predominantly late viral P7.5 promoter (Figure S2A). Control viruses were created with *A52R* and *TK* gene deletions, or just the *TK* deletion, but without the addition of any transgenes (Figure S2A). Initially, we confirmed expression of transgenes; *CCR2* mRNA levels were significantly higher in ASP1012-infected cells ($p < 0.0001$, Figure S2B); Leptin-IL2 expression and secretion was confirmed by ELISA for both IL2 ($p < 0.0001$, Figure S2C) and Leptin ($p < 0.001$, Figure S2D) in supernatant. The ability of ASP1012 to replicate in B cells and express *CCR2* protein was also confirmed (Figures S3A and S3B), and functional activity of the *CCR2* was confirmed with ASP1012-infected lymphocytes (B cells) demonstrating increased migration toward the chemokine CCL2 relative to B cells infected with WR.TK- virus ($p < 0.05$; Figure 2A).

ASP1012 transgenes provide both systemic delivery and therapeutic advantages

Viral *CCR2* expression could result in targeted movement of any cells infected *in situ* after intravenous delivery toward tumors expressing the chemokine CCL2. We initially identified syngeneic mouse tumor models that expressed CCL2. Lewis lung carcinoma (LLC) tumors demonstrated the greatest CCL2 expression *in vivo* (Figure S4A) and were chosen for subsequent studies. MC-38 also expressed CCL2, but at lower levels, while Renca and EMT-6 expressed negligible levels of CCL2 (Figure S4A).

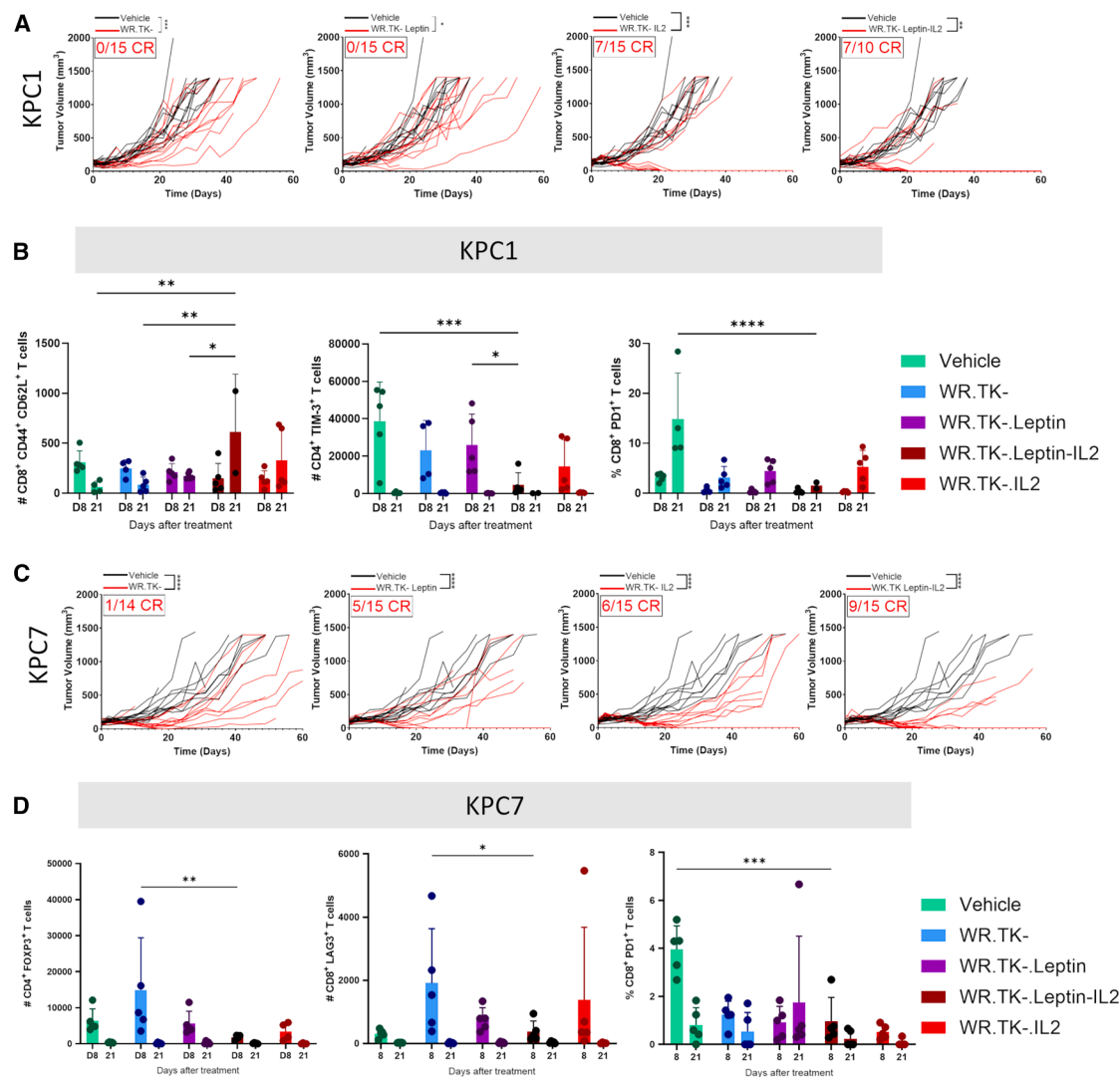


Figure 1. Leptin-IL2 demonstrates enhanced therapeutic activity against CD11b-high “cold” KPC1 and CD3-high “hot” KPC7 tumors

(A) Growth of KPC1 CD11b+ high cold tumors ($n = 15$) treated with viral buffer vehicle control (vehicle) was compared to a single intratumoral (1×10^7 PFU) treatment of (1) thymidine kinase deletion (WR.TK-), (2) WR.TK-.Leptin virus, (3) WR.TK-.IL2 virus, and (4) WR.TK-.Leptin-IL2 virus. Indicated differences in survival between groups were assessed by Kaplan-Meier analysis with Mantel-Cox test (multiple comparisons controlled via Holm-Šidák); subjects with complete responses (CRs) out of total assessable subjects are enumerated within each panel; vehicle treatment produced zero (of 15) CRs. (B) Tumors collected at indicated times post treatment were dissociated and immune cell subsets analyzed by flow cytometry to compare treatment-associated changes to central memory CD8+ T cells (CD8+ CD44+ CD62L+), expression of exhaustion markers (CD4+ TIM3+ T cells), and expression of the PD-1 immune checkpoint marker (CD8+ PD1+ T cells). Each individual measurement is plotted as a point; bars mean and SD. Comparisons were performed by two-way ANOVA with Dunnett's *post hoc*. (C) Growth of KPC7 CD3+ high hot tumors ($n = 15$) treated with viral buffer vehicle control was compared to a single intratumoral (1×10^7 PFU) treatment of (1) thymidine kinase deletion (WR.TK-), (2) WR.TK-.Leptin virus, (3) WR.TK-.IL2 virus, or (4) WR.TK-.Leptin-IL2 virus; analysis was performed and presented as in (A); vehicle, zero (of 15) CR. (D) Tumors collected at indicated times post treatment were dissociated and immune cell subsets analyzed by flow cytometry. Treatment-associated differences in regulatory T cells (CD4+ FOXP3+) and immune checkpoint-expressing T cells (either LAG3+ or PD1+ cytotoxic T cells) were measured; all individual samples are shown accompanied by mean and SD. Analysis was performed as in (B). WR.TK-, thymidine kinase deletion; WR.TK-.Leptin, thymidine kinase deletion virus containing Leptin; WR.TK-.Leptin-IL2, thymidine kinase deletion virus containing murine Leptin-IL2; WR.TK-.IL2, thymidine kinase deletion virus containing murine IL2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Note: only flow cytometry plots where WR.TK-leptin-IL2 showed a significant difference to other treatment groups are shown.

To test the role of CCR2 in delivery of virus to the tumor, C57BL/6 mice were subcutaneously implanted with LLC tumors ($>50 \text{ mm}^3$), and then treated intravenously with vehicle buffer or 1×10^7 PFU of different vi-

rus treatments; thymidine kinase deleted virus (WR.TK-); TK-.A52R-virus with human CCR2 transgene inserted into the A52R gene deletion region (WR.TK-.A52R-CCR2+); TK-.A52R- double-deleted

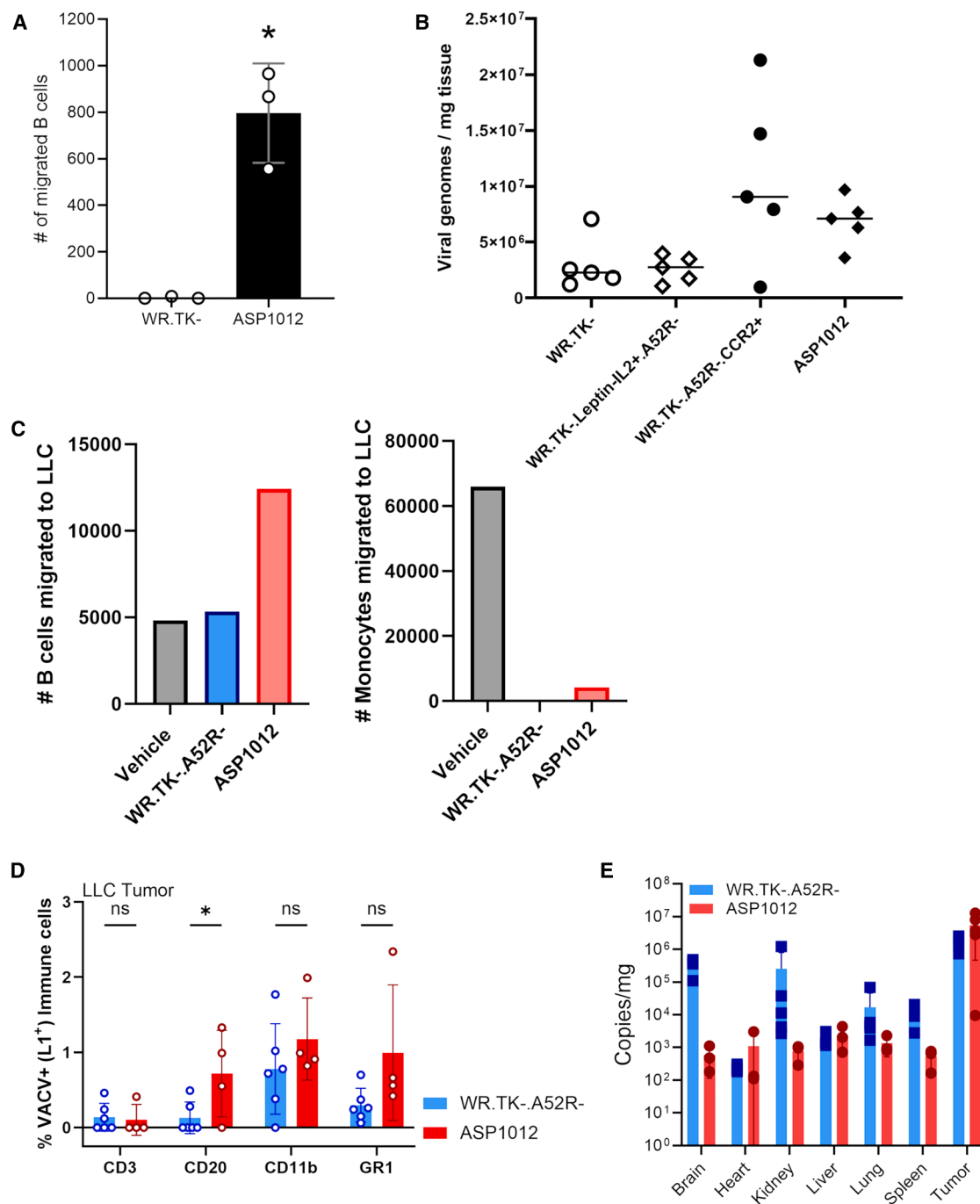


Figure 2. ASP1012 expressing CCR2 demonstrates enhanced systemic delivery to CCL2-expressing tumors

(A) B cells isolated from human PBMC and infected with WR.TK- virus or ASP1012 at MOI of 5 for 24 h were allowed to migrate in Transwell plates to 100 ng/mL CCL2 ($n = 3$ wells per group). Migrated cell counts are shown with means and SD, and the effect of treatment was compared by t test ($p = 0.0227$). (B) qPCR of viral genomes per mg LLC tumor tissue ($n = 5$) for tumors recovered 18 h after intravenous delivery of 1×10^7 PFU of the indicated viruses (C57BL/6 mice bearing subcutaneous tumors $>50 \text{ mm}^3$). Viruses containing CCR2 (filled symbols) contained significantly higher ($p = 0.0036$ by Student's t test) viral genomes per mg tumor tissue in comparison to viruses without CCR2 (open symbols); lines indicate median genome copies/mg tumor tissue. (C) *In vitro* trafficking of PBMCs after infection (at MOI = 5) with indicated virus. Numbers of migrated B cells (CD20+) and monocytes (CD14+) are shown as bars; displayed data are representative of two experiments. (D) Infected lymphocyte populations recovered from LLC tumors *in vivo*. Bars indicate mean percentage, with SD, of vaccinia virus-infected (%VACV L1+) immune cells in tumors of mice 18 h after intravenous treatment

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virus with the *Leptin-IL2* fusion gene inserted into the TK- deletion region (WR.TK-.A52R-.Leptin-IL2+); or ASP1012, TK-.A52R- containing both CCR2 and Leptin-IL2 human transgenes. Systemic delivery of virus to the tumor was quantified by measuring viral genomes in the tumor by qPCR 18 h after intravenous treatment, before release of progeny virions from cells infected directly by the virus inoculum. This time point was selected to best separate measurement of delivery from potentially confounding viral replication in tumor cells, which would occur efficiently after delivery. It was observed that viruses expressing CCR2 (either alone or in combination with *Leptin-IL2* in ASP1012) produced increased systemic delivery of virus to the tumor (Figure 2B). This was also seen for MC-38 tumors that express only low-level CCL2 (Figure S4B) but not for Renca that does not express CCL2 (Figure S4C).

It was predicted that some portion of the intravenously delivered vaccinia virus naturally infects lymphocytes or other cell types while in circulation.¹⁶ Because the CCR2 transgene is expressed from a transient, early promoter, it is expressed prior to the commencement of viral replication, before the virus has taken over the cellular machinery.^{17,18} It is believed, therefore, that this allows the virus-infected cells to express CCR2 and actively migrate toward CCL2-expressing tumors in a targeted fashion prior to viral replication and release of progeny virus into the tumor microenvironment.

This mechanism of enhanced systemic delivery was examined in several ways. ASP1012, expressing CCR2, no longer demonstrated enhanced systemic delivery to Renca (which does not express CCL2) (Figure S4C). Renca tumors instead express the chemokine CXCL12, which binds to CXCR4. When a virus matched to ASP1012, but expressing CXCR4 instead of CCR2, was tested (WR.TK-.A52R-.CXCR4+.Leptin-IL2+), enhanced delivery was demonstrated in the Renca tumor model (Figure S4C). It appears, therefore, that expression of different unique chemokine receptors results in enhanced systemic trafficking of viruses toward tumors expressing the matching chemokine ligand(s). Further, the promoter driving the chemokine receptor was found to be of critical importance. Optimal delivery was achieved only when the promoter used was transient, early-only, and weaker strength (Figure S4D), indicating chemokine receptor expression needs to occur prior to viral takeover of the cellular machinery.

The increased delivery of ASP1012 to tumor tissue may be mediated by B cell carriers

Expression of the CCR2 transgene in ASP1012 increased systemic delivery of the virus to CCL2-expressing tumors (Figure 2B). Several experiments were run to identify what cell population(s) are mediating this. Initial studies used human peripheral blood mononuclear cells (PBMCs) infected with ASP1012 or a control virus (without

CCR2) in a Transwell assay to identify lymphocyte populations that migrated to CCL2-expressing tumor cells (Figure 2C). In this assay that compares B cells with monocytes, it was found that B cells uniquely demonstrated a selective increase in migration when infected with virus (ASP1012) expressing CCR2 (Figure 2C). In the uninfected control group, monocytes demonstrated the greatest level of trafficking (which is unsurprising as macrophages are known to express CCR2). However, these cells were lost after viral infection, possibly due to apoptosis or infection-induced lysis caused by the high multiplicity of infections (MOIs) employed for this *in vitro* experiment (Figure 2C).

To examine whether monocytes might also be trafficking prior to lysis and release of virus, the experiment was repeated, and viral-infected tumor cells quantified after migration (to see whether migration could also result in “hand off” of virus from infected lymphocytes to the tumor itself) (Figure S4E). It was observed that use of ASP1012-infected PBMCs resulted in greater numbers of infected tumor cells relative to WR.TK-.A52R- virus (without CCR2 expression). Further, there was no loss in delivery of ASP1012 to tumor cells when CD14+ monocytes were depleted from the PBMC population, but there was a reduction when CD20+ B cells were depleted (Figure S4E). No significant changes were observed in delivery of the control virus without CCR2 expression after depletion of either cell type. These data together indicate that B cells appear to be important for increased systemic delivery seen with chemokine receptor expression.

Further *in vivo* experiments were run to identify lymphocyte populations additionally expressing viral (L1) protein recovered from CCL2-expressing tumors after systemic viral dosing. In LLC tumors after intravenous delivery, ASP1012-treated tumors had a significantly greater percentage of L1+ (viral) positive B cells in comparison to treatment with buffer alone (vehicle) or WR.TK-.A52R- virus without CCR2 expression ($p < 0.05$, Figures 2D and S4F). No other immune cell type displayed a significant change in the number of virally infected cells in the tumor when CCR2 was expressed. Non-significant trends between treatment with CCR2-lacking control virus versus ASP1012 (t test) were seen with both CD11b+ ($p = 0.3186$) and GR1+ ($p = 0.0991$) cells (Figure 2D). Therefore, although we cannot rule out the involvement of other cell types, B cells do appear to play a critical role in systemic trafficking of CCR2-expressing virus toward (and subsequent infection of) CCL2-expressing tumors both *in vitro* and *in vivo*.

To confirm that the enhanced ASP1012 delivery to tumor tissue is dependent on both CCL2 and CCR2, transfection was used to express CCL2 from 4T1 tumor cells that do not naturally express this chemokine. 4T1 WT (4T1) or 4T1 cells expressing CCL2

with WR.TK-.A52R- virus or ASP1012. For each cell marker, the effect of treatment was compared by t test; CD20, $p = 0.04705$. (E) Biodistribution of viruses (concentrations plotted on logarithmic scale) after intravenous delivery of different viruses to LLC tumor-bearing C57BL/6 mice ($n = 5$). Ns, not significant; vehicle, virus formulation buffer; WR.TK-, thymidine kinase deletion; WR.TK-.A52R-, control virus; WR.TK-.A52R-.CCR2+, thymidine kinase A52R deletion virus containing CCR2; WR.TK-.A52R-.Leptin-IL2+, thymidine kinase A52R deletion virus containing murine Leptin-IL2; ASP1012, ASP1012 containing murine Leptin-IL2.

(4T1+CCL2) were implanted on opposite flanks in a bilateral tumor model and viruses with CCR2 (ASP1012) or without CCR2 (WR.TK-.A52R-) expression were delivered intravenously via tail vein, and relative delivery was quantified by bioluminescence imaging of viral gene expression. When CCR2 was expressed from the virus, increased relative delivery of virus to the CCL2-expressing tumor was seen (Figure S4G). These results demonstrate that both the CCR2 transgene in the virus and secretion of CCL2 from the tumor and/or associated cells are required for enhanced delivery of ASP1012 to tumors.

To examine how or whether CCR2 expression impacts the delivery of ASP1012 virus in other organs or tissues in addition to the tumor, different mouse organs were harvested and analyzed for viral genome copies present (18 h after treatment). As seen previously (Figure 2B), ASP1012 showed an increase in viral copies per mg of tissue recovered from tumors after intravenous treatment in comparison to WR.TK-.A52R- virus (Figure 2E). However, the same was not true for other organs. Instead, these tissues generally showed decreased genome copies per mg for ASP1012 relative to WR.TK-.A52R- virus (Figure 2E). This indicates that the increased delivery of ASP1012 was uniquely targeted to the tumor and apparently away from other organs and tissues.

ASP1012 transgenes and gene deletions provide a therapeutic advantage in LLC tumor models after intravenous delivery

To confirm the importance of each of the modifications incorporated into ASP1012, component viruses were tested in LLC tumor models after intravenous delivery. As expected, WR.TK- virus treatment alone resulted in some therapeutic advantage over vehicle controls (Figure 3A). However, in comparison to WR.TK- treatment, the additional modifications incorporated into ASP1012 each resulted in additional further therapeutic benefit; WR.TK-.A52R- provided a small but significant therapeutic benefit over WR.TK- at early times after treatment (one-way ANOVA on tumor volumes with Tukey's *post hoc*, 10 days post treatment $p = 0.0201$). Further addition of CCR2 to WR.TK-.A52R- resulted in further delayed tumor growth at these earlier times after treatment. Addition of Leptin-IL2 alone to WR.TK-.A52R- virus resulted in significant tumor growth delay over an extended time frame (one-way ANOVA with Tukey's *post hoc*, $p = 0.0005$ at 21 days post treatment), presumably due to ongoing immune effects even once viral load would be expected to have been reduced. All virus treatments produced distinct and statistically different median survival probabilities (Kaplan-Meier survival analysis with Tukey's *post hoc*, $p < 0.0001$ for all pairs), with ASP1012 (with both viral gene deletions and both transgenes expressed) presenting the most favorable outcome (Figure 3A). In this model, ASP1012 was the only treatment to produce CRs, with 47% of animals demonstrating CRs (Figure 3A).

ASP1012 intravenous treatment results in a dose-dependent response in LLC tumors

To determine the minimum effective dose, a series of decreasing murine ASP1012 doses (two doses at 1×10^7 ; two doses at 2×10^6 ; two

doses at 4×10^5 PFU) were administered intravenously to mice with LLC tumors and compared to vehicle and WR.TK- virus. Both viruses provided significant benefit to survival probability (Kaplan-Meier survival analysis with Tukey's *post hoc*, $p < 0.0001$ for every dose; Figure 3B). At all doses tested, ASP1012 treatment resulted in significant tumor growth inhibition when compared against vehicle-treated subjects (two-way ANOVA for specified groups; days 10, 14, and 17 post treatment with Tukey's *post hoc*, $p < 0.0001$), with a 13% CR (two responders of 15 evaluable) at the mid-dose level (Figure 3B middle right panels) and a 53% CR (eight of 15) again seen at the higher dose (Figure 3B bottom right panels), whereas WR.TK- virus, despite showing tumor growth inhibition at these early measurement points (two-way ANOVA for specified groups; days 10, 14, and 17 post treatment, $p < 0.0001$), did not produce any CRs at any dose (Figure 3B left panels). Similarly, no CRs were observed in vehicle-treated mice. Of note, the 1×10^7 PFU dose is not considered a maximum tolerated dose in mice for ASP1012, as there was minimal weight loss and no observed signs of toxicity at this dose. This dose was used, as it is in the range of the typical human clinical dose used with different oncolytic vaccinia virus strains¹⁹ when converted on a PFU/kg basis. These results indicate that ASP1012 produced a dose-dependent response in LLC tumors. Further, ASP1012-treated mice that underwent CRs were able to reject re-challenge with the same LLC tumor line (Figure S5A).

ASP1012 treatment demonstrates increased activity in multiple tumor models

The efficacy of intravenous ASP1012 treatment was tested in several additional mouse tumor models, including MC-38 (C57BL/6 colorectal cancer), which is traditionally more responsive to immunotherapy but expressed lower levels of CCL2 relative to LLC, and EMT-6 (BALB/c mammary cancer), which does not express CCL2. 1×10^7 PFU of ASP1012 treatments were administered systemically to mice bearing MC-38 or EMT-6 tumors. In comparison to treatment with vehicle alone, treatment of MC-38 tumors with ASP1012 resulted in significant (Welch's *t* test, day 10 post-treatment tumor volumes, $p < 0.0001$) tumor growth inhibition and a 60% (six of 10) CR (Figure 3C), treatment of EMT-6 tumors resulted in a CR in 31% (four of 13) of animals (Figure 3D). In contrast, vehicle treatment of MC-38 yielded no CR and all subjects were deceased by day 31 post treatment, while vehicle treatment of EMT-6 tumor-bearing mice yielded two survivors with volumes exceeding $1,000 \text{ mm}^3$ at the end of study. These results demonstrate ASP1012, with the *Leptin-IL2* transgene, remains a viable therapy across different tumor types including in tumors that express little or no CCL2 (Figures 1A, 1C, 3C, and 3D). Of note, although the enhanced systemic delivery of ASP1012 is lost when CCL2 is not expressed from the tumor, systemic delivery is still inherently possible, resulting in multiple CRs in multiple mouse tumor models.

ASP1012 retained systemic delivery and therapeutic activity in previously immunized mice

In order for intravenous delivery of OVI to become routinely used in the clinic, it will be necessary not only to increase delivery in

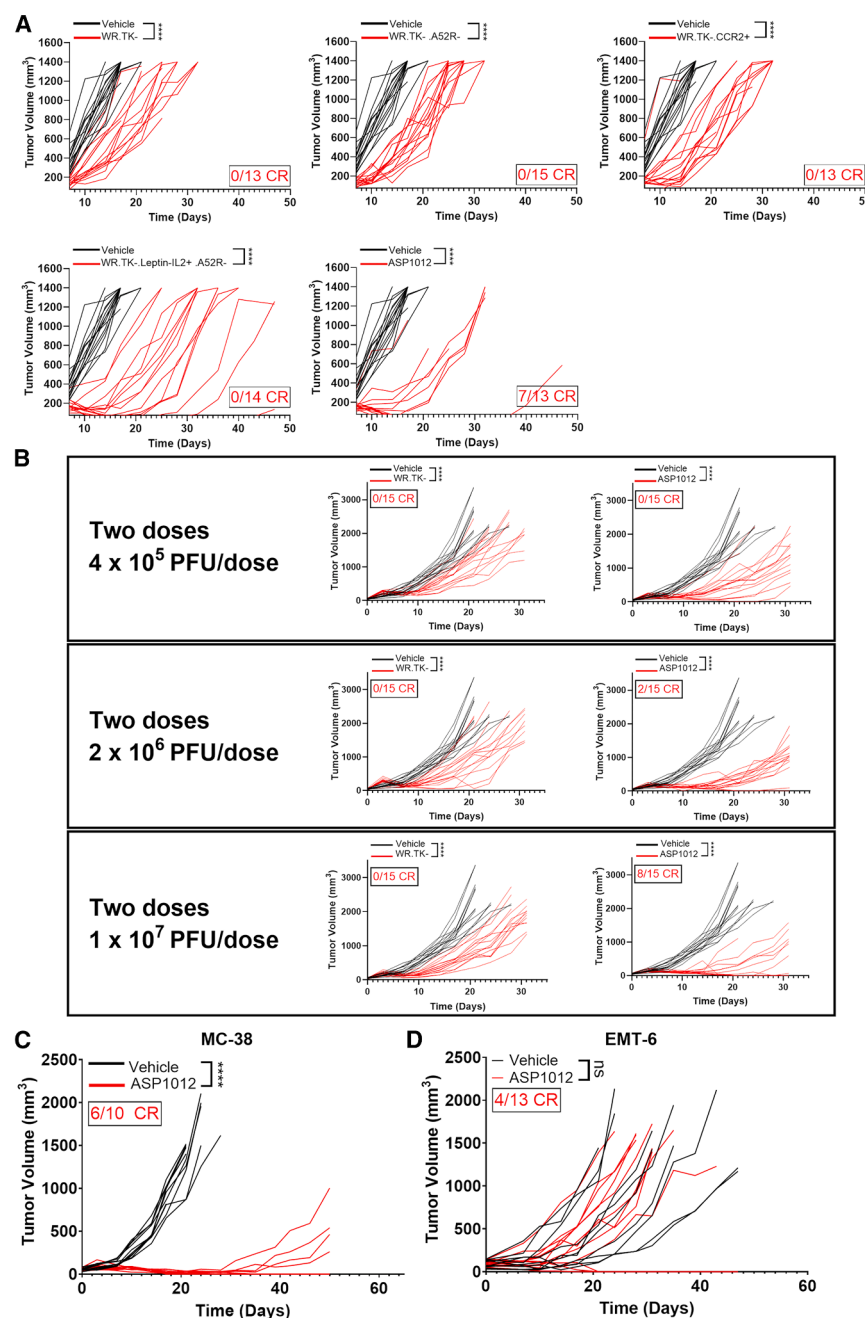


Figure 3. Intravenous treatment with ASP1012 results in therapeutic effects in multiple tumor models

(A) Spider plots of tumor growth after intravenous treatments (twice at 1×10^7 PFU/dose) of vehicle-treated (black lines) mice relative to ASP1012 or component virus treatments (red lines) in the LLC tumor model ($n = 15$). Treatment with ASP1012 results in a 54% CR outcome (seven of 13 evaluable, tallied at end of study). No other treatment generated CRs in this model. Tests for the effect of each treatment (versus vehicle) on survival were performed via Kaplan-Meier analysis with Mantel-Cox test. Multiple comparisons were controlled via Holm-Sidak; differences are indicated for each virus-to-vehicle plot, **** $p < 0.0001$. (B) LLC tumor model spider plots ($n = 15$ per group) comparing intravenous treatment with viral buffer vehicle control (black lines) to WR.TK-virus (red lines) and ASP1012 (red lines, murine Leptin-IL2) after two doses of 4×10^5 PFU (top), two doses of 2×10^6 PFU (middle), or two doses of 1×10^7 PFU (bottom). CRs out of total evaluable subjects are indicated in boxes; survival comparisons were performed and displayed as described in (A). (C) Tumor growth after intravenous treatment with vehicle control (black lines) was compared to treatment twice with 1×10^7 PFU/dose of ASP1012 (murine Leptin-IL2, red lines) in C57BL/6 mice with subcutaneous MC-38 tumors $>50 \text{ mm}^3$ ($n = 10$ per group). Treatment with ASP1012 (murine Leptin-IL2) results in a 60% CR outcome (six of 10 evaluable, tallied at end of study); survival comparison ($p < 0.0001$) per (A) without Holm-Sidak. (D) BALB/c mice bearing subcutaneous EMT-6 tumors treated intravenously as before with vehicle ($n = 10$) or ASP1012 ($n = 13$). Treatment with ASP1012 (murine Leptin-IL2) results in a 31% CR outcome (four of 13 evaluable, tallied at end of study); survival comparison ($p = 0.5830$) per (A) without Holm-Sidak. Vehicle, virus formulation buffer control; WR.TK-, thymidine kinase deletion; ASP1012, ASP1012 containing murine Leptin-IL2.

naive patients but also to achieve repeat delivery, even after induction of anti-viral immunity. Because the mechanism of ASP1012 delivery is within infected lymphocytes prior to viral replication, it is possible this could lead to evasion of anti-viral immunity.

ASP1012 treatments in LLC or MC-38 tumor-bearing mice were therefore compared between non-immunized and previously immunized mice (Figures 4A and 4B). Mice were initially immu-

nized with 1×10^3 PFU of WR. TK- virus 28 days prior to tumor implantation, and the existence of circulating neutralizing antibodies was confirmed prior to treatment (Figure S5B) at levels equivalent to treatment with ASP1012 (Figure S5C). This model was used as the time taken for full antibody induction was otherwise too long to be induced in mice already bearing tumors. Virus within the tumor was quantified by qPCR 18 h after treatment (Figures S5D and S5E). In LLC tumor-bearing mice, pre-immunization did not significantly reduce viral delivery for ASP1012 (Figure S5D). Similarly, no significant difference in delivery efficacy was observed in the MC-38 tumor model, which expresses lower levels of CCL2 (Figure S5E). The systemic delivery of CCR2-equipped virus to CCL2-producing tumors in pre-immunized mice exceeded even that achieved

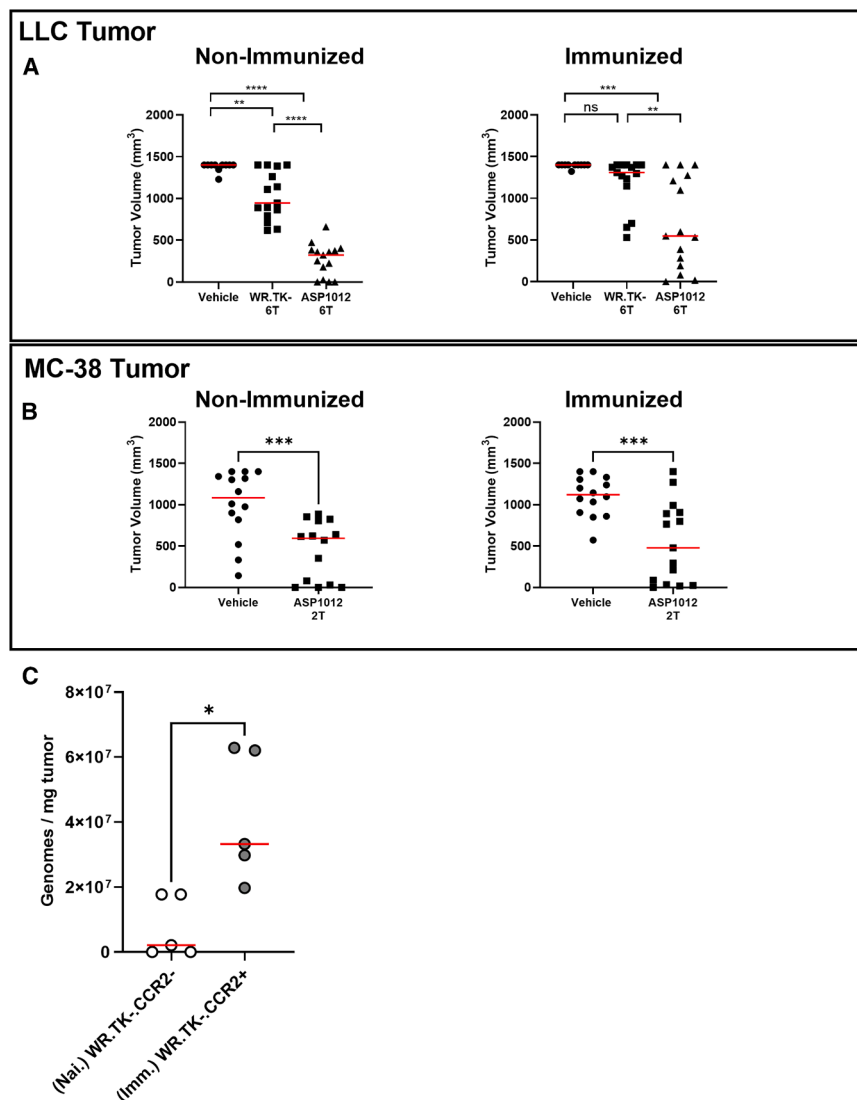


Figure 4. ASP1012 intravenous treatment demonstrates delivery and efficacy against pre-immunized mice

(A) C57BL/6 mice bearing subcutaneous LLC tumors ($>50\text{mm}^3$), and previously immunized with vaccinia (1×10^3 PFU 28 days earlier) or non-immunized (naïve) were treated intravenously with six doses ("6T," two per week) of viral buffer control (vehicle) ($n = 10$) or 1×10^7 PFU of ASP1012 (murine Leptin-IL2) or WR.TK- virus treatments (each $n = 15$). Individual mouse tumor burdens are shown as individual dots 21 days after first treatment with lines indicating group medians. Volume differences between treatment groups were assessed by one-way ANOVA with Tukey's *post hoc* analysis: $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. (B) Comparison of non-immunized and immunized C57BL/6 mice with subcutaneous MC-38 tumors ($>50\text{mm}^3$) after 21 days ($n = 15$) treated with two doses ("2T") of vehicle or 1×10^7 PFU/dose ASP1012. Individual mouse tumor burdens are shown as individual dots 21 days after first treatment with lines indicating group median; group volumes were compared using the Welch's unpaired *t* test, immunized $***p = 0.0007$, naïve $***p = 0.0009$. (C) As in (A), previously immunized mice ($n = 5$) bearing LLC tumors were treated intravenously with CCR2-equipped virus "WR.TK-CCR2+." In parallel, additional naïve mice ($n = 5$) were treated with an equivalent control virus lacking CCR2 "WR.TK-CCR2-"; viral genome copies in the tumor were quantified by qPCR from samples collected 24 h post treatment and values shown (lines: median) are normalized against tumor mass; Welch's *t* test $p = 0.0142$. Vehicle, virus formulation buffer; WR.TK-, thymidine kinase deletion; ASP1012, ASP1012 containing murine Leptin-IL2.

by an analogous but CCR2-lacking virus in naïve mice (Figure 4C), indicating the importance of CCR2-mediated systemic trafficking for delivery in the face of pre-existing anti-viral immunity.

In the LLC model, six consecutive treatments of ASP1012 (the typical clinical regimen for an oncolytic vaccinia virus)^{20,21} significantly reduced tumor burden when compared to vehicle treatment in both non-immunized and immunized mice (one-way ANOVAs with Tukey's *post hoc*, non-immunized $p < 0.0001$, immunized $p < 0.001$, at day 21 post start of treatment; Figure 4A). WR.TK- virus only showed significant tumor growth inhibition relative to vehicle control in the non-immunized setting ($p < 0.01$; Figure 4A). Two treatments of ASP1012 also showed similar and significant therapeutic effects between non-immunized or immunized mice bearing MC-38 tumors (naïve $p =$

0.0009; immunized $p = 0.0007$) at day 21 post start of treatment (Figure 4B).

ASP1012 induces enhancement of effector memory T cells and downregulation of suppressive immune gene signatures within the tumor microenvironment

NanoString analysis was performed on LLC tumors collected 72 h after systemic treatment with vehicle, WR.TK-.A52R-, or ASP1012 to examine the mechanism of action driving the increased activity of ASP1012. ASP1012 treatment resulted in the upregulation of 65 genes (including *GZMA*, *GZMB*, *STAT1*, *41BBL*, and *GITR*) that were primarily associated with activity of effector T cells and downregulation of 11 genes (*IDO2*, *TGFB3*, *CBX2*, *SNAIL*, etc.) that were primarily associated with immune suppression (Figures S6A and S6B).

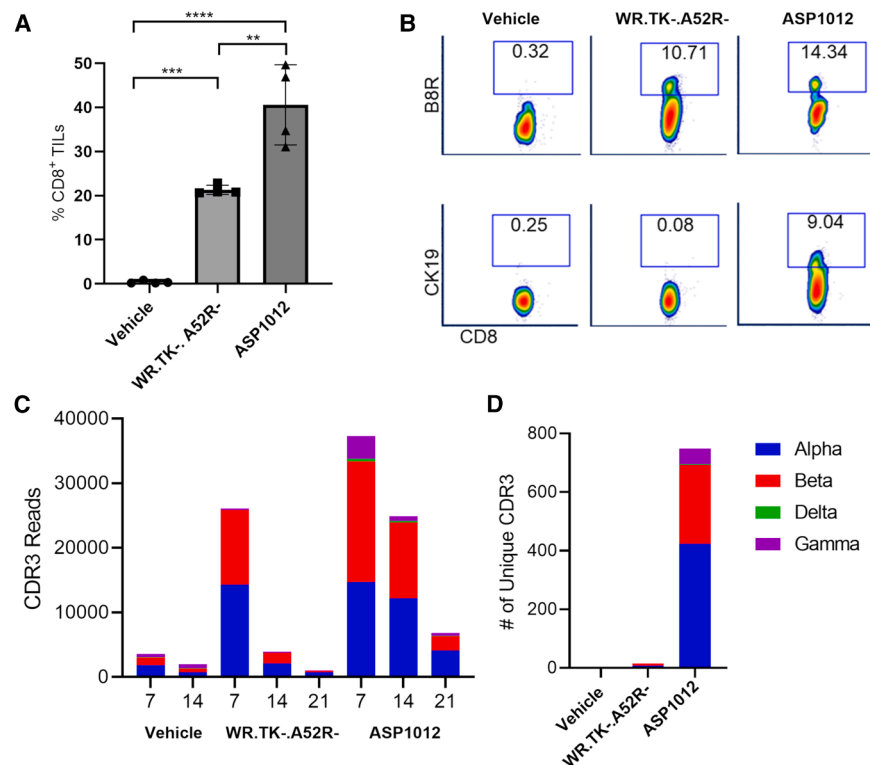


Figure 5. Effects of ASP1012 on T cell response

(A) Percentage of CD8⁺ tumor-infiltrating lymphocytes (mean with SD) in live gated CD45⁺ cells from dissociated tumors analyzed 14 days after vehicle or a single 1×10^7 PFU intravenous treatment of WR.TK-.A52R-virus or ASP1012 (murine Leptin-IL2) of C57BL/6 mice bearing subcutaneous LLC tumors ($n = 4$); groups were compared by one-way ANOVA with Tukey's *post hoc* analysis: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) Percentage of B8 (viral antigen) and CK19 (tumor antigen) tetramer-positive CD8⁺ T cells analyzed on D14 after treatment as in (A); data shown illustrate the gating strategy to identify reactive cells, and the observed response is representative of five individual samples per condition. Bar graphs displaying (C) frequencies of total CDR3 alpha, beta, delta, and gamma TCR chains in the same treatment groups on days 7, 14, and 21 after treatment (D) or unique CDR3 alpha, beta, delta, and gamma TCR chains shared within treatment groups. Data presented in (C) and (D): each bar displays the count from one subject. Vehicle, virus formulation buffer; WR.TK-.A52R-, control virus; ASP1012, ASP1012 containing murine Leptin-IL2.

Treatment with ASP1012 enhances number, tumor specificity and T cell receptor repertoire of CD8⁺ tumor-infiltrating lymphocytes

Additional studies were run to further define the effects of the Leptin-IL2 fusion on the CD8⁺ T cell response in the tumor. Initially CD8⁺ T cell infiltration was measured in tumors treated with vehicle, WR.TK-.A52R-, or ASP1012 14 days post treatment by flow cytometry on dissociated tumors. Although WR.TK-.A52R- virus alone was able to significantly increase the levels of CD8⁺ T cell infiltration into the tumor, ASP1012 treatment resulted in a further significant, 2-fold increase in the levels of CD8⁺ tumor-infiltrating lymphocytes ($p < 0.001$, Figure 5A).

Furthermore, major histocompatibility complex (MHC) class I tetramers specific for vaccinia (B8) and tumor (CK19) antigens were used to define the relative levels of anti-viral and anti-tumor adaptive T cell immune response. As expected, T cells from vehicle-treated tumors did not show any anti-viral or anti-tumor T cell adaptive immune response; when WR.TK-.A52R- was used, an anti-viral immune response was induced, but there was no detectable cross-presentation and induction of immune response against the CK19 tumor antigen. However, when ASP1012 treatment was used, an additional anti-tumor immune response was also raised (Figure 5B).

iRepertoire analysis was used to determine the level and clonality of the T cell receptor (TCR) CDR3 reads after different treatments. The use of ASP1012 resulted in the greatest number and clonality of TCR

response in alpha-beta T cells (relative to WR.TK-.A52R-), a response that also persisted even after the expected clearance of the virus (Figures 5C and 5D). Interestingly, ASP1012 was also uniquely able to increase the number, clonality, and persistence of TCR in gamma delta T cells, which have been associated with a more robust anti-tumor response (Figures 5C and 5D).

Spatial changes in the immune tumor microenvironment induced by ASP1012

10x Xenium spatial transcriptomics analysis was conducted to further study changes in the tumor microenvironment induced by ASP1012, relative to WR.TK-.A52R- control virus. Single cells from LLC tumors were classified into eight cell subsets (Figures S7A and S7B). Myeloid cells were further classified into six subgroups (Figures S7C and S7D). Likewise, CD8⁺ T cells were further classified into four subgroups (Figures S8A and S8B). Finally, all cell subsets were mapped across each tumor section (Figure S8C).

Treatment with ASP1012 decreased cancer cells and increased necrotic cells on days 7 and 14, whereas tumors treated with WR.TK-.A52R- showed only a partial response, with a cancer-rich area still evident on day 7 that had regrown throughout the tissue on day 14 (Figures 6A and 6B), consistent with the superior anti-tumor effects of ASP1012 seen in this model.

Interestingly, the cancer cell-rich areas in WR.TK-.A52R- virus-treated tumors colocalized with M2-like macrophages and appeared

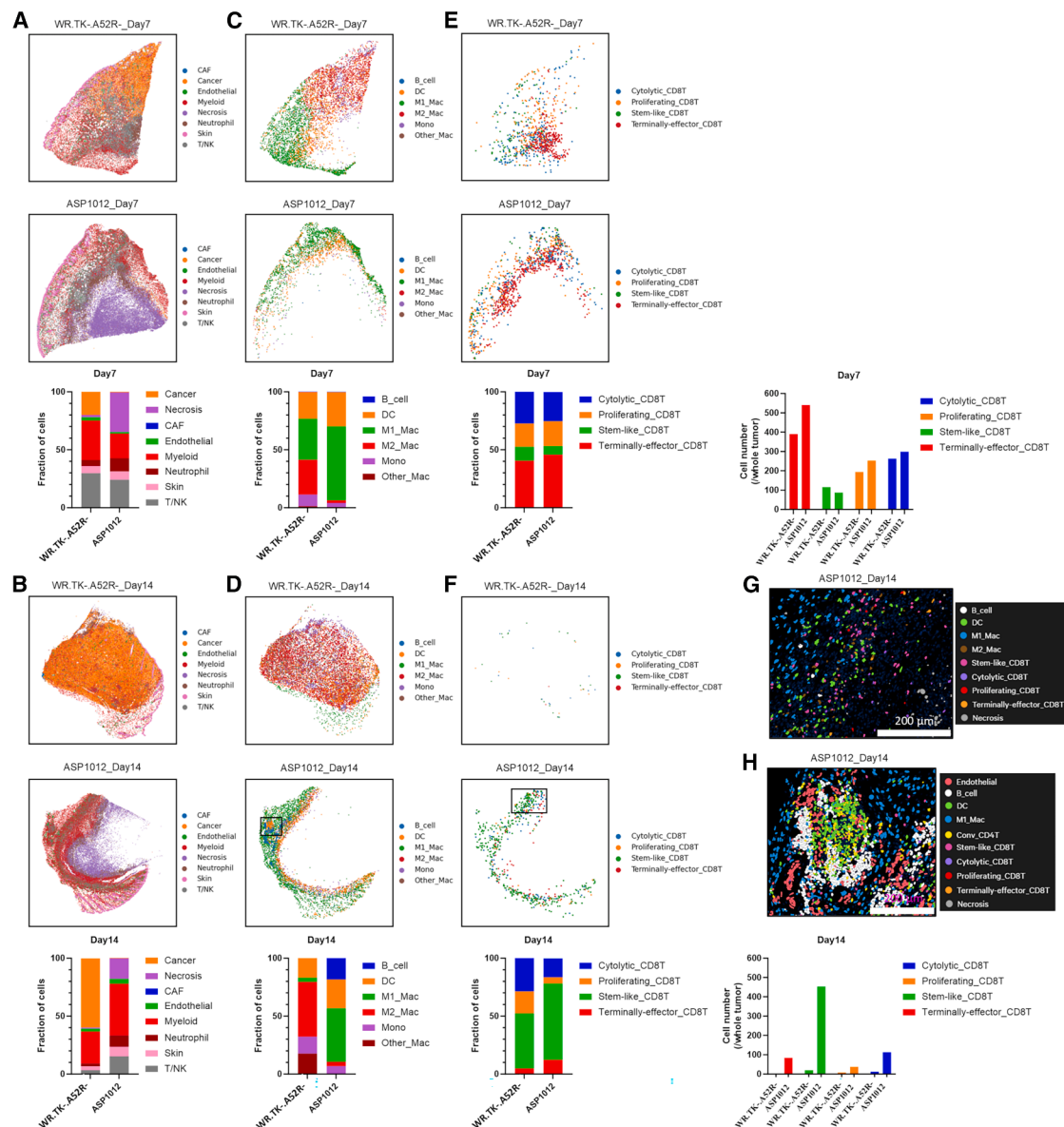


Figure 6. Spatial tumor microenvironment changes induced by ASP1012 versus a control virus

(A and B) Spatial mapping of different cell types after the initial clustering. Upper, WR.TK-A52R-treated (1×10^7 PFU intravenous) tumor (C57BL/6 with subcutaneous LLC tumor $n = 1$); middle, ASP1012-treated (1×10^7 PFU intravenous) tumor (C57BL/6 with subcutaneous LLC tumor $n = 1$); lower, the proportions of each cell type on each tumor tissue sample ($n = 1$) at (A) 7 days and (B) 14 days after the treatment. (C and D) Spatial mapping of the myeloid cell types after the clustering of myeloid cell population. Upper, WR.TK-A52R-treated tumor ($n = 1$); middle, ASP1012-treated tumor ($n = 1$); lower, the proportions of each myeloid cell type on each tumor tissue sample at (C) 7 days and (D) 14 days after the treatment. (E and F) Spatial mapping of the CD8 T cell types after the clustering of CD8 T cell population. Upper, WR.TK-A52R-treated tumor ($n = 1$); middle, ASP1012-treated tumor ($n = 1$); lower, the proportions of each CD8 T cell type on each tumor tissue sample at (E) 7 days and (F) 14 days after the treatment. (G and H) Spatial visualization of selected cell types on Xenium explorer in regions highlighted in (D) and (F). The results of cell annotation from spatial transcriptome analysis on Python were extracted and visualized on the Xenium explorer. WR.TK-A52R-, control virus; ASP1012, ASP1012 containing murine Leptin-IL2.

to exclude M1-like macrophages on both day 7 and day 14 (Figures 6C and 6D). While a small number of cytolytic CD8+ T cells and proliferating CD8+ T cells were dispersed on day 7, the most common T cell population were terminally effector CD8+ T cells that were excluded from cancer-rich regions. Almost no

T cells were present by day 14 after this treatment (Figures 6E and 6F). However, following ASP1012 treatment, a very different pattern was observed. At day 7, enriched M1-like macrophages were found in the periphery of the necrotic areas, surrounding effector CD8+ T cells. By day 14, these immune cells had apparently formed even

more structured bands around the necrotic areas, with cytolytic and many stem-like CD8+ T cells closest to the necrotic region, mixing with dendritic cells (DCs) in regions proximal to the necrotic areas, while M1-macrophages formed an outer shell (Figures 6D–6F and 6G). Furthermore, whereas terminally effector CD8+ T cells were the primary infiltrating subpopulation in the ASP1012-treated tumor on day 7, stem-like CD8+ T cells became dominant on day 14, suggesting a transition of CD8+ T cells from the effector phase to the memory phase, as we had observed previously (Figures 1B and 1D). ASP1012 greatly increased the number of stem-like CD8+ T cells compared to WR.TK-.A52R- virus treatment.

Additionally, an apparently organized immune structure with features similar to a tertiary lymphoid structure enriched with B cells, DCs, and CD4+ T cells was found in the periphery of the ASP1012-treated tumor at day 14 (Figure 6H).

DISCUSSION

Vaccinia-based OVI has demonstrated significant potential in the clinic,^{22,23} especially with respect to the platform's ability to attract T cells into even cold tumors.²⁴ However, the approach has frequently fallen short due to the tumor's ability to subvert or exhaust the T cell response and has been limited practically by the need to directly inject vectors into the tumor itself.

In order to improve delivery, we have developed a platform approach (VET) to create oncolytic vaccinia vectors that can be reliably delivered intravenously. ASP1012 contains the first reported VET backbone, which is combined with expression of a Leptin-IL2 fusion protein as a therapeutic transgene (Figure S2A).

A critical and unique VET modification in ASP1012 is the expression of CCR2 to achieve targeted systemic delivery of virus to CCL2-expressing tumors. Vaccinia can replicate in some hematopoietic cell lineages found in circulation,²⁵ and so it is unsurprising that some amount of intravenously delivered vaccinia virus will infect these cells in circulation. Macrophages and B cells in particular have been shown to support vaccinia replication, while other lineages, including natural killer (NK) and T cells, do not.¹⁶ It is unclear whether infected macrophages and B cells, which can both act as antigen-presenting cells, would normally then present the virus to the immune response or whether this is a mechanism the virus has evolved to spread within a host.

Here, it was determined that expression of chemokine receptors, such as CCR2 or CXCR4, under control of a transient early promoter (such that gene expression occurs during the viral eclipse phase, prior to viral takeover of the cellular machinery),¹⁷ makes it possible to redirect these naturally infected lymphocyte populations toward the cognate chemokine. The chemokine receptor CCR2 was selected as its ligand, CCL2, is frequently upregulated across the majority of human solid tumor types (The Cancer Genome Atlas [TCGA] database), including melanoma,²⁶ with only low-level expression in normal tissues (Protein Atlas). Here, we added early CCR2 expres-

sion into a Western Reserve vaccinia strain lacking TK. Removal of viral TK yields a strain that replicates well only in cancerous cells, and at most low levels—incapable of causing severe disease—in some healthy cells. By doing so, we found it was possible to increase the systemic delivery of the vaccinia vector to tumors expressing CCL2 by around 2- to almost 10-fold (Figures 2B and 4C). This is likely to be significant in a clinical setting, where systemic delivery of oncolytic vaccinia has been demonstrated, but viral accumulation in the tumor is still often below the levels needed to produce reliable therapeutic responses. Sacrifice of B cells to achieve tumor-specific delivery poses low risk for patient safety: therapeutic doses of ASP1012—entailing infusion of up to 1×10^{10} infectious particles—remain orders of magnitude lower than the amount of cells in circulation, and the degree of B cell loss here is negligible in comparison to B cell-depletion therapy used clinically to treat autoimmune disorders. Therefore, the small fraction of B cells “consumed” for delivery is unlikely to be consequential, and these lost cells are rapidly replenished. Enhanced delivery was selective for the tumor, as delivery was reduced in most normal tissues, which may provide additional safety. The liver showed equivalent uptake between control virus and ASP1012, likely due to reticuloendothelial system's removal of virus from circulation, but historically there has been limited indications of associated liver toxicity with oncolytic vaccinia. Finally, because the virus is delivered to the tumor within a carrier cell during the eclipse phase of viral replication, delivery was possible even in pre-immunized mice (Figure 4; Figures S5D and S5E). This will be critical to achieve repeated intravenous treatments in a clinical setting.

Effector T cells that may be primed and clonally expanded in draining lymph nodes often become exhausted or subverted to a suppressive phenotype by environmental cues after arrival in tumors. Prior work established that leptin signaling induces metabolic reprogramming within CD8+ T cells to increase their resistance to exhaustion while simultaneously favoring the expansion of memory cells.² These studies also demonstrated the feasibility of using recombinant vaccinia to produce leptin *in situ* from infected tumor cells. Building upon these studies, we present here the efficacy of combining leptin with IL2 as a flexibly linked fusion protein to ensure prolonged exhaustion-resistant activation of CD8+ T cells already residing in targeted tumors or those recruited by localized vaccinia-induced inflammation. This dual-signal strategy addresses one of the limitations found in the clinical use of IL2: its affinity for, and activity toward, regulatory T cells, which also express high levels of the IL2 receptor.¹¹ Leptin binding has opposite effects on effector T cells (T helper 1 [Th1] polarization, proliferation, secretion of stimulatory cytokines) and regulatory T cells (anergy and loss of suppressive response), meaning the Leptin-IL2 fusion may both better target leptin to T cells, while also abrogating the negative effects of IL2 binding to regulatory T cells. Indeed, Leptin-IL2 provided greater therapeutic response than either IL2 or Leptin alone in both hot and cold tumor models (Figure 1). The greater therapeutic effects of Leptin-IL2 were associated with alterations in the T cell population within the tumor. It is also possible the Leptin-IL2 fusion will increase the stability of

IL2 and help retain it in the tumor microenvironment. It is noteworthy that the response to ASP1012 appears to be as good or better in a cold tumor model relative to a hot tumor. This may be related to differences in the immune escape mechanisms employed by these different tumors. If this is reproducible, the fact that CCL2-expressing tumors tend to be cold may be a benefit.

The resulting vector combining both *CCR2* and *Leptin-IL2* expression, ASP1012, was found to be therapeutically active in multiple mouse tumor models after intravenous delivery. After two doses at a PFU/kg in the range of the expected human clinical dose,²¹ many CRs were produced in LLC, MC-38, and EMT-6, while control viruses with no transgenes did not produce any CRs (Figure 3). Of note, LLC and MC-38 express high and low CCL2 respectively, with both showing enhanced ASP1012 delivery as well as therapeutic activity. EMT-6, however, does not express CCL2, and so response in this model relies on inherent systemic delivery of vaccinia, demonstrating that, although CCL2 expression provides an advantage, it is not a requirement for therapeutic activity with ASP1012. Furthermore, systemic delivery and activity of ASP1012 were retained even in pre-immunized mice (Figure 4). The effects of ASP1012 on the tumor microenvironment were examined, with multiple beneficial effects seen, including expression of pro-effector T cell and loss of suppressive gene signatures (Figures S6A and S6B); increased CD8+ T cell numbers in the tumor (Figure 5A); unique induction of an anti-tumor adaptive T cell response (Figure 5B); and increased number, clonality, and persistence of TCRs, notably including gamma delta T cells (Figures 5C and 5D). Finally, spatial transcriptomics analyses demonstrated a significantly more organized immune response around the tumor (relative to a control virus without transgenes) (Figure 6). In addition, a structure with similarities to a tertiary lymphoid structure, which have been associated with clinical responses to immune checkpoint inhibitors, was found after treatment with ASP1012 (Figure 6H); however, more studies would be needed to confirm the production of functional TLS.

ASP1012 therefore addresses many shortcomings of OVI and warrants further testing in the clinic.

MATERIALS AND METHODS

Virus and cell lines

Vaccinia strain Western Reserve was obtained from ATCC BEI resources. HeLa, B16, LLC, EMT-6, 2PK-3, WEHI-231, and Renca cells were purchased from ATCC. MC-38 cells were kindly donated by Dr. Antoni Ribas, University of California, Los Angeles (UCLA).

Generation of ASP1012 and other vaccinia virus strains

Cloning plasmids containing the pUC57 backbone (Azenta) were designed to recombine into the Western Reserve vaccinia virus genome and replace either the *A52R* (*VACWR178*) or thymidine kinase/*J2R* (*VACWR094*) viral genes. In this way the human *CCR2* gene replaced the *A52R* gene under the control of the native *A52R* promoter. The *Leptin-IL2* fusion replaced the *TK/J2R* gene and was under the control of the vaccinia p7.5 promoter. Recombination and selection

were via standard techniques. Two versions of ASP1012 were constructed: WO0428N.001 expresses a murine Leptin-IL2 fusion protein and KV0467N.001 expresses a human Leptin-IL2 fusion protein. Both constructs express human *CCR2a*. KV0467N.001 was used throughout this work unless specified otherwise.

In addition, control viruses containing just the *TK/J2R* deletion (*TK-*) or the *A52R* and the *TK/J2R* deletion (*WR.TK-.A52R-*) were constructed without transgenes, and viruses with both *A52R* and *TK/J2R* deletions were constructed with either human *CCR2A* alone or murine Leptin-IL2 fusion alone. Viruses were expanded in HeLa cells and purified through sucrose gradients and titered by plaque assay on U-2 OS cells.

qPCR of CCR2

HeLa cells were infected with control virus or ASP1012 at a MOI of 5 for 24 h. The cells were then harvested and RNA extracted using miRNeasy kit (Qiagen). cDNA synthesis was then performed using an iScript cDNA synthesis kit (Bio-Rad). A qPCR mastermix was prepared by adding human GAPDH or human *CCR2* primers (Bio-Rad) and SSO advanced Sybr mix (Bio-Rad). The qPCR was run on an Applied Biosystems Quantstudio 6 Flex qPCR system. Data were analyzed using QuantStudio 6 analysis software. *CCR2* fold-change expression levels over GAPDH were plotted.

Chemotaxis

Human B cells were isolated using a human B cell enrichment kit (#19054, Stem Cell Technologies) according to manufacturer's specifications. Isolated B cells were infected with *WR.TK-.A52R-* or ASP1012 for 24 h at an MOI of 5. Twenty-four hours after infection, B cells were harvested, placed in the upper chamber of a 24-well Transwell plate (#3422, Costar), and allowed to migrate to 100 ng/mL of recombinant human CCL2 (#279-MC-010, R&D Systems) in the bottom chamber for 90 min at 37° C. The migrated cells in the bottom chamber were harvested and quantified on an Attune flow cytometer.

IL2 and Leptin ELISA

HeLa cells were infected at an MOI of 5 for 24 h as described above. At 24 h post infection, supernatants were harvested for ELISA. To detect and quantify recombinant human IL2 produced by ASP1012, EIA plates (#3590, Costar) were coated with anti-IL2 primary antibody (#500-p119-100, Peprotech) overnight at 4° C. All later incubations were performed at room temperature. The plates were blocked in PBS (#20012027, Gibco) with 2% BSA (#A30075, Research Products International) for 1 h followed by addition of serially diluted recombinant IL2 standards (#210-12-10UG, Peprotech)/samples and incubation for 2 h. This was followed by incubation with a human IL2 biotinylated antibody (#500-P155GBT-50UG, Peprotech) for 1 h. Streptavidin-horseradish peroxidase (HRP) (#N100, Thermo Fisher Scientific) was allowed to bind to the biotinylated secondary antibodies for 30 min and detected with a TMB/peroxide substrate (#1854060, 1854050, Thermo Fisher Scientific) followed by addition of 50 μ L of 1 M H₂SO₄ (#S25597, Fisher Scientific).

Education) to stop the reaction. Plates were read at 450 nm using a Tecan Spark 10M plate reader. Leptin/Leptin-IL2 levels were determined using a human Leptin ELISA kit from R&D systems according to manufacturer's specifications.

Flow cytometry

In vitro cultured CD8⁺ T cells or single-cell suspension from tumor were stained with Zombie Yellow (#423103, BioLegend) live/dead dye for 15 min then washed and stained in BD stain buffer (#554656, BD Sciences) with 2 μ L of CD45-APC Fire-750 (30-F11) (#103154, BioLegend), CD8-A488 (53-6.7) (#100723, BioLegend), CD44-A647 (IM7) (#103018, BioLegend), CD62L-PE-Cy7 (MEL14) (#104418, BioLegend) antibodies for surface immune cell markers or with VACV B8R TSYKFESV-PE (#TB-M538-1, MBL international) or CK19 SQLSMKAAL-PE (#TBCM3-DBI-1 MBL international) tetramers for 30 min. The cells were washed twice in BD stain buffer. For intracellular staining of GZMB-PECy7 (#B352429, BioLegend), cells were fixed and permeabilized with Cytostain fix and perm kit (#426803, BioLegend) and then stained with anti-GZMB antibodies. The cells were washed twice in BD stain buffer, and the stained cells were analyzed using Attune flow cytometer.

In vivo studies

All animal studies were performed at the AAALAC accredited Hilltop Animal labs (Scottdale, PA) according to Institutional Animal Care and Use Committee (IACUC)-approved protocols. Power analysis was used to determine animal sample sizes. Animals were group housed in cages. When animals were ready to enroll for viral treatments, animals were stratified into treatment groups for the same average tumor measurements per treatment group. Tumor measurements were performed twice weekly by Hilltop staff who were masked to study conditions and treatment groups. Volume measurements were terminated for subjects deemed non-evaluable because they fulfilled any of the following conditions: tumor ulceration caused reduction in tumor volume, clear evidence that a tumor was eaten by cage mates, and tumor volume exceeded 1,400 mm³ requiring the subject to be sacrificed per IACUC. Non-evaluable subjects were never counted as CRs.

KPC mice

Homozygous and heterozygous tamoxifen-inducible KPC mice were purchased from Jackson laboratories and housed at Hilltop Animal Laboratories. Mice were allowed to acclimate and age to breeding age. Animals WT or heterozygous for Kras were paired as follows: heterozygous females paired with WT males or all WT females paired with heterozygous Kras males. Dams were dosed with 300 μ L of 20mg/mL tamoxifen (Thermo Fisher Scientific) on days 0 (new litter), 1, 2, and 4. Litters were genotyped by Hilltop Animal Laboratories staff.

Development of KPC tumor cell lines

To create tumor cell lines from KPC tumors, KPC mice ($n = 10$) that developed tumors were harvested and homogenized individually using an octo dissociator (Miltenyi Biotec). Each tumor sample was

numbered randomly from 1 to 10. Homogenized samples were filtered through a cell strainer, and the cell pellet was resuspended in 5 mL of ACK lysis buffer (Life Technologies) and incubated for 5 min at room temperature. Next, 10 mL of RP10 complete medium was added to each tube and tubes were centrifuged at 1,200 rpm for 5 min at room temperature. The supernatant was discarded, and the cell pellets were resuspended in 10–20 mL of RP10 complete medium based on the size of the cell pellet. Cell counts were performed using 0.4% trypan blue. Cells and medium were added to T175 tissue culture flasks at 2e6 cells/flask and incubated at 37°C + 5% CO₂ for 3 days.

Growth and CD11b and CD3 staining of KPC cell line tumors

To test the ability of KPC cell lines to form tumors *in vivo*, KPC cell lines were implanted into 8- to 10-week-old female C57BL/6 mice at 4e5 cells/mouse at Hilltop Animal Laboratories. Once five tumors for each cell line type (KPC1–10) reached 200 mm³, tumors were harvested for immune infiltrate analysis and frozen at –80°C. Samples from each tumor were sectioned (10 μ m thick) onto slides using a cryostat and stored at –80°C until ready for staining. For staining, samples were submerged in 4% paraformaldehyde (PFA) for 30 min then washed with 1 $\times$ PBS and then dried. Next, samples were covered with background suppressor for 15 min then blocked with PBS/1% BSA/0.5% Triton X-100 for 30 min. Diluted CD11b and CD3 antibodies were added to corresponding slides and samples were incubated overnight at 4°C. Slides were washed 10 $\times$ with PBS then dilutions of streptavidin-HRP conjugate were added to each slide. Samples were washed 10 $\times$ with PBS, each slide was mounted with 20–50 μ L of Everbrite Mounting Medium with DAPI, and coverslips were placed on each slide. Samples were imaged on a CellInsight CX7 LZR microscope (Thermo Fisher Scientific) at 4 $\times$ and 20 $\times$ magnification. The percentage CD11b and CD3 cell populations for each KPC cell line were analyzed.

To determine the efficacy of KPC1 and KPC7 tumors treated with component viruses *in vivo*, 8- to 10-week-old C57BL/6 mice ($n = 15$) were implanted with 4e5 KPC1 or KPC7 cells. When tumors reached ~100 mm³, animals were intratumoral treated with virus formulation buffer (vehicle) or 1 $\times$ 10⁷ PFU WR.TK-, WR.TK-.Leptin, WR.TK-.IL2, or WR.TK-.Leptin-IL2 component viruses. Tumor growth was monitored blinded for 60 days. To analyze tumor cell populations after treatment by fluorescence-activated cell sorting (FACS) a smaller subset of KPC1 and KPC7 tumor-bearing animals ($n = 5$) were harvested on D8 and D21. Tumor samples were transferred to gentleMAC C tubes and homogenized as described above. The final cell pellets were resuspended in 1 mL, pipetted into a 96-well plate, and centrifuged at 1,200 rpm for 5 min. The supernatant was discarded and cells were incubated with Zombie Yellow to determine viability and immune antibody panels that corresponded to CD4⁺ and CD8⁺ T cell populations.

LLC, MC-38, EMT-6, and Renca tumor models

Mice were implanted subcutaneously with 4 $\times$ 10⁵ LLC, 5 $\times$ 10⁵ MC-38 (C57BL/6), or 4 $\times$ 10⁵ EMT-6 or Renca (BALB/c) cells and treated

via tail vein or intratumoral injection with viruses once tumors were 50–100 mm³. All mouse studies were repeated at least one time.

qPCR of animal tissues

Tumors and other tissues were recovered and homogenized using a FASTPrep-24 tissue homogenizer (Fisher Scientific) at speed #6 for intervals of 4 × 30 s treated with proteinase K. Vaccinia virus was inactivated by incubating samples at 65°C for 4 h. DNA was isolated using a DNEasy kit (Qiagen). Samples were run on an Applied Biosystems Quantstudio 6 Flex qPCR system using a viral standard curve E9L pUC Kana plasmid DNA (Genewiz) and primers forward AGAAATTCATCCATGGTGGCA and reverse AAAATGCTCCGTCGCAGA (IDT). Virus was quantified using the Taqman probe 56-FAM/TGCTGCGTT/ZEN/TCTTTGGCTCTTCT/3IABkFQ (IDT). Data were analyzed using QuantStudio 6 analysis software.

Pre-immunization study

28 days prior to implantation with LLC tumor cells, 10-week-old C57BL/6 female mice were pre-immunized with 1 × 10³ PFU WR.TK- virus by intraperitoneal injection.

Neutralizing antibody assays

Neutralizing antibody titer was determined using U-2 OS cells seeded in 96-well plates (#3595 Costar) at a density of 1.2 × 10⁴ cells per well. Serum samples from virus-treated and naive mice were serially diluted, initially 1:10 then 2-fold with 1,000 PFU of TK deleted virus and added to U-2OS cells. Plates were incubated for 4 days at 37°C in 5% CO₂. The spent medium was removed using a pipette, and wells were replaced with PBS containing 4.5 g/L glucose. Twenty microliters of MTS reagent (Promega) was added to each well, and samples were incubated for 3 h at 37°C in 5% CO₂. Plates were read at 490 nm on a Tecan Spark plate reader. Dilutions that neutralized 50% of virus (EC50) were recorded.

NanoString and RNA-seq

Tumors were harvested 3 days post treatment for NanoString and RNA sequencing (RNA-seq) analysis. Tumor tissues were homogenized in Qiazol (Qiagen) using lysing matrix D tubes and a bead mill as described above.

RNA from cells and tumor tissues was isolated by lysis in Qiazol (Qiagen) followed by extraction using an RNeasy kit (#74104, Qiagen). cDNA synthesis was performed using an iScript cDNA synthesis kit (#1708891, Bio-Rad) according to manufacturer's specifications.

RNA extracted from mouse tumor tissues was sent to NanoString (Seattle, WA) and was analyzed with an nCounter Mouse Immunology gene panel.

RNA samples were sent to Azenta (Genewiz) Life Sciences for RNA-seq. RNA-seq libraries were prepared using a NEBNext Ultra II RNA Library Prep kit (New England Biolabs). First- and second-strand cDNA was subsequently synthesized. cDNA fragments were end-re-

paired and adenylated at 3' ends, and universal adapters were ligated to cDNA fragments followed by index addition and library enrichment by PCR. Sequencing libraries were validated on an Agilent TapeStation (Agilent Technologies) and quantified using a Qubit 2 Fluorometer (Thermo Fisher Scientific) as well as by qPCR (KAPA Biosystems). Sequencing libraries were run on an Illumina HiSeq instrument according to manufacturer's instructions.

iRepertoire analysis

RNA extracted from tumors was used to make TCR α, β, γ, and δ chain libraries using mouse RepSeq with four chain cassettes (iR + panT-MLRI-C), using the IR-Flex system and sent to iRepertoire for sequencing. Data were analyzed on Irweb, and tree maps and CDR3 reads were tabulated.

Mass spectrometry

B cell lines infected with different vaccinia strains (MOI 1.0) were lysed after 24 h and supernatants sent to the University of California (UC) Davis Proteomics Core Facility for processing, liquid chromatography-mass spectrometry (LC-MS), and data analysis.

Spatial transcriptomics

Sample preparation for Xenium analysis

LLC tumor-bearing mice were intravenously treated with virus formulation buffer ($n = 2$), 1 × 10⁷ PFU of WR.TK-.A52R- virus ($n = 2$), or 1 × 10⁷ PFU of ASP1012 ($n = 2$) on days 0 and 3. On day 7 and day 14, tumors were collected ($n = 1$ per time point), and formalin-fixed, paraffin-embedded (FFPE) tumor blocks were prepared. 5-μm sections were prepared from the FFPE samples using a microtome (Thermo Scientific, HM355S). The Xenium workflow was conducted according to the manufacturer's protocol (10× Genomics, CG000578) using Slides & Sample Prep Reagents (10× Genomics). Briefly, the sectioned samples were put on Xenium slides, followed by deparaffinization and decrosslinking to make the RNA accessible. After probe hybridization, probes were ligated. During this ligation, a rolling circle amplification (RCA) primer was also annealed. The circularized probes were amplified to generate multiple copies of the gene-specific barcode for each RNA. After the amplification, cell segmentation staining was conducted according to the manufacturer's protocol (10× Genomics, CG000749) using Cell Segmentation Staining Reagents (10× Genomics), followed by chemical autofluorescence quenching. After nuclear staining, the Xenium slides were subjected to gene expression analysis using the Xenium Analyzer instrument (10× Genomics). For gene detection, 284 murine genes of interest were selected for the targeted analysis of anti-tumor immunity (10× Genomics, Xenium Add-on Custom Panel).

Data analysis after Xenium analysis

The spatial *in situ* data were analyzed using Python Scanpy and Squidpy on Jupyter Lab (4.0.11). All transcriptomic data of vehicle, WR.TK-.A52R- virus, and ASP1012-treated tumor samples were integrated, and principal-component analysis (PCA) was used to reduce dimensionality. For visualization, UMAP was used to project the data down to two dimensions. After the initial clustering and

annotation, we subset the T/NK cell population and the myeloid cell population and then performed several rounds of the clustering and annotation for each population to identify the granular clusters for the CD8 T cell subpopulations and the myeloid cell subpopulations. The whole-cell-annotation dataset was exported from Python to visualize populations on Xenium explorer (Version 3, 10× Genomics).

Statistical analysis

Students' T test and one-way ANOVA with Tukey's *post hoc* analysis were used to determine statistical significance. The Cox-Mantel statistical test was used to determine the significance in Kaplan-Meier estimator plots. $p < 0.05$ was used to define statistical significance. All graphs were plotted using GraphPad Prism version 9 (La Jolla, CA).

DATA AND CODE AVAILABILITY

- All data will be publicly available and researchers may request access to any data.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Antoni Ribas for the MC-38 cell line and Gerald Koelsch for critical review and editing of the manuscript. Studies were funded in part by National Cancer Institute grant R44 CA224737-01(S.H.T.).

AUTHOR CONTRIBUTIONS

Conceptualization, S.H.T. and S.N.; methodology, S.H.T., R.M., C.C., M.Z., and P.S.; investigation, R.M., C.C., M.Z., P.S., G.A.-B., J.M., R.B., D.D., N.M., K.B., M.C., B.W., M.H., and S.A.; visualization, R.M., C.C., M.Z., P.S., G.A.-B., J.M., R.B., D.D., N.M., K.B., M.C., B.W., M.H., S.A., K.M.B., and S.H.T.; writing – original draft, R.M., M.H., K.M.B., S.H.T., and S.N.; writing – review and editing, R.M., C.C., M.Z., P.S., K.M.B., S.H.T., and S.N.

DECLARATION OF INTERESTS

R.M., C.C., M.Z., P.S., G.A.-B., J.M., R.B., D.D., N.M., K.B., M.C., B.W., S.A., K.M.B., and S.H.T. are employed by Kalivir Immunotherapeutics. S.N. and M.H. are employed by Astellas Pharma.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.ymthe.2026.07.005>.

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