Molecular Therapy
Oncolytics
Brief Report

Oncolytic myxoma virus is effective in murine models of triple negative breast cancer despite poor rates of infection

Raquela J. Thomas,[1] Mee Y. Bartee,[1] Miriam Valenzuela-Cardenas,[1] and Eric Bartee[1]

[1]Department of Internal Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Oncolytic viruses are being heavily investigated as novel methods to treat cancers; however, predicting their therapeutic efficacy remains challenging. The most commonly used predictive tests involve determining the *in vitro* susceptibility of a tumor's malignant cells to infection with an oncolytic agent. Whether these tests are truly predictive of *in vivo* efficacy, however, remains unclear. Here we demonstrate that a recombinant, oncolytic myxoma virus shows efficacy in two murine models of triple negative breast cancer despite extremely low permissivity of these models to viral infection. These data demonstrate that *in vitro* infectivity studies are not an accurate surrogate for therapeutic efficacy and suggest that other tests need to be developed.

INTRODUCTION

A large body of literature demonstrates that oncolytic virotherapy (OV) is effective in numerous murine tumor models.[1–4] This efficacy frequently requires the infecting oncolytic agent to be replication competent[5,6] demonstrating that *in vivo* replication plays a critical role in these therapies. Because of this, the first step of many OV studies is to demonstrate the permissivity of the target malignancy to infection with the oncolytic agent. However, a variety of non-replicative viral vectors have also been shown to be effective as cancer therapeutics.[7–10] Therefore, whether the replicative potential of oncolytic viruses truly predict an agent's *in vivo* efficacy remains controversial.[11–13]

Our lab focuses on the use of oncolytic myxoma virus (MYXV). This virus is extremely promiscuous *in vitro* and has been shown to productively infect a wide variety of cancer types, including glioblastoma, lung adenocarcinoma, melanoma, ovarian cancer, colon cancer, gall bladder cancer, osteosarcoma, renal cancer, and prostate cancer.[6,14–16] However, it displays poor infectivity against triple negative breast cancer (TNBC) cells.[16] In the current work, we confirm that murine TNBC cells are poorly permissive to infection with MYXV *in vitro* but demonstrate that the virus remains therapeutically active against these cancers *in vivo*.

RESULTS

Murine TNBC cells are poorly infectible with oncolytic MYXV

We have previously described a recombinant MYXV that expresses both a soluble PD1 inhibitor and interleukin-12 (vPD1/IL12) and is highly active in a variety of tumor models.[14] The efficacy of this virus against TNBC, however, has not been tested. We therefore performed a series of *in vitro* experiments to determine whether vPD1/IL12 could infect a panel of TNBC cells. The results demonstrated that all three TNBC lines tested displayed extremely low rates of viral infection (Figures 1A and 1B), produced significantly fewer infectious particles (Figure 1C), and allowed for only minimal viral spread (Figure 1D) compared with permissive BSC40 cells. Additionally, while infection with high MOIs could reduce the total number of cells in TNBC cultures (Figure S1A), this appeared to be the result of virally induced cytostasis and not acute cell killing since the membranes of infected cells remained intact and their overall morphology was largely unchanged (Figures S1B and S1C). Taken together, these data suggest that TNBC cell lines are largely resistant to both infection and killing by oncolytic MYXV.

vPD1/IL12 treatment delays tumor growth and improves survival in murine models of TNBC

Although our previous results found that TNBC cells were largely non-permissive to infection with vPD1/IL12, we still investigated the efficacy of this virus against TNBC *in vivo*. Syngeneic mice were implanted with EO771 cells and then treated with either saline or three doses of 1×10^7 ffu of vPD1/IL12 delivered directly intratumorally (IT) over 5 days (Figure 2A). Tumors were then measured every other day and animals euthanized when tumors reached 15 mm in any direction. Interestingly, despite poor *in vitro* infectivity, vPD1/IL12-treated mice displayed significantly delayed tumor growth (Figure 2B) and improved overall survival (Figure 2C) compared with controls. Similar results were observed in the spontaneously metastatic 4T1 model (Figure 3A) with vPD1/IL12 therapy delaying primary tumor growth (Figure 3B), improving overall survival (Figure 3C), and reducing metastatic tumor burden in the lungs (Figures 3D and 3F). Taken together, these results demonstrate that vPD1/IL12 is an effective therapeutic agent against TNBC *in vivo*, despite extremely poor rates of infection *in vitro*.

https://doi.org/10.1016/j.omto.2023.08.014.

Correspondence: Eric Bartee, University of New Mexico Health Sciences Center, 1 University of New Mexico, Albuquerque, NM 87131, USA.
E-mail: ebartee@salud.unm.edu

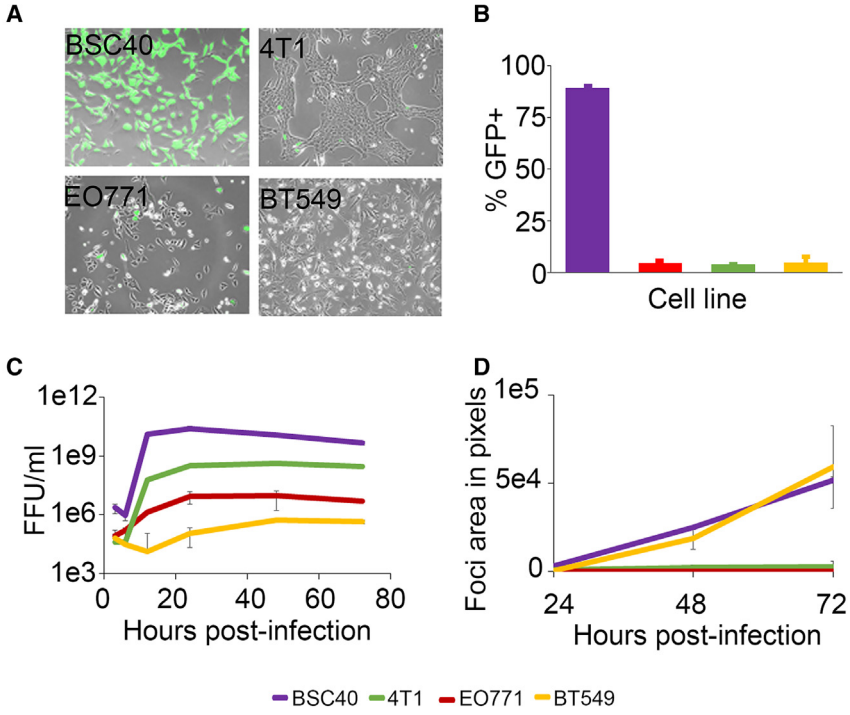

Figure 1. TNBC cells are poorly infectible with a recombinant myxoma virus *in vitro*

TNBC cell lines were infected with vPD1/IL12 at an MOI of 10. (A) Fluorescent images of infected cells at 48 h post-infection. (B) Quantitation of the numbers of infected cells from the cultures in (A). (C) Single-step growth curves showing viral titer at indicated time points. (D) Quantification of average foci size at indicated time points. Data are presented as mean ± SEM and are pooled from two separate experiments with two replicates each.

DISCUSSION

Previous work has suggested that two human breast cancer cell lines (MCF-7 and MDA-MB435), were non-permissive to MYXV infection due to having no detectable endogenous p-Akt.[16] This finding is consistent with our current observations that murine TNBC cell lines are poorly permissive to MYXV infection. Interestingly, this mechanism of restriction is unique to MYXV, although the closely related poxvirus, vaccinia, has been shown to successfully infect human breast GI 101A cells *in vitro*.[17] The low levels of infection in TNBC cells by MXYV might therefore be unique to this oncolytic platform.

Interestingly, *in vitro* infectability did not predict the responsiveness of TNBC tumors to vPD1/IL12 treatment *in vivo*. This is most likely explained by the current oncolytic paradigm in which the immune system plays a major role in OV function. Indeed, our data, taken together with the observation of cytostatic vs. cytolytic activity, the abscopal effect on the lungs, and literature supporting a role for the immune system in OV efficacy indicate that vPD1/IL12 functions through a systemic mechanism that does not require high infection rates. Previous work on other non-replicative viral therapeutics suggests that this could be mediated by either the activation of pattern recognition receptors in infected cells leading to the induction of an interferon response[8–10] or through the section of vPD1/IL12's encoded transgenes (which occurs despite the poor rate of infection, Figure S2).[7] This would explain how the virus can show efficacy *in vivo* despite poor infection *in vitro*.

MATERIALS AND METHODS

Cell lines and reagents

BSC40 (catalog no. BRL-2761), 4T1 (catalog no. CRL-2539), and EO771 (catalog no. CRL-3461) cells were purchased from the

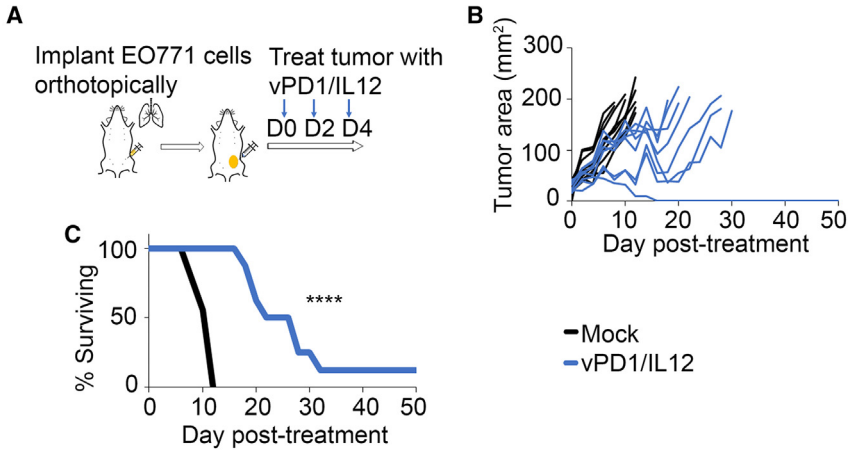

Figure 2. vPD1/IL12 treatment delays tumor growth and improves survival of mice bearing E0771 tumors

(A) Schematic diagram of experimental procedure. Mice were implanted orthotopically with EO771 cells, followed by intratumoral (IT) injection of vPD1/IL12 every 2 days for a total of three treatments. (B) Tumor sizes of mock- or vPD1/IL12-treated mice. (C) Overall survival of mice implanted with EO771 TNBC cells and treated with mock or vPD1/IL12. Significance was determined using log rank analysis (****$p < 0.0001$).

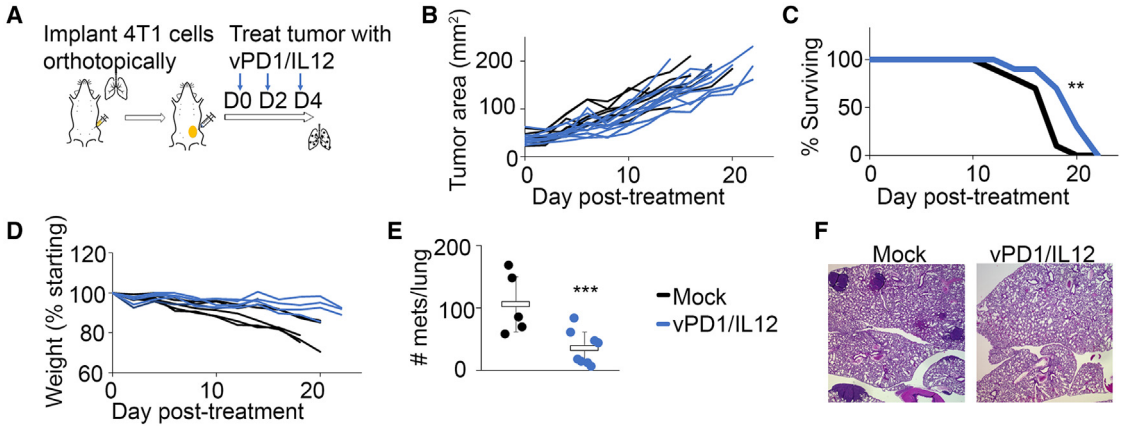

Figure 3. vPD1/IL12 treatment decreases lung metastases and improves survival of mice bearing 4T1 tumors

(A) Schematic diagram of experimental procedure. Mice were injected orthotopically with 4T1 cells followed by IT injection of vPD1/IL12 every 2 days for a total of three treatments. (B) Overall survival of mice implanted with 4T1 TNBC cells and treated with mock or vPD1/IL12. (C) Tumor sizes of mock- or vPD1/IL12-treated mice. Significance was determined using log rank analysis (**$p < 0.01$). (D) Weights of animals as a percentage of weight at day 0. n = 10 mice per group. (E) Number of metastases per lung. n = 5–8 mice per group. Data are presented as mean ± SD. Significance was determined using Student's t test (***$p < 0.001$). (F) Representative micrographs of H&E-stained lungs.

American Type Culture Collection (Manassas, VA, USA). BT549 cells were a kind gift from Dr Eric Prossnitz at the University of New Mexico. BSC40 cells were cultured in DMEM + 10% fetal bovine serum + 1× penicillin-streptomycin-L-glutamine (Mediatech, Manassas, VA, USA); EO771 cells were cultured in the same + 20 mM HEPES (Gibco, Long Island, NY, USA). 4T1 cells were cultured in RPMI-1640 + 10% fetal bovine serum + 1× penicillin-streptomycin-L-glutamine (Mediatech); BT549 cells were cultured in the same + 2.5 mg/mL insulin. All cells were passaged for less than 6 months prior to use.

Virus and infections

The oncolytic viral construct expressing the soluble ectodomain of PD1 and IL-12 is described elsewhere.[14] All *in vitro* assays were carried about by adsorbing virus to cells for 30 min at room temperature then removing viral inoculation media, washing twice with PBS and once with complete media, and finally replacing with new complete growth media. Single-step viral growth curves and foci-forming assays were conducted as previously described.[18,19]

Animal models

All animal protocols were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee. All mice used in these studies were between 6 and 8 weeks of age. BALBc mice were implanted with 1×10^6 TNBC cells in 50 μL PBS orthotopically into the fourth left mammary fat pad. Once all tumors were ~25 mm^2 (~8–10 days), 1×10^7 ffu of vPD1/IL12 in 30 μL PBS per mouse was injected IT on days 0, 2, and 4. Tumor area was measured every 2 days using digital calipers and is presented as tumor area (mm^2) determined using the formula (area = length × width). Weights in grams were measured every 2 days by a digital scale. Euthanasia criteria was a loss of ≥10% of body weight coupled with a decline in body condition score. For lung analyses, mice were euthanized and lungs harvested on day 26. Lungs were embedded in paraffin, sectioned at 8 μm and stained by H&E.

DATA AND CODE AVAILABILITY

No large datasets were generated in the current manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.08.014.

ACKNOWLEDGMENTS

This work was funded by an intramural grant to E.B. from the University of New Mexico, NIH-K12-2GM088021 grant to R.J.T., and UNM Comprehensive Cancer Center grant (P30-CA118100). The authors would like to thank the University of New Mexico Health Sciences Center Flow Cytometry Shared Resource and Human Tissue Repository and Tissue Analysis Shared Resource.

AUTHOR CONTRIBUTIONS

R.J.T.: designed and performed experiments and wrote the paper; E.B.: designed experiments and provided edits and rewrites; M.B. and M.V.C.: performed experiments.

DECLARATION OF INTERESTS

E.B. holds intellectual property concerning the use of the vPD1/IL12 virus, as well as additional intellectual property concerning other recombinant oncolytic viruses that is not relevant to the current manuscript.

REFERENCES

1. Kirn, D.H., and Thorne, S.H. (2009). Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. Nat. Rev. Cancer *9*, 64–71.
2. Martinez-Quintanilla, J., Seah, I., Chua, M., and Shah, K. (2019). Oncolytic viruses: overcoming translational challenges. J. Clin. Invest. *129*, 1407–1418.
3. Rahman, M.M., and McFadden, G. (2020). Oncolytic Virotherapy with Myxoma Virus. J. Clin. Med. *9*, 171.
4. Sitta, J., Claudio, P.P., and Howard, C.M. (2022). Virus-Based Immuno-Oncology Models. Biomedicines *10*, 1441.
5. Allen, C., Opyrchal, M., Aderca, I., Schroeder, M.A., Sarkaria, J.N., Domingo, E., Federspiel, M.J., and Galanis, E. (2013). Oncolytic measles virus strains have significant antitumor activity against glioma stem cells. Gene Ther. *20*, 444–449.
6. Lun, X., Yang, W., Alain, T., Shi, Z.Q., Muzik, H., Barrett, J.W., McFadden, G., Bell, J., Hamilton, M.G., Senger, D.L., and Forsyth, P.A. (2005). Myxoma virus is a novel oncolytic virus with significant antitumor activity against experimental human gliomas. Cancer Res. *65*, 9982–9990.
7. Hofbauer, G.F.L., Baur, T., Bonnet, M.C., Tartour, E., Burg, G., Berinstein, N.L., and Dummer, R. (2008). Clinical phase I intratumoral administration of two recombinant ALVAC canarypox viruses expressing human granulocyte-macrophage colony-stimulating factor or interleukin-2: the transgene determines the composition of the inflammatory infiltrate. Melanoma Res. *18*, 104–111.
8. Yang, N., Garcia, A., Meyer, C., Tuschl, T., Merghoub, T., Wolchok, J.D., and Deng, L. (2022). Heat-inactivated modified vaccinia virus Ankara boosts Th1 cellular and humoral immunity as a vaccine adjuvant. NPJ Vaccin. *7*, 120.
9. Dai, P., Wang, W., Yang, N., Serna-Tamayo, C., Ricca, J.M., Zamarin, D., Shuman, S., Merghoub, T., Wolchok, J.D., and Deng, L. (2017). Intratumoral delivery of inactivated modified vaccinia virus Ankara (iMVA) induces systemic antitumor immunity via STING and Batf3-dependent dendritic cells. Sci. Immunol. *2*, eaal1713.
10. Wang, Y., Jin, J., Li, Y., Zhou, Q., Yao, R., Wu, Z., Hu, H., Fang, Z., Dong, S., Cai, Q., et al. (2022). NK cell tumor therapy modulated by UV-inactivated oncolytic herpes simplex virus type 2 and checkpoint inhibitors. Transl. Res. *240*, 64–86.
11. Madlambayan, G.J., Bartee, E., Kim, M., Rahman, M.M., Meacham, A., Scott, E.W., McFadden, G., and Cogle, C.R. (2012). Acute myeloid leukemia targeting by myxoma virus in vivo depends on cell binding but not permissiveness to infection in vitro. Leuk. Res. *36*, 619–624.
12. O'Riordan, C.R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S.C., Smith, A.E., and Francis, G.E. (1999). PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. Hum. Gene Ther. *10*, 1349–1358.
13. Yarde, D.N., Nace, R.A., and Russell, S.J. (2013). Oncolytic vesicular stomatitis virus and bortezomib are antagonistic against myeloma cells in vitro but have additive anti-myeloma activity in vivo. Exp. Hematol. *41*, 1038–1049.
14. Valenzuela-Cardenas, M., Gowan, C., Dryja, P., Bartee, M.Y., and Bartee, E. (2022). TNF blockade enhances the efficacy of myxoma virus-based oncolytic virotherapy. J. Immunother. Cancer *10*, e004770.
15. Weng, M., Gong, W., Ma, M., Chu, B., Qin, Y., Zhang, M., Lun, X., McFadden, G., Forsyth, P., Yang, Y., and Quan, Z. (2014). Targeting gallbladder cancer: oncolytic virotherapy with myxoma virus is enhanced by rapamycin in vitro and further improved by hyaluronan in vivo. Mol. Cancer *13*, 82.
16. Wang, G., Barrett, J.W., Stanford, M., Werden, S.J., Johnston, J.B., Gao, X., Sun, M., Cheng, J.Q., and McFadden, G. (2006). Infection of human cancer cells with myxoma virus requires Akt activation via interaction with a viral ankyrin-repeat host range factor. Proc. Natl. Acad. Sci. USA *103*, 4640–4645.
17. Hofmann, E., Grummt, F., and Szalay, A.A. (2011). Vaccinia virus GLV-1h237 carrying a Walker A motif mutation of mouse Cdc6 protein enhances human breast tumor therapy in mouse xenografts. Int. J. Oncol. *38*, 871–878.
18. Flores, E.B., Bartee, M.Y., and Bartee, E. (2020). Reduced cellular binding affinity has profoundly different impacts on the spread of distinct poxviruses. PLoS One *15*, e0231977.
19. Wolfe, A.M., Dunlap, K.M., Smith, A.C., Bartee, M.Y., and Bartee, E. (2018). Myxoma Virus M083 Is a Virulence Factor Which Mediates Systemic Dissemination. J. Virol. *92*, e02186-17.