Deletion of immunomodulatory genes as a novel approach to oncolytic vaccinia virus development

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INTRODUCTION

Vaccinia virus (VV) has emerged as a promising platform for oncolytic virotherapy. Many clinical VV candidates, such as the double-deleted VV, vvDD, are engineered with deletions that enhance viral tumor selectivity based on cellular proliferation rates. An alternative approach is to exploit the dampened interferon-based innate immune responses of tumor cells by deleting one of the many VV immunomodulatory genes expressed to dismantle the antiviral response. We hypothesized that such a VV mutant would be attenuated in non-tumor cells but retain the ability to effectively propagate in and kill tumor cells, yielding a tumor-selective oncolytic VV with significant anti-tumor potency. In this study, we demonstrated that VVs with a deletion in one of several VV immunomodulatory genes (N1L, K1L, K3L, A46R, or A52R) have similar or improved in vitro replication, spread, and cytotoxicity in colon and ovarian cancer cells compared to vvDD. These deletion mutants are tumor selective, and the best performing candidates (ΔK1L, ΔA46R, and ΔA52R VV) are associated with significant improvement in survival, as well as immunomodulation, within the tumor environment. Overall, we show that exploiting the diminished antiviral responses in tumors serves as an effective strategy for generating tumor-selective and potent oncolytic VVs, with important implications in future oncolytic virus (OV) design.

Received 9 October 2020; accepted 12 May 2021; https://doi.org/10.1016/j.omto.2021.05.007.

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Candidate VV immunomodulatory gene roles

| VV gene | Role |
|---------|------|
| N1L | Inhibits NF-κB and IRF3 activation via interaction with the IKK (complex) and TBK1<br>● Inhibits pro-apoptotic proteins (Bad/Bax/Bid) by binding to their BH3 motifs |
| K1L | Prevents regulatory protein IkBα degradation and subsequent release of NF-κB into the nucleus<br>● Blocks early and intermediate viral RNA from triggering PKR-dependent NF-κB activation<br>● Inhibits acetylation of NF-κB subunit RelA<br>● Binds to ACAP2<br>● Acts as a host range protein |
| A46R | Inhibits TLR and IL-1 signaling by binding to TIR-domain-containing proteins (i.e., MyD88, TRIF, MAL, TRAM) thereby inhibiting NF-κB, MAPK, and IRF3 activation |
| A52R | Activates p38 MAPK to drive TLR-4 mediated IL-10 expression<br>● Activates MAPK/AP-1 (c-Jun) pathway |

Abbreviations: IRF3, interferon-regulatory factor 3; IKK (complex), IκB kinase (complex); BH3, B cell lymphoma 2 homology 3; PKR, protein kinase R; eIF2α, α subunit of eukaryotic initiation factor 2; IL-1, interleukin-1; TIR, Toll/IL-1 receptor; MyD88, myeloid differentiation factor 88; TRIF, Toll-receptor-associated activator of interferon; MAL, MyD88 adaptor-like protein; TRAM, TRIF-related adaptor molecule; MAPK, mitogen-activated protein kinase; IRAK2, IL-1 receptor-associated kinase 2; TRAF6, tumor-necrosis-factor-receptor-associated-factor 6; AP-1, activator protein 1.

Table 1. Candidate VV immunomodulatory gene roles

nucleotide pools, it is not optimal for slow-growing malignancies and fails to capitalize on the dysfunctional antiviral response that characterizes many tumor cells.5

VV expresses several immunomodulatory genes to evade the host immune response,5,18 and single or multiple gene deletions may attenuate its ability to replicate in non-tumor cells without affecting its virulence in transformed cells. Using the Western Reserve VV strain, which has enhanced tumor specificity and anti-tumor potency at baseline,19 we generated a panel of oncolytic VVs with gene deletions related to the inhibition of host cell interferon (IFN) induction and signaling pathways. These included N1L, K1L, K3L, A46R, and A52R. Previous studies have suggested that single gene deletions of A46R, A52R, N1L, and K1L lead to reduced virulence in BALB/c mice compared to vvDD.20 When K3L was deleted, viral replication was maintained in HeLa cells, but not BHK cells.26 As such, we postulated that a single gene deletion of N1L, K1L, K3L, A46R, or A52R from Western Reserve VV would generate a tumor-selective oncolytic virus with improved tumor-potency compared to vvDD.

RESULTS

Creation of candidate VV deletion mutants

In order to determine whether VVs with a dampened ability to block type I IFN responses would exhibit tumor-specific replication and potent oncolytic activity, we generated a panel of Western Reserve VVs with a deletion in one of several VV immunomodulatory genes. These included N1L, K1L, K3L, A46R, or A52R. Gene candidates were rationally selected based on their role in inhibiting major steps in IFN induction and signaling pathways (Table 1). Homologous recombination was facilitated by flanking an expression cassette with DNA sequences associated with each gene of interest. Primers (Table 2) were designed based on gene sequences described in GenBank accession number AY243312. The resultant candidate VV deletion mutants have each immunomodulatory gene interrupted by an expression cassette containing the R2R segment and a xanthine-guanine phosphoribosyltransferase (xprt) gene (Figure 1). Viruses were confirmed by red fluorescent protein (RFP) expression and PCR (data not shown).

Candidate VV deletion mutants exhibit cytotoxicity, replication, and spread similar to or better than vvDD in monolayers of colon and ovarian cancer cell lines

The in vitro oncolytic potential of our candidate VV deletion mutants was compared to vvDD16,27 in terms of tumor cell cytotoxicity, viral replication, and viral spread in MC38, DLD-1, and A2780 cancer cell lines. Cytotoxicity was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTS) assay in monolayer cultures infected at an MOI of 1. The cytotoxicity of each of our candidate VV deletion mutants was either equal to or higher that of vvDD in the colon cancer cell lines MC38 and DLD-1 (Figure 2). The difference in cytotoxicity of all candidate VVs compared to vvDD was most dramatic in MC38 cells, where cell viability of samples infected with candidate VVs was reduced to approximately half that of vvDD-treated cells at 72 hpi (p < 0.05; Figure 2A). Ovarian cancer A2780 cells were the most susceptible to all VVs, where the relative survival was less than 50% by 48 h in all treatment groups (Figure 2C). Among all candidate VV deletion mutants, the ΔΔA46R VV was the most cytotoxic with a mean cell viability of 40.1% ± 0.3%, 40.3% ± 2.0%, and 4.8% ± 1.1% at 72 h in MC38, DLD-1, and A2780 cells, respectively. While some of the difference in cytotoxicity may be explained by the presence of TK and VGF genes, the fact that there was significant variation in replication and spread between candidate VVs (Figures 2G–2I and 3A and 3B) as described below, and that the most cytotoxic candidates did not display the highest replication levels, suggests other mechanisms were likely at play as well.
The replication and spread of the VVs in all cancer cell lines were assessed by infecting cancer cells at a low MOI of 0.1. The replication of vvDD and VV deletion mutants in all cell lines resulted in a 2–3 log-fold increase in total virus by 72 hpi (Figures 2D–2I). In general, all candidate VV deletion mutants exhibited similar or better viral replication compared to vvDD in MC38, DLD-1, and A2780 cells at peak viral titers, with the exception of ΔA46R VV (Figures 2D–2I). However, the replication efficiency of all VVs in A2780 cells was generally higher compared to the colon carcinoma cells (Figure 2G–2I). Among them, the ΔK1L VV demonstrated the most efficient replication ability across all cell lines, with the average fold change in replication being 2.8, 13.9, and 2.3 times higher than the average fold change of vvDD in MC38, DLD-1, and A2780 cells, respectively (Figures 2G–2I, p < 0.05).

The spread of all candidate VVs was similar or better than vvDD in all cell lines tested, as confirmed by both visual assessment and quantitative analysis (Figure 3). Visual assessment confirmed superior viral spread for ΔK1L VV, ΔN1L VV, and ΔA52R VV in all cell lines. The best performer, ΔA52R VV, demonstrated consistently superior viral spread in all cell lines, infecting 55.6 ± 6.1%, 39.7 ± 8.5%, and 65.5 ± 4.1% of monolayers of MC38, DLD-1, and A2780 cells, respectively (Figure 3). As such, mean ΔA52R VV spread was 11.8%–33.7% better relative to vvDD at peak time points in all tested cell lines (Figure 3, p < 0.05).

**Candidate VVs exhibit equal or better viral spread and cytotoxicity in tumor spheroids compared to vvDD**

We used MC38 and DLD-1 tumor spheroids to mimic the 3D structure and microenvironment of a tumor mass and allow for more relevant in vitro investigation (Figure 4). At 72 hpi, the majority of infection was concentrated at the spheroid periphery. In comparing vvDD to candidate viruses, only minimal amounts of RFP expression associated with vvDD infection could be seen in MC38 spheroids at 72 hpi. In contrast, the ΔN1L, ΔK1L, ΔA46R, and ΔA52R VVs infected most of the rim and exhibited some penetration into the MC38 spheroids. The DLD-1 spheroids were more resistant to infection by VVs, resulting in small, dispersed foci of RFP by 72 hpi. However, ΔA52R VV was able to infect a larger portion of the DLD-1 spheroids. Out of all viruses tested, ΔA52R VV demonstrated the most substantial spread in both MC38 and DLD-1 spheroids (Figure 4A). Moreover, virus-induced cytotoxicity of all VVs resulted in ≤3.3% and ≤1.3% surviving fraction in MC38 and DLD-1 spheroids, respectively, at 96 hpi as measured by a clonogenic assay (Figures 4B and 4C).

**At therapeutic doses, candidate VVs have preserved tumor specificity and significant survival benefit**

The 3 best-performing candidate VVs in vitro were chosen for subsequent in vivo investigation against vvDD. These were ΔK1L VV, ΔA52R VV, and ΔA52R VV.

Maximum tolerable doses (MTDs) were determined by injecting increasing doses of VV intraperitoneally (i.p.) into non-tumor-bearing mice. The highest dose at which mice survived for 4 weeks or more was deemed the maximum tolerable dose and used for subsequent tumor survival studies (data not shown). For immunocompetent C57BL/6 mice, the i.p. MTD for the ΔK1L VV was 5 × 10⁷ PFU, while the MTD of ΔA46R VV and ΔA52R VV was 1 × 10⁷ PFU. The i.p. MTD of all candidate VVs in nude mice was 10⁶ PFU. In contrast, the established i.p. dose of vvDD is 10⁵ PFU, where only transient weight loss after injection is observed.29

The anti-tumor efficacy and tumor-selectivity of i.p.-delivered candidate VVs and vvDD were compared in tumor-bearing models of PC. Specifically, a syngeneic model of C57BL/6 mice bearing i.p. MC38 tumors and xenograft models in nude (NU/NU) mice bearing i.p. A2780 or DLD-1 tumors were used. Mice were injected i.p. with tumor cells and treated with i.p. VV 12 days later at indicated doses.

Using the MTD for each virus to evaluate clinically relevant anti-tumor efficacy, candidate VV deletion mutants improved survival similar to or better than vvDD with respect to mock-treated controls. In the MC38 tumor-bearing C57BL/6 syngeneic model (Figure 5A), the ΔN1L, ΔK1L, ΔA46R, and ΔA52R VVs significantly improved survival compared to vvDD. The ΔA52R VV was the most potent, with a 47.3% survival benefit at the MTD. These results demonstrate that candidate VVs can demonstrate clinical activity comparable to that of vvDD, with improved tumor specificity and survival benefit.

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**Table 2. Primers for producing inserts for the shuttle plasmid**

| Gene | Fwd | Left flanking insert | Rev | Right flanking insert |
|------|-----|----------------------|-----|----------------------|
| N1L  | Fwd | 5'-gatccaaatgcttccatca-3' | Rev | 5'-gatccgctagcttccatca-3' |
| K1L  | Fwd | 5'-gatccaaatgcttccatca-3' | Rev | 5'-gatccgctagcttccatca-3' |
| K3L  | Fwd | 5'-gatccaaatgcttccatca-3' | Rev | 5'-gatccgctagcttccatca-3' |
| A46R | Fwd | 5'-gatccaaatgcttccatca-3' | Rev | 5'-gatccgctagcttccatca-3' |
| A52R | Fwd | 5'-gatccaaatgcttccatca-3' | Rev | 5'-gatccgctagcttccatca-3' |

Abbreviations: Fwd, forward; Rev, reverse.
vvDD- and ΔA46R-treated mice showed a trend toward improved survival over mock-treated mice. On the other hand, a significant improvement in median survival time compared to mock-treated mice was observed in ΔK1L-treated mice (35 days vs. 28.5 days, p = 0.0058), not observed in vvDD, and ΔA52R VV-associated median survival was also longer than vvDD or mock treatment (32.5 days).

There was a trend toward improved survival over vvDD in xenograft models of PC using DLD-1 tumor-bearing mice (Figure 5B), with long-term survival at 160 dpi observed in 12.5%, 25%, and 37.5% of vvDD-, ΔA46R VV-, and ΔA52R VV-treated mice, respectively. Finally, in A2780 tumor-bearing mice (Figure 5C), ΔK1L VV treatment, unlike vvDD or the other candidate VVs, significantly improved median survival time compared to mock-treated mice (53.5 days versus 40 days, p = 0.0021). A 12.5% long-term survival rate was associated with vvDD, ΔK1L, and ΔA52R treatment.

VV replication preferentially targeted the tumor and the ovaries in all cancer mouse models tested (Figure 6). In C57BL/6 mice bearing MC38 tumors (Figure 6A), 2–4 log-fold less candidate VV deletion mutant titer was detected in the tumor compared to vvDD. With the exception of the ovaries, all viruses displayed several log-fold titer reductions in other non-tumor tissues compared to tumor. Most impressively, ΔA52R VV was undetectable in the bowel, spleen, liver, heart, and brain. In DLD-1 and A2780 tumor-bearing NU/NU mice (Figures 6B and 6C), the tumor viral load of candidate VVs compared to vvDD was more similar, ranging within approximately 1 log of each other. In general, the concentration of infectious particles of all candidate VV deletion mutants and vvDD was lower in non-tumor tissues, except the ovary, compared to tumor.

Candidate VVs and vvDD treatment are associated with increased immune infiltration into tumors

A portion of the tumors harvested from the MC38 and DLD-1 tumor mouse models were used for immunohistochemistry and stained for B220 (B cells), CD3 (T cells), F4/80 (macrophages), and Ly6G (neutrophils/PMN-MDSCs; Figure 7). There were no significant differences in B220 staining between all treatment groups for both tumor models (Figure 7A) whereas all candidate VVs were associated with increased T cell infiltration into MC38 tumors (ΔK1L VV, 7.18% ± 0.78%; ΔA46R VV, 6.79% ± 0.81%; ΔA52R VV, 9.96% ± 0.30%) compared to mock-treatment (0.15% ± 0.06%), but not vvDD (3.56% ± 0.89%; Figure 7B). Moreover, the percent F4/80 staining in MC38 tumors treated with ΔA52R VV was at least 3 times higher than both mock- and vvDD-treated tumors. Similarly, ΔA52R VV treatment in DLD-1 tumors was associated with an increased mean percent of F4/80 staining compared to mock treatment (Figures 7C and 7E). In contrast, no increase in neutrophil/MDSC infiltration was seen with any of the viruses compared to mock (Figure 7D); however, there was a significant decrease seen in ΔK1L VV compared to vvDD.

DISCUSSION

Genetic alterations in tumor cells can disrupt host antiviral pathways and render immunomodulatory viral genes redundant for successful viral infection and propagation. Mutations affecting IFN responses are common in tumors, with an estimated 65%–70% of cancer cell lines harboring defective IFN responses.\(^6\) Other groups have designed OVVs to exploit this phenomenon. For example, Kirn et al.\(^{30}\) investigated a VV with a deletion in its IFN-β antagonist, B18R, as a potential oncolytic VV. Here, we describe a panel of Western Reserve VVs with a gene deletion in a VV antagonist of TLRs (A46R, A52R), nuclear factor-κB (NF-κB; N1L, K1L), or PKR (K3L; Table 1). To assess the potential of these VVs as oncolytic agents, we compared the candidate VVs to a clinical candidate oncolytic VV, vvDD.\(^{16,27}\) We hypothesized that our panel of VVs with single VV immunomodulatory gene deletions would have improved potency against colon and ovarian cancer while retaining equal or better attenuation in normal tissue compared to vvDD.

Overall, the candidate VVs performed equally or better than vvDD in our in vitro assessments in terms of cytotoxicity, replication, and spread in the colon cancer cell lines MC38 and DLD1, and the ovarian cancer cell line A2780. These cell lines have been found to be moderately to poorly responsive to IFN.\(^{30–35}\) Interestingly, the ΔK1L, ΔA46R, and ΔA52R VV consistently demonstrated superior viral replication, tumor cytotoxicity, and viral spread. This suggests that the redundancy of the deleted IFN-related genes in cancer cells may indeed support VV propagation and cell-killing in vitro, although further investigation, for instance comparing IFN-deficient and IFN-intact tumor cell lines, is needed. The deletions in vvDD (TK and VGF) render the virus reliant on the cell-intrinsic pool of nucleotides\(^8\) while the candidate VVs still express TK and VGF. This may be problematic for vvDD spread within the tumor environment, reflected here in tumor spheroids, where, for example, the nutrient gradient renders tumor cells near the center of the spheroid quiescent.\(^{33}\) However, vvDD can better defend itself against host cell antiviral mechanisms as it still expresses the full armamentarium of VV immunomodulatory genes.

The improved performance of candidate VV’s over vvDD in vitro may speak to both the presence of TK and VGF genes and the comparative degree of reduced fitness that results from immunomodulatory gene deletion as opposed to TK and VGF deletions. Given that
we demonstrated variability in the in vitro activity of each candidate VV, this would support a role for the redundancy of VV IFN genes in these tumor cell lines above and beyond only the presence of TK and VGF. However, further investigation into the complex interactions between VV and host cells is necessary to more fully explain the observed phenomena.

The ΔA46R VV demonstrated superior in vitro tumor cytotoxicity as measured by the MTS assay. The product of the A46R gene, A46, inhibits TLR signaling through Toll/interleukin 1 receptor (TIR) domain-containing proteins, ultimately decreasing NF-κB, IFN regulatory factor 3 (IRF3), and mitogen-activated protein kinase (MAPK) activation.20 Among the protein targets of A46, TIR-domain-containing adaptor-inducing IFN-β (TRIF) can induce apoptosis through a protein motif that can also activate NF-κB.34 Thus, we hypothesize that the deletion of A46R enables TRIF-induced apoptosis in response to the VV infection leading to the higher cell death compared to other candidate VVs and vvDD. While a deeper investigation of the molecular mechanisms at play here was outside the scope of this study, further evaluation of specific VV deletion mutants is planned to confirm the degree to which changes in components of the IFN pathway such as TRIF and types of tumor cell death are involved.

The ΔK1L VV consistently replicated to the highest titer within 72 h following in vitro infections. While constitutive expression of NF-κB is characteristic of many tumor types, including the cell lines used in this study,35–40 other common perturbations in malignant cells such as Ras/MEK activation nonetheless suppress downstream expression of various IFN response genes.32 Based on the above findings for our ΔK1L VV, one explanation is that this gene is the most redundant in the face of this repression of IFN response in the tumor lines evaluated.

Among the two genes with direct effects on NF-κB, N1L also has anti-apoptotic properties,38 which may be important for prolonging host cell survival to allow more VV replication. Thus, we hypothesize that the K1L was the most redundant VV gene among our candidate genes for enhancing VV replication in monolayer cultures of tumor cells, leading to the superior viral replication seen compared to candidate VVs and vvDD.

Lastly, the ΔA52R VV demonstrated the best in vitro spread for all cell lines in both monolayer and spheroid culture. In addition to inhibiting TLR signaling, the expression of A52R leads to c-Jun N-terminal kinase (JNK)21 and AP-1 activation.39 During VV infection, JNK activation is important for cytoskeleton reorganization. In JNK1/2 knockout cells, early cell contractibility in response to Western Reserve (WR) VV infection is abrogated and plaque size, trafficking of early virions to the cell periphery, and the number of extracellular enveloped virions released are increased.40 Thus, the reduced JNK activation during ΔA52R VV infection may lead to increased extracellular enveloped virus (EEV) release, thereby increasing VV spread. Pereira et al.41 hypothesized that WR VV induces cytoskeleton rearrangements in order to congregate immature virions and...
reduce immune detection by the host, thereby increasing VV replication and spread. Perhaps this mechanism is redundant in our tumor cell lines and the possible increase in EEV release in the absence of A52R provided more benefit.

We subsequently investigated our top performing candidate VVs (ΔK1L, ΔA46R, and ΔA52R VVs) from our in vitro studies in mouse models of peritoneal carcinomatosis. At doses that would likely be used in the clinical setting, the tumor selectivity of our candidate VVs was similar to vvDD. In agreement with literature,8,29,41 the viral replication of vvDD localized to the tumors and ovaries. Likewise, the candidate VVs showed a similar biodistribution in all mouse models. Both engineered and intrinsic properties of VV would have contributed to the biodistribution of candidate VV and vvDD replication. The deletions in candidate VVs dampen their ability to inhibit host innate antiviral mechanisms, allowing normal tissues to more effectively defend against successful candidate VV propagation compared to tumor cells, which often have defective antiviral responses. Intrinsically, VVs are large viruses; hence, the virions may accumulate in tissues with leaky vasculature that allow VV to exit the bloodstream, such as tumors.42 Though the viral load in the ovaries for all VVs was high, this may be a mouse-specific phenomenon as vvDD was undetectable in non-human primate ovaries 6 days following intravenous (i.v.) treatment.43 Further, the amount of replicating WR VV found in the ovaries of rhesus macaques (3,646 PFU/mg) was comparably lower than the viral load of candidate VVs in our mouse ovaries.43 A possible explanation is that the estrous cycle in mice, involving periods of increased cell proliferation and angiogenesis in mouse ovaries,44 contributed to the high VV load in our models.

Figure 3. VV spread in monolayer tumor cell lines
Tumor cell lines were mock-infected or infected with either vvDD or a candidate VV deletion mutant at an MOI of 0.1 and evaluated for viral spread based on RFP marker expression. (A) Representative bright-field and fluorescent images (Cy3) at 10× magnification at peak fluorescence at 72 hpi (MC38, DLD-1) or 48 hpi (A2780; n = 3). (B–D) Quantification of percent infected area (n = 3). Data are a combination of two independent experiments, each with three replicates, and values are mean ± SEM. *p < 0.05 compared to vvDD.
A

| vvDD | ΔN1L | ΔK1L | ΔK3L | ΔA46R | ΔA52R | Mock |
|------|------|------|------|-------|-------|------|
| ![Image of vvDD] | ![Image of ΔN1L] | ![Image of ΔK1L] | ![Image of ΔK3L] | ![Image of ΔA46R] | ![Image of ΔA52R] | ![Image of Mock] |

MC38

2h: ![Image of MC38 at 2h]
72h: ![Image of MC38 at 72h]

DLD-1

2h: ![Image of DLD-1 at 2h]
72h: ![Image of DLD-1 at 72h]

B

**MC38**

![Bar graph of Surviving Fraction for MC38]

C

**DLD-1**

![Bar graph of Surviving Fraction for DLD-1]

(legend on next page)
Treatment with the candidate VVs demonstrated a survival benefit in both immunocompetent and immunocompromised tumor-bearing mouse models at dosages chosen through MTD determination. The maximum doses at which non-tumor-bearing mice survived 4 weeks following infection were deemed treatment doses for survival studies (data not shown). Compared to the established vvDD dose of $10^9$ PFU, candidate VV treatment doses were 20–100 times lower in C57BL/6 mice ($\Delta K1L$ VV, $5 \times 10^7$ PFU; $\Delta A46R$ and $\Delta A52R$ VV, $1 \times 10^7$ PFU) and 1,000 times lower in NU/NU mice ($1 \times 10^6$ PFU). Lower MTDs of the candidate VVs were expected. Unlike vvDD, the deletions in the VV immunomodulatory genes render our candidate VVs unable to inhibit the antiviral signaling cascade leading to pro-inflammatory cytokine and IFN activation. Possibly, a cytokine storm in response to the candidate VV infection resulted in a heightened immune response that contributed more to the tissue damage and subsequent deaths than the VV infection alone. Further studies measuring cytokine and IFN induction following candidate VV treatment will be necessary to confirm this speculation. Nonetheless, given our biodistribution and anti-tumor data for candidate VVs, the reduced dosing compared to vvDD appears to be both safe and effective at this initial stage of evaluation.

In our MC38-bearing immunocompetent model, only the $\Delta K1L$ VV treatment was associated with a statistically significant improvement in median survival compared to mock ($p = 0.0058$). Moreover, the viral load in tumors was the lowest among mice in the $\Delta K1L$-treated group by 6 days post-treatment. It is well-established that OV's have multiple mechanisms of action against cancer including direct oncolysis, disruption of vasculature, and induction of anti-cancer immunity. Immunohistochemistry results show that $\Delta K1L$ VV treatment increased T cell infiltration compared to mock-treatment even though neutrophil and MDSC infiltration was decreased compared to vvDD treatment in this model. This is similar to the intradermal $\Delta K1L$ VV infection model by Cruz et al. wherein innate immune cell infiltration was decreased but VV-specific CD8+ T cell infiltration and activation were elevated compared to wild-type WR VV. Thus I.p. $\Delta K1L$-VV treatment in our immunocompetent mice may have also resulted in improved tumor-specific adaptive T cell response, which contributed, in conjunction with direct viral lysis, to the improved anti-tumor efficacy.

Similar to findings associated with the NYVAC VV strain, our $\Delta A46R$ and $\Delta A52R$ WR VV treatments also increased T cell infiltration into tumors. However, these vectors offered no statistically significant improvement in the median tumor survival compared to mock- and vvDD-treated groups. Perhaps the initial higher replication efficiency of $\Delta K1L$ VV demonstrated in the in vitro assays was translated to this mouse model and triggered a more robust anti-cancer immune response with more T cell activation compared to $\Delta A46R$ VV, $\Delta A52R$ VV, and vvDD. A more in-depth investigation of the immune response will be necessary to uncover the mechanism of anti-tumor efficacy of the candidate VVs in an immunocompetent model.

Candidate VVs also improved the survival of nude mice implanted with human ovarian or colon cancer xenografts. In DLD-1 tumor-bearing mice, the $\Delta A52R$ VV treatment was associated with complete response and long-term survival in the highest proportion of mice (37.5%), followed by $\Delta A46R$ VV (25%) and vvDD (12.5%). There were no differences in immune infiltrates between VVs in DLD-1 tumor-bearing mice except for an increase in F480+ cells in $\Delta A52R$ VV-treated tumors compared to the mock treatment. In A2780 tumor-bearing mice, $\Delta K1L$ VV improved mean survival, but both $\Delta K1L$ and $\Delta A52R$ VV treatment conferred complete responses and long-term survival (12.5%). In both models, immunocompromised nude mice were used to allow for human tumor development. Hence, the major contributors of tumor efficacy were potentially direct viral oncolysis, vasculature disruption, and the innate immune response. As in the immunocompetent model, the factors that influenced candidate VV efficacy could also apply to the efficacy observed in the nude mouse models. The superior $\Delta A52R$ VV spread, demonstrated in monolayer and spheroid culture, may have played a major role in its therapeutic efficacy in the immunocompromised models, especially in the DLD-1 tumor-bearing mice as DLD-1 is relatively resistant to VV infection and spread. The redundancy of a particular VV gene deletion in promoting successful VV propagation is important for anti-tumor efficacy in these models. In addition, the induction of innate immunity by candidate VV or vvDD infection, though dysregulated in tumor cells, may have also contributed to anti-tumor efficacy. Other groups have investigated innate immune induction from candidate VV gene-deleted vectors and vvDD in different models. Further work will be necessary to elucidate the innate immune response and cytokine induction in our tumor-bearing nude mouse models and correlate them to the anti-tumor efficacy presented in this study. Finally, in the DLD-1 model, all viruses, including vvDD, demonstrated an early non-significant increased death rate over control treatment. While this may have been due to the increased pro-inflammatory cytokine response discussed above in the candidate VVs, given that this was also present in vvDD suggests otherwise. Certainly, all VV groups in this model also displayed a trend toward improved survival over mock-treated mice, consistent with MC38 and A2780 models.

In summary, we have generated a panel of novel VVs with deletions in VV immunomodulatory genes (N1L, K1L, K3L, A46R, and A52R) and investigated the potential of these vectors as anti-tumor agents compared to vvDD. The candidate VVs demonstrated equal or figure 4. VV spread and cytotoxicity in multicellular tumor spheroids MC38 and DLD-1 tumor spheroids mock infected or infected with either vvDD or a candidate VV deletion mutant at an MOI of 2 (n = 5). (A) Representative bright-field and confocal fluorescent images (Cy3) at 150 μm depth. Scale bar, 100 μm; 10× magnification (n = 4). Clonogenic assay of (B) MC38 and (C) DLD-1 tumor spheroids at 96 dpi. Surviving fraction is the number of colonies (>50) after 10 days of incubation relative to the number originally seeded. Values are means of triplicates ± SEM corresponding to a representative experiment out of 2 independent experiments.
improved in vitro viral replication, spread, and tumor cell cytotoxicity in both monolayers and spheroid cultures of colon and ovarian cancer cells. Moreover, superior candidate VVs from the in vitro assays (ΔK1L VV, ΔA46R VV, and ΔA52R VV) demonstrated a significant anti-tumor effect, comparable tumor selectivity, and a trend toward improved survival compared to vvDD when using a clinically relevant dosing approach. Thus, we have demonstrated that exploiting the dampened IFN response in tumors is a viable mechanism of tumor selectivity for generating potential OVVs. It would be interesting to investigate the interactions between the candidate VVs, tumor, and
the immune system that lead to improved therapeutic efficacy. A better understanding of the mechanisms would inform possible combination treatments to further improve survival. Additionally, our findings support further investigation of anti-tumor potency of the candidate VVs in other common malignancies. Overall, the immunomodulatory gene deleted VVs described herein are promising OVs for development as clinical cancer therapies.

MATERIALS AND METHODS

Cell lines
Human colorectal adenocarcinoma cell line DLD-1, human ovarian cancer cell line A2780, and normal monkey kidney fibroblast cell line CV-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MC38 murine colorectal adenocarcinoma cell line and C57BL/6 murine sarcoma cell line 24JK were

Figure 7. Immune infiltrate of VV-treated tumors
C57BL/6 (n = 3) and NU/NU (n = 3) mice were injected i.p. with MC38 or DLD1 tumor cells, respectively, and treated i.p. with HBSS, vvDD, or candidate VVs 12 days later as follows: MC38-bearing C57BL/6 mice (vvDD = 1 × 10^9 PFU, ΔK1L = 5 × 10^7 PFU, ΔA46R = 1 × 10^7 PFU, or ΔA52R = 1 × 10^7 PFU, and DLD-1-bearing NU/NU mice (all VVs: 5 × 10^6 PFU). Mice were sacrificed 6 days post-treatment and 2–3 tumors per mouse were harvested and fixed in formalin for analysis by immunohistochemistry. Quantification of immunohistochemistry staining for (A) B220, (B) CD3 (MC38-bearing C57BL/6 model only), (C) F4/80, and (D) Ly6G. Data depict percent of positively stained pixels relative to total number of pixels per tumor. (E) Representative images of tumors stained for F4/80. Values are means of triplicates ± SEM, *p < 0.05 compared to mock-treated (HBSS); †p < 0.05 compared to vvDD-treated.
obtained from the National Institutes of Health (NIH; Bethesda, MD, USA). Cells were cultured at 37°C and 5% CO₂ in media supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Etobicoke, ON, Canada) and 1% penicillin-streptomycin (Invitrogen, Gibco, Grand Island, NY, USA). All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with the exception of A2780 cells, which were maintained in Roswell Park Memorial Institute medium (RPMI 1640; in-house Toronto Medical Discovery Tower media, University Health Network, Toronto, ON, Canada).

**Vaccinia viruses**

Recombinant Western Reserve VV F13L⁺, a wild-type virus with lacZ insertions,⁴⁹ and vvDD-R2R-Luc were used in this study. vvDD-R2R-Luc expresses an R2R segment, with a RFP insertion conjoined with Renilla luciferase via a foot-and-mouth disease virus 2A motif.⁵⁰ vvDD-R2R-Luc is derived from a double-deleted Western Reserve VV platform lacking TK and VGF.⁸

**Creation of candidate VV deletion mutants**

Wild-type Western Reserve VV F13L⁺ was used as a backbone virus to generate the VV deletion mutants via homologous recombination. Using the primers listed in Table 2, segments of DNA (300–500 bp) flanking the left and right regions of wild-type N1L, K1L, K3L, A46R, or A52R VV genes were amplified by PCR. They were then inserted into a shuttle plasmid to flank an expression cassette consisting of an R2R segment and a xgprt gene under the regulation of the synthetic late pSyn/late promoter and the early/late p7.5 promoter, respectively (Figure 1). Monolayers of CV-1 cells at 80% confluence were infected with F13L⁺ (MOI = 0.05) in DMEM-2.5% FBS for 2 h. Liposomal transfection (Lipofectamine 2000; Invitrogen, GIBCO) was then conducted as per the manufacturer’s protocol using 2.5 μg of each individual shuttle plasmid DNA and 10 μL of liposome solution per well. Subsequent selection for xgprt expression from recombinant viruses in drugged DMEM (250 μg/mL xanthine, 14.9 μg/mL hypoxanthine, 25 μg/mL mycophenolic acid; Sigma Aldrich) was performed for at least 5 rounds in CV-1 cells under an agarose overlay and confirmed for RFP expression. Final recombinant VV clones were expanded and DNA was extracted by Proteinase K digestion as previously described.⁸ PCR confirmation was performed with the forward primer of the left flanking insert and the reverse primer of the right flanking insert of the corresponding viruses (Table 2).

**Viral replication and spread in monolayers**

Tumor cells were grown in 6-well plates overnight (5 × 10⁵ cells/well for MC38; 1 × 10⁶ cells/well for DLD-1 and A2780) and mock-treated or infected with candidate VVs or vvDD (MOI = 1) in low-serum media (2.5% FBS) for 2 h and then supplemented with complete media (10% FBS). Viral cytotoxicity was evaluated using the MTS cell viability assay (CellTiter96 Aqueous One Solution, Promega, Madison, WI, USA) according to the manufacturer’s protocol, with cell viability presented relative to mock-treated samples.

**Tumor spheroid model**

Spheroids were generated in 96-well round-bottomed plates coated with 1% polyHEMA (Sigma Aldrich) as previously described and grown for 72 h. Spheroids were mock-infected or infected with candidate VVs or vvDD (MOI = 2) in DMEM-2.5% FBS for 2 h and then supplemented with DMEM-10% FBS. Bright-field and fluorescent confocal images were obtained with the LSM 700 Confocal Microscope (Carl Zeiss). Clonogenic assays were conducted at 96 hpi as previously described.⁵⁵ Surviving fraction was calculated as the number of colonies (>50 cells) divided by the number of cells originally seeded.

**Mice**

Female 6- to 8-week-old C57BL/6 and NU/NU athymic nude mice (Charles River Laboratories; Saint-Constant, QC, Canada) were used. All mice were housed under standard conditions and given food and water ad lib. Experimental protocols were approved by the Animal Care Centre, University Health Network, Toronto. Mice were euthanized when signs of morbidity including weight loss of >20%, a subcutaneous tumor >1.5 cm, extreme abdominal distention, difficulty breathing, moribund condition, or inability to obtain food or water, were present.

**In vivo toxicity**

Female C57BL/6 mice or NU/NU athymic nude mice (n = 6) were injected i.p. with the indicated doses of virus in Hank’s balanced salt solution (HBSS; Invitrogen, GIBCO) or HBSS alone and monitored for signs of morbidity as above.

**In vivo tumor models**

Female C57BL/6 mice were injected i.p. with 10⁵ MC38 cells in 2 mL serum-free media. Female NU/NU athymic nude mice were injected i.p. with 5 × 10⁶ DLD-1 cells or 10⁷ A2780 cells in 2 mL serum-free media. 12 days later, mice were injected i.p. with virus in 1 mL HBSS or control HBSS alone. Mice were followed for morbidity (n = 8) or sacrificed 6 days post-infection for biodistribution studies or immunohistochemistry (n = 3).
For biodistribution studies, tumor and non-tumor tissues were harvested into HBSS. Samples were homogenized with the TissueLyser II (QIAGEN, Hilden, Germany), subjected to 3 freeze-thaw cycles, and sonicated prior to viral quantification by plaque assay with CV-1 cells. Titers were normalized to total protein per sample measured with the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). For immunohistochemistry, tumor tissues were fixed in 10% formalin for 48–72 h, transferred to 70% ethanol, and then embedded in paraffin. Tissue sections were stained with monoclonal rat anti-mouse F4/80 (cat # MCA497R, clone A3-1, dilution 1/2,000; Serotec/Bio-Rad, Hercules, CA, USA), CD3 (cat # A0452, clone F7.2.38 dilution 1/500; Dako, Santa Clara, CA, USA), B220 (cat # 553084, clone RA3-6B2, dilution 1/2,000, BD PharMingen, San Jose, CA, USA), or monoclonal anti-mouse Ly6G (cat # 127601, clone 1A8, dilution 1/1,500; BioLegend, Santa Clara, CA, USA).

**Statistical analysis**

Data were analyzed by a two-tailed independent samples Student’s t test between candidate VVs and vvDD where applicable. Survival curves were evaluated with the log-rank test. Graphs were generated with the Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Values are presented as mean ± SEM (standard error of the mean) and p values < 0.05 were considered significant.

**ACKNOWLEDGMENTS**

This project was funded by the Lotte and John Hecht Foundation Innovation Grant of the Canadian Cancer Society Research Institute.

**AUTHOR CONTRIBUTIONS**

T.Y.H. was the primary researcher for all experiments and the primary author of the manuscript. J.A.M. was the senior researcher and author of the manuscript. D.F.S. was involved throughout the work and provided assistance with hypothesis development, experimental design, interpretation of findings, and manuscript editing. D.M. provided assistance with laboratory work and manuscript editing. I.O. provided laboratory technician support throughout the entirety of the research.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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