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Oncolytic virotherapy

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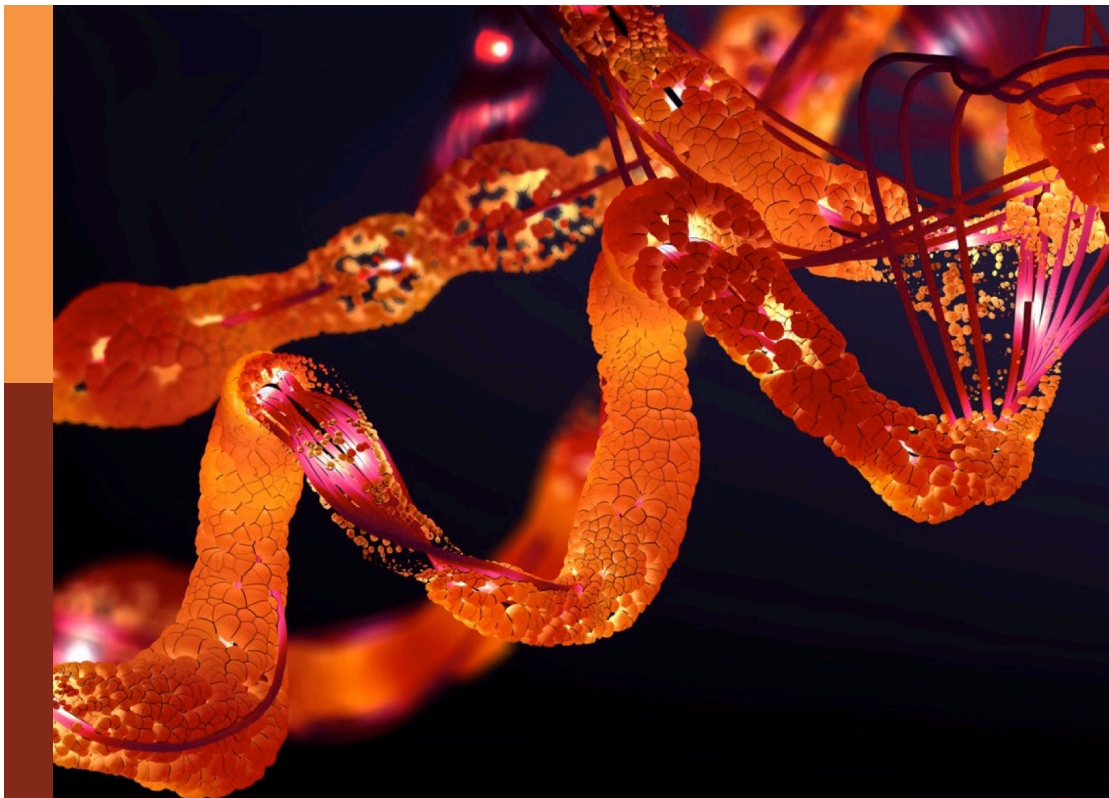

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Oncolytic virotherapy

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Editorial: Oncolytic virotherapy

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Editorial on the Research Topic
Oncolytic virotherapy

1 Background and purpose of the Research Topic

Using viruses to treat cancer is an established concept, and many viruses have shown promising antitumor efficacies (Vähä-Koskela et al., 2007). Oncolytic viruses are safe and well characterized pathogens with a stable genome (Maroun et al., 2017). The outstanding clinical results of oncolytic virotherapy deserve serious attention and consideration to make it a treatment option alongside classical cancer therapeutics (Russell et al., 2022). Virotherapy uses replication-competent oncolytic viruses to replicate and destroy cancer cells selectively. The transformed nature of cancer cells offers a permissive environment for the replication of some viruses and to complement viral mutations (Nemunaitis and Edelman, 2002). *In situ* amplification and spread within the tumor mass are the key benefits of such replication-competent viruses. Oncolytic virotherapy is divided into two main groups, according to tumor specificity: naturally oncolytic viruses to replicate in human cancer cells and gene-modified viruses engineered to accomplish selective oncolysis (Driever and Rabkin, 2001). OV kills cancer cells through several mechanisms (Figure 1), including cell lyses, due to virus replication (Al-Shammari et al., 2021).

In this Research Topic, 15 articles related to oncolytic virotherapy were submitted, including 8 reviews, one method article, and six original research articles. These articles shed light on recent and promising research on oncolytic virotherapy and ways to enhance its efficacy against cancer.

The articles were divided into five subtopics: 1) cancer models to investigate the efficacy of oncolytic viruses; 2) OVs as cancer immunotherapeutic agents; 3) novel viral platforms; 4) combination therapies; and 5) methods to develop OV quantification.

2 Cancer models investigate the efficacy of oncolytic viruses

Carter and colleagues (Carter et al.) used stable organoid cell cultures derived from breast cancer tissue to develop a protocol to study the effects of oncolytic viruses. They used an established three-dimensional organoid model derived from tissue from 10 patients with primary breast cancer.

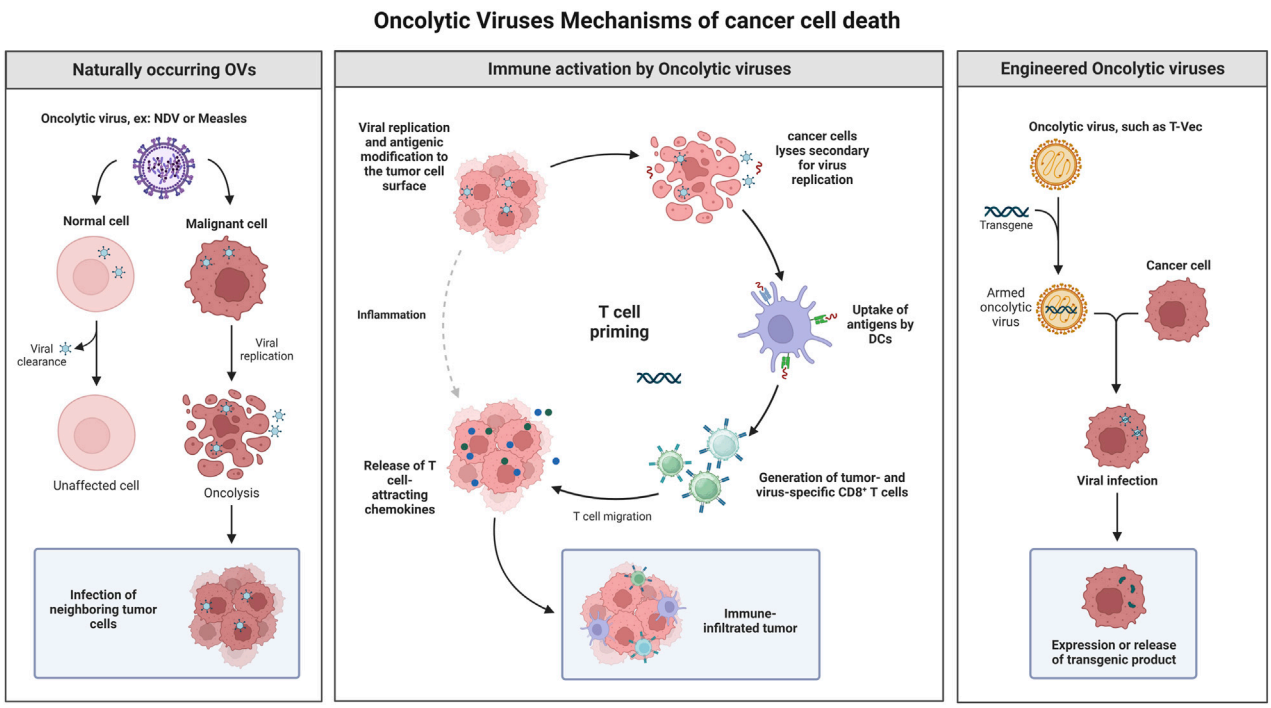

FIGURE 1
Oncolytic virotherapy is divided into two main groups, according to tumor specificity: naturally oncolytic viruses replicating in human cancer cells and gene-modified viruses engineered to accomplish selective oncolysis. OV kills cancer cells through several mechanisms, including cell lyses, due to virus replication, apoptosis induction, induction of specific antitumor immune response, and transgene expression that lead for cancer cell death and many others. Created with BioRender.com.

They developed an investigational protocol for oncolytic viruses' infection of organoid cultures. They compared the oncolytic effects of the measles vaccine virus (MeV) and a vaccinia virus (GLV) genetically engineered to express different transgenes. The most significant oncolytic effects were observed with oncolytic viruses expressing a suicide gene combined with the prodrug 5-FC. Therefore, the *in vitro* cancer model offers testing methods for new virotherapeutic vectors for treating breast cancer for further use *in vivo*.

Salman, Al-Shammari and colleagues (Salman et al.) established 3D coculture spheres *in vitro* consisting of two types of cells: the first type is derived from the breast cancer cell line (MCF-7 or AMJ13), and the second type is normal adipose tissue-derived mesenchymal stem cells. The floater culture plate was used to culture the cells so they could form spheroids, which were transferred to a special scaffold dish. The newly formed 3D culture spheroids were used to assess the oncolytic activity of the Newcastle disease virus AMHA1 strain by labeling the Newcastle disease virus (NDV) with a fluorescent PKH67 linker to track virus infection. They found that introducing chemical fluorescent dye into NDV particles is an effective strategy to identify virus particles in the infected co-culture spheroid model. Their results revealed that the oncolytic strain of NDV AMHA1 replicates effectively in cancer cells but not in normal cells in the 3D coculture system. This combined model of 3D coculture plus fluorescent tracking indicates that the NDV AMHA1 strain is selective and effective in antitumor virotherapy.

Howard and his associates (Howard et al.) evaluated the systemic delivery of HSV1716 in multiple mouse models of breast cancer. They found a direct relationship between virus tolerability and mouse strains. They tested the C57/B16, FVB, and Balb/c strains and found different responses. Intravenous administration of OV induces a lethal side effect in Balb/c mice, while C57/B16 is the most tolerant. These differences in response to OV in mouse strains may produce confusing results, which are mainly due to the interaction between OV and the immune system of these different strains. Eventually, this leads to a decrease in predictive value and low clinical efficacy. Howard and associates treated Balb/c mice with immunomodulators before injecting OV to decrease side effects. However, they could not estimate whether the immunomodulators increased virus tolerability. However, this work presented data to support therapeutic modulation of immune subsets with the aim of promoting a pro-inflammatory reaction, particularly by increasing CD8 + T cell levels that combat tumor growth. Finally, they are stating that the inconsistencies found in mouse models will help to have a larger picture, making it applicable, possibly to heterogenic human populations, which will lead to the development of translational oncolytic virotherapy.

3 Oncolytic viruses as cancer immunotherapeutic agents

Cerqueira and colleagues (Cerqueira et al.) reviewed possible immunotherapies that can be combined with oncolytic viruses for

the treatment of Melanoma according to its molecular characteristics. They explored molecular changes in Melanoma that can be targeted taking into consideration that melanoma has a high mutation rate, leading to the appearance of tumor-specific antigens (TSA) and infiltration of lymphocytes, facilitating the utilization of therapeutic technologies that elicit novel or reinstate preexisting responses from the immune system. Immune checkpoint inhibitors are one of these innovative therapeutic technologies for melanoma treatment that could be combined with oncolytic virotherapy for synergistic action. They propose that a virus's oncolytic action is due to virus replication and activation of innate and adaptive antiviral immune responses. This may be just as important, if not more so, than viral replication.

A research work by Uche et al. engineered recombinant oncolytic HSV-1 (oHSV) VC2-OVA that expresses a fragment of ovalbumin (OVA) as a fusion protein with the virion capsid protein VP26. They evaluated the efficacy of VC2-OVA to work as a vector capable of stimulating specific antitumor immunity in a syngeneic murine melanoma model. The administration of VC2-OVA through therapeutic vaccination resulted in a notable decrease in the presence of tumor cells in the lungs of mice that were intravenously exposed to B16cOVA cells. Furthermore, the administration of VC2-OVA resulted in strong preventive antitumor activity and prolonged the survival of mice that were intradermally implanted with B16cOVA tumors compared to mice inoculated with a control virus. Their findings demonstrate the efficacy of VC2 as an oncolytic virotherapy, showing promise for its potential application as a combined oncolytic virotherapy and personalized vaccine in the treatment of human and animal malignancies.

In the review by Kaufman et al., they described the progress made in advancing Talimogene laherparepvec (T-VEC) to the most proper melanoma patients, expansion to patients with non-melanoma cancers, and clinical trial results of T-VEC combination studies. T-VEC is a modified oncolytic herpes Simplex virus type 1 (HSV-1) that encodes granulocyte-macrophage colony stimulating factor (GM-CSF). T-VEC is adapted for selective replication in melanoma cells, and GM-CSF was expressed to augment host antitumor immunity. T-VEC is indicated for the local treatment of recurrent melanoma after primary surgery and is the first-in-class oncolytic virus to achieve FDA approval in 2015. Its tumor cell selective replication was improved by careful deletion of the two viral infected cell protein (ICP) 34.5 genes, which encodes the neurovirulence factor. Furthermore, deletion of ICP47 is believed to promote antitumor immunity as it facilitates MHCI loading of tumor-associated antigens. It showed very promising clinical outcomes. Additionally, it appeared that T-VEC could be used in combination with or sequentially to checkpoint blockade, without influence on therapeutic responses. T-VEC may be an important consideration for older patients with melanoma who may not be able to tolerate other systemic options.

According to the findings reported by Kaufman et al., there is evidence indicating the existence of specific patient subsets within the melanoma population who may exhibit a higher likelihood of seeing therapeutic benefits from T-VEC treatment. Mostly, patients with head and neck melanoma appeared to have higher response rates. Despite the higher mutation load due to Sun exposure being postulated, no demonstration of this was offered. Other potentially interesting settings are allotransplanted patients who cannot receive potent immunotherapy, such as immune checkpoint blockade, due to the risk of rejection of allograft, as well as patients with early-stage I-II melanoma patients, such as neoadjuvant treatment. As far as other cancers are concerned, T-VEC showed *ex vivo* preclinical activity, and then clinical trials have been planned. In general, accessible tumors for intratumoral injection have been a priority, and this has included head and neck squamous cell carcinoma, soft tissue sarcoma, and breast cancer. Other studies have been conducted to evaluate T-VEC in pancreatic cancer, hepatocellular carcinoma, and non-melanoma skin cancers. The combination with CTLA4 blockers seems promising, but further investigation is needed. It should be noted that some data support the role of elements of the antiviral interferon signaling machinery in tumor cells as possible predictive biomarkers of oncolytic virus (OV) activity and merit further clinical investigation (Kaufman et al.).

4 Novel viral platforms

A review article (Cristi et al.), described genetic modifications that were done to OVs to improve the killing ability of tumor cells directly, to dismantle the tumor microenvironment, or to alter tumor cell signaling and enhance antitumor immunity. Although many OVs have progressed to human clinical trials, their performance as monotherapy has not been as successful as expected. Importantly, recent literature suggests that the oncolytic potential of these viruses can be further increased by genetically modifying the viruses.

These advances are particularly important to increase virus spread and reduce metastasis, as demonstrated in animal models. The extracellular matrix (ECM) in a tumor does not have the same characteristics as that in normal tissues. In the tumor, the ECM is more rigid, abundant and dense. Because of this, tumor ECM acts as a barrier for therapeutic agents such as OV. At the same time, the barrier impairs oxygen and nutrients supply, activating apoptosis and senescence. Thus, ECM is a candidate cancer therapeutic target. Since at the onset of metastasis, during the invasion process, remodeling of the ECM is mainly done by metalloproteases (MMPs), both adenovirus- and vaccinia-based OVs have been genetically modified to exploit the natural functions of MMPs and enhance virus dissemination. Other attempts have been made by adding relaxin, hyaluronidase, or exonucleases. Overall, it resulted in increased virus spread and reduced tumor growth, including metastases in some cases (Cristi et al.).

Recently, Li and colleagues (Li et al.) showed that Ad-Apoptin-hTERTp-E1a (Ad-VT), a bispecific oncolytic adenovirus, can effectively induce cell death of breast cancer cells and has a better effect when used in combination with chemotherapy drugs (1–2). Ad-VT has no cytotoxicity in normal cells, with the advantage of specifically inducing tumor cell apoptosis, through the expression of the apoptin protein (Xiao et al., 2010). In their studies, the authors showed that the cytotoxic effect of Ad-VT was present in anthracycline resistant breast cancer cell lines and that Ad-VT could restore anthracycline sensitivity by down-regulating MRP1 expression (Li et al.). On the contrary, MDR1 and BCRP levels remain unchanged. Furthermore, since Ad-VT can induce cell

death through either apoptosis or autophagy, the authors also explored the second mechanism. They found that MRP1 expression was significantly affected by autophagy inhibition, while it was not modified by apoptosis inhibitors. Therefore, they concluded that Ad-VT restored sensitivity to anthracyclines through autophagy induction. Clearly, the effect was shown to be mediated by mTOR inhibition; When the authors analyzed the expression of the mTOR protein after adding an autophagy inhibitor, they found that inhibition of autophagy significantly increased mTOR activation and that treatment of MCF-7/ADR cells with 60 MOI Ad-VT treatment reversed the effects caused by autophagy inhibitors. This effect was likely mediated by the AMPK pathway and more specifically by the AMPK-mTOR-eIF4F signaling axis. Traditionally, the relationship between autophagy and drug resistance has been divided into two distinct mechanisms and their related effects: one is associated with its protective mechanism against tumor drug resistance, and the other is related to autophagy-induced cell death, which increases tumor sensitivity to apoptosis. In their study, Li et al. could effectively highlight both effects in MCF-7/ADR treated with Ad-VT. It would certainly be interesting to assess whether similar effects can be induced in other types of cancer cells.

Another review article (Lundstrom) on genetically engineered alphavirus vectors, which have been evaluated for prophylactic and therapeutic use for a broad range of cancer indications in various animal models and in several clinical. Although, based on numerous vaccine studies, it has not been possible to demonstrate superiority of any alphavirus system with respect to immune responses or therapeutic efficacy. In most cases, robust immune responses have been obtained, including humoral and cellular responses. Th1-biased immunogenicity confirmed the potential of alphavirus-based cancer vaccine. The possibility to include alphavirus-based delivery of cytotoxic genes, antitumor genes, immunostimulatory genes, apoptosis induced by alphaviruses, and RNA interference in the form of short interfering RNAs and microRNAs expands the possibilities of therapeutic interventions. Moreover, alphavirus vectors can be applied as recombinant viral particles, including replication-deficient, replication-proficient, and oncolytic viruses, as well as RNA replicons and DNA replicons. It has been demonstrated that the stability of RNA and its resistance against degradation can be improved by RNA encapsulation in lipid nanoparticles. Several studies have also confirmed that due to the presence of alphavirus replicons, both RNA replicons and DNA replicons can induce the same immune response at 100 to 1,000 times lower doses compared to synthetic mRNA and conventional DNA plasmids, respectively. Although alphaviruses have shown good safety and efficacy in various animal models, transfer to humans has often generated disappointingly weak immune responses in clinical trials. Several issues such as targeting, delivery, dose optimization, and potential combination therapy need to be addressed.

Newcastle disease virus was one of the novel platforms reviewed by Huang et al., they discussed the biological properties of NDV, the molecular mechanisms of antitumor of oncolytic NDV, and its application in the field of tumor therapy. NDV is among the limited number of viruses that have shown the ability to elicit partial or even complete responses after treatment with a single drug. The enduring nature of these reactions implies that the therapeutic impact of the virus might be based not only on direct oncolysis, but also on its capacity to facilitate long-term immunity. Recent research findings on NDV demonstrate significant potential in both preclinical and clinical trials.

The process of NDV replication takes place inside the cytoplasmic region of the host cell, without integrating into the host genome. This mechanism ensures the preservation of the integrity and safety of the parental virus. The oncolytic nature of Newcastle disease virus (NDV) can be classified as either lytic or nonlytic, as it selectively infects cells that possess a compromised interferon system. This characteristic enhances the safety profile of NDV when utilized as a vaccine. Incorporation of foreign genes is not necessary for NDV to exhibit a potent anticancer impact and maintain persistent expression of foreign genes. The integration of NDV viral therapy with conventional and emerging tumor treatment modalities has been documented and holds significant potential for widespread implementation. However, many inquiries about NDV therapy, similar to other oncolytic viruses (OV), persist without definitive answers. These unsolved questions encompass the practical methodologies for administering NDV therapy, optimal genetic engineering approaches, the therapeutic sequence for immune checkpoint inhibitors, and the most effective combination partners for NDV therapy. At present, there is a lack of established consensus on the most effective and appropriate approach for patients to utilize the virus, both in terms of methodology and timing. The presence of a tumor microenvironmental barrier and the cytoplasmic matrix in solid tumors may limit and suppress virus entry and multiplication, hence reducing its oncolytic efficacy. The presence of an excessive number of foreign genes may have an impact on the replication process of Newcastle Disease Virus (NDV). Furthermore, the NDV purification process requires extensive measures to achieve a clinical-grade virus product. Integrating NDV therapy with conventional and alternative medicines has the potential to emerge as an innovative approach in the field of cancer treatment. The potential increase in the anticancer effect can be achieved by integrating NDV viral therapy with existing immunotherapy, using the immunomodulatory impact of NDV. Consequently, NDV will emerge as a promising candidate for tumor therapy in the near future.

In the next review (Corbett et al.) discussed one such promising oncolytic virus called the Seneca Valley Virus (SVV-001) and its therapeutic implications. SVV development has seen seismic evolution over the past decade and now boasts of being the only OV with a practically applicable biomarker for viral tropism. We discuss relevant preclinical and clinical data involving SVV and how bioselecting for TEM8/ANTXR1, a negative tumor prognosticator, can lead to first of its kind biomarker-driven oncolytic viral cancer therapy. The initial discovery of SVV-001 revealed its specificity for neuroendocrine tumors, highlighting its remarkable capacity to revolutionize the field of neuroendocrine neoplasm therapies. This novel treatment has shown the ability to induce a substantial tumor response, even in cases where immunotherapy was previously believed to be ineffective. However, initial investigations were limited due to the absence of a biomarker that could be used to identify patients who were susceptible to severe viral vasculitis (SVV). The discovery of TEM8/ANTXR1 as a receptor for SVV-001, a therapeutic agent that can be administered through intratumoral injections, in a patient population with a high

prevalence of biomarkers, and in conjunction with dual checkpoint blockade to enhance treatment responses, established the foundation for future clinical trials utilizing SVV-001 with a more targeted and strategic approach. The treatment paradigm under consideration was originally designed to address neuroendocrine neoplasms. However, recent advances in understanding the plasticity of neuroendocrine transformation in various solid tumor types, as well as studies revealing widespread upregulation of TEM8/ANTXR1, indicate that SVV-001 may have the capability to target numerous other tumor types that exhibit high resistance to therapy and are associated with high mortality rates. Gaining a greater understanding of the specific immune tumor microenvironment associated with upregulation of TEM8/ANTXR1 in high-grade neuroendocrine carcinoma, well differentiated neuroendocrine tumors, and related tumor types is crucial for effectively using SVV-001 as a therapeutic approach for these conditions. Furthermore, this understanding is essential for the advancement of novel agents that can be used in conjunction with SVV-001.

5 Combination therapies

In research work by Obaid et al. employed acarbose (ACA), a specific alpha-glucosidase inhibitor, to induce glucose deficit combined with oncolytic Newcastle disease virus (NDV) to enhance antitumor action. In this study, a murine model of breast cancer was used, in which mammary adenocarcinoma tumor cells (AN3) were subjected to treatment with ACA, NDV, and a combined administration of both compounds. The research includes an investigation of various parameters, including antitumor efficacy, relative body weight, glucose level, hexokinase level (HK-1) determined by enzyme-linked immunosorbent assay (ELISA), glycolysis product (pyruvate), total adenosine triphosphate (ATP), oxidative stress markers (reactive oxygen species and reduced glutathione), and apoptosis assessed by immunohistochemistry. The findings demonstrated an important level of antitumor efficacy after the administration of combination therapy. The observed antitumor activity was associated with a drop in body weight and glucose levels, downregulation of HK-1, inhibition of glycolysis products such as pyruvate and total ATP, activation of oxidative stress characterized by an increase in reactive oxygen species (ROS) and a decrease in reduced glutathione, as well as the appearance of apoptotic cell death. The results suggest a novel approach to combating breast cancer by targeting glycolysis suppression, glucose deprivation, oxidative stress, and apoptosis, with potential therapeutic applications.

In summary, the findings of this investigation provide solid evidence in favor of the innovative hypothesis that ACA triggers glucose restriction, while virotherapy acts synergistically to improve metabolic oxidative stress and induce apoptosis. This study presents novel findings that indicate that ACA-induced glucose restriction acts in synergy with oncolytic NDV, which demonstrates a promising therapeutic approach that targets the glycolysis pathway for enhanced efficacy and safety. The integration of many therapeutic approaches into this unique treatment modality demonstrates a strong potential for application in clinical therapy.

The next article on this Research Topic described that the latest discoveries related to oncolytic adenoviruses (OAds) have the potential to offer a novel approach to improve the outcomes of individuals affected by triple negative breast cancer (TNBC) and other forms of breast cancer (BC) (Green-Tripp et al.). Oncolytic adenoviruses (OAds) have been genetically modified to exhibit the ability to specifically induce lysis, elimination, and activation of host antitumor immune responses, while protecting normal cells from injury. The common modifications observed involve the removal of certain components within the early gene products, such as the E1B55 KDa protein and particular segments of the E1A protein. Alternatively, the introduction of tumor-specific promoters can also be employed as a modification strategy. The efficacy of oncolytic adenoviruses (OAds) in the treatment of several types of adenocarcinomas in patients with breast cancer (BC) has not been adequately evaluated in clinical trials. Preclinical research showed effectiveness in breast cancer cell lines, namely, triple negative breast cancer cells, using innovative adenoviral mutants that showed encouraging results. In this review, Green-Tripp et al. examined the results described for the most promising oncolytic adenoviruses (OAds) in preclinical investigations and clinical trials, both as standalone treatments and in combination with established conventional therapies or emerging therapeutic approaches. The present focus of research involves investigating the efficacy of combining OAds with small molecule medications that target the epidermal growth factor receptor (EGFR), androgen receptor (AR), and DNA damage repair through new PARP inhibitors. These combinations have been shown to exhibit improved efficacy. The co-administration of Olaparib, a PARP inhibitor, with oncolytic adenoviruses (OAds) demonstrated a significant and significant anti-neoplastic response. The most encouraging results have been observed to date when oncolytic adenoviruses (OAds) are used in combination with antibodies targeting immunological checkpoints or expressing cytokines derived from the viral backbone. Although multiple clinical trials and preclinical research have provided evidence of the safety and efficacy of cancer-selective oncolytic adenoviruses (OAds), additional advances are required to effectively eradicate metastatic lesions, enhance immune activation, and promote intratumoral viral dissemination.

In the next review (Shao et al.) concluding the presence of relatively good results of studies in the field of treatment of solid cancers such as gastric cancer using oncolytic viruses, it seems that these viruses can be used more widely in combination therapies to increase the efficiency and effectiveness of cancer treatment. Nevertheless, this therapeutic method has its difficulties and requires more studies. In peritoneal metastasis gastric cancer, virotherapy can limit peritoneal metastasis and tumor metastasis to the peritoneum in diverse ways, such as direct oncolysis of tumor cells, as well as inhibition of mechanisms and molecules involved in angiogenesis. Alternatively, inserting genes with antitumor function into the genome of oncolytic viruses for expression in virus-infected tumor cells can enhance therapeutic effect. Viruses seem to have a wide range of unknown functions, and due to their extraordinary capabilities, such as their ability to replicate in hypoxic conditions, which is one of the drawbacks of cancer therapy, shortly, they can be used to treat cancers to the maximum benefit performance.

6 Methods to develop OV quantification

Despite careful quality control, during high titer production, "wild-type-" like replication-competent adenovirus (RCA) contaminants can be generated through recombination events due to the DNA sequence similarity between OV and host cells Gao et al.. These RCA contaminants raise various safety concerns in clinics and detection methods have been developed. Cell culture-based methods have been developed to detect RCA contaminants in replication-deficient adenovirus vectors. These methods were based on the fact that only RCA contaminants, but not the vectors, can grow in and lyse the test cell line. However, these methods are not suitable to distinguish RCA contaminants from oncolytic adenovirus products because both can replicate in test cell lines. The presence of RCA contaminants is then manually judged by microscopic observation, and thus the results may not always be accurate and quantitative. More recently, Gao et al. developed a qPCR-based method to detect and quantify oncolytic adenovirus products. This system takes advantage of the common use of E1B-deleted oncolytic adenoviruses in clinics. Therefore, specific primers have been designed to differentiate between RCA contaminants and E1B-deleted OV. The system turned out to be robust, accurate, and able to detect an extremely small number of RCA contaminants among high-concentration viral particles. In perspective, simply optimizing primers, the use of this tool could be implemented and expanded (Gao et al.).

7 In conclusion

It is obvious that oncolytic virotherapy is progressing in steady and wide steps toward being among conventional cancer therapies. Since the approval of more than one type of OV in clinical use and many in clinical trials, more research is expected to focus on increasing the efficacy to be used as a first-line therapy. The development of oncolytic viruses that target specific types of mutations and genetic alterations in cancer cells will help treat difficult tumors clinically and give more hope to cancer patients.

Author contributions

AA-S: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. PP: Writing–original draft, Writing–review and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ad-Apoptin-hTERTp-E1a Regulates Autophagy Through the AMPK-mTOR-eIF4F Signaling Axis to Reduce Drug Resistance of MCF-7/ADR Cells

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Ad-VT (Ad-Apoptin-hTERTp-E1a) is a type of oncolytic adenovirus with dual specific tumor cell death ability. It can effectively induce cell death of breast cancer cells and has better effect when used in combination with chemotherapy drugs. However, it has not been reported whether Ad-VT reduces the resistance of breast cancer cells to chemotherapy drugs. The purpose of this study is to investigate the effect of Ad-VT on drug resistance of Adriamycin-resistant breast cancer cells. For this, the effects of different doses of Ad-VT on the resistance of breast cancer cells to Adriamycin were analyzed using qualitative and quantitative experiments *in vitro* and *in vivo*. The Ad-VT can reduce the resistance of MCF-7/ADR to adriamycin, which is caused by the reduction of MRP1 protein level in MCF-7/ADR cells after treatment with Ad-VT, and MRP1 can be interfered with by autophagy inhibitors. Subsequently, the upstream signal of autophagy was analyzed and it was found that Ad-VT reduced the resistance of cells to doxorubicin by reducing the level of mTOR, and then the analysis of the upstream and downstream proteins of mTOR found that Ad-VT increased the sensitivity of MCF-7/ADR cells to adriamycin by activating AMPK-mTOR-eIF4F signaling axis. Ad-VT can not only significantly induce cell death in MCF-7/ADR cells, but also improved their sensitivity to Adriamycin. Therefore, the combination of Ad-VT and chemotherapy drugs may become a new strategy for the treatment of breast cancer in overcoming Adriamycin resistance.

Keywords: adriamycin resistance, oncolytic adenovirus, breast cancer, toxicity, autophagy

INTRODUCTION

Cancer is one of the main causes of human death. In 2020, there will be 19.3 million cancer patients diagnosed worldwide, and 10 million people will die from cancer (Sung et al., 2021). The number of new cases of breast cancer (BC) is 2.26 million, surpassing lung cancer highest incidence rate. The mortality rate of breast cancer also ranks first among women. At present, the main treatment for breast cancer is a combination of surgery, radiotherapy, and chemotherapy. However, these

mainstream therapies have serious side effects, are ineffective for metastatic patients, and have a post-therapeutic risk of recurrence. One of the most important reasons of recurrence is the development of resistance to chemotherapy drugs. Therefore, identifying the molecular mechanisms of drug resistance will have a great research significance and clinical value for the treatment of recurrent breast cancer.

At present, several studies showed that the overexpression of some ABC transporters is one of the important causes of tumor multidrug resistance (MDR) that represents the main obstacle for successful chemotherapies (Pluchino et al., 2012; Finlay et al., 2015). MDR does not only refer to drug resistance associated with one chemotherapeutic drug, but also implies drug resistance to other chemotherapeutic drugs with different functions, physical and chemical properties, and action targets. The multi-drug resistance that is mediated by the ABC family of transporters is the most important and crucial pathway that is used by tumor cells to resist chemotherapy. The representative MDR members of the ABC family include multidrug resistance gene 1 (MDR1), multidrug resistance associated protein (MRP1), and breast cancer resistance protein (BCRP) that is also known as ABCG2 (ABC transporter subfamily G) (Srinivasan et al., 2009). The common feature of these proteins is their capacity to provide energy through ATP hydrolysis, which helps tumor cells pump out a variety of anticancer drugs by reverse concentration gradient, leading to the decrease in the concentration of intracellular chemotherapeutic and the development of tumor cells' drug resistance (Gottesman et al., 2002). Many studies have shown the presence of high expression levels of MDR 1, MPR 1, and BCRP in breast and lung cancers and leukemia, that were associated with poor chemotherapeutic responses (Coley, 2008; Corich et al., 2009; Li et al., 2010a). A clinical follow-up study showed that high expression levels of MDR1, MPR1, and BCRP were closely related to the prognosis of cancer patients (Li et al., 2009).

With the advances in molecular and cellular biology, and virology, gene therapy has become a new approach in cancer treatment. Oncolytic virus therapy shows great advantages and is expected to be a reliable method for breast cancer treatment. In a previous study, our team constructed a bispecific oncolytic adenovirus Ad-VT (Ad-Apoptin-hTERTp-E1a), which could specifically replicate and express the Apoptin gene in tumor cells (Li et al., 2010b). Apoptin is a type of apoptosis-inducing protein that is derived from chicken anemia virus (CAV). The oncolytic adenovirus Ad-VT has no cytotoxicity in normal cells, with the advantage of specifically inducing tumor cell apoptosis (Liu et al., 2012; Zhang et al., 2013; Qi et al., 2014; Yang et al., 2015).

The length and activity of human telomerase reverse transcriptase (hTERT) are related to cell senescence and immortalization. Most normal human cells lack telomerase activity due to the rate limitation of telomerase reverse transcriptase (hTERT) gene and the tight transcriptional inhibition of catalytic components. However, the expression of hTERT and the activation of telomerase are observed in up to 90% of human malignant tumors, resulting in unlimited proliferation (Shay and Bacchetti, 1997). Therefore, Ad-VT can only replicate and specifically kill a variety of tumor cells. Ad-VT showed excellent killing effects in a variety of tumor cells, including breast cancer cells (Chen et al., 2019; Wang et al., 2020). Meanwhile, when used in combination with different chemotherapy drugs, the anti-tumoral effect is improved. Therefore, we speculate that the combination of Ad-VT and chemotherapy drugs can result in synergistic effects, indicating that the oncolytic virus can reduce tumor cells' drug resistance.

In this study, we analyzed the changes in different drug-resistant proteins in Adriamycin (ADR)-resistant MCF-7/ADR cells that were infected by Ad-VT to explore the effect of Ad-VT on Adriamycin-mediated resistance in breast cancer cells and the cellular pathways involved. The results of the study provide a new theoretical basis for the treatment of breast cancer using a treatment combination of oncolytic adenovirus and chemotherapy.

MATERIALS AND METHODS

Reagent

All antibodies were purchased from CST and all inhibitors were acquired from MCE.

Viruses, Cells, and Transfection

The BC cell line MCF-7 cell was purchased from the Chinese Academy of Sciences (cat. SCSP-669) and MCF-7/ADR cell was purchased from Beijing Beina Chuanglian Institute of Biotechnology (cat. BNCC340584). MCF-7 cells were cultured in DMEM medium, and MCF-7/ADR cells in RPMI 1640 medium with 500 ng/ml Adriamycin. The media were supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 U/mL streptomycin and cultured in an incubator at 37°C containing 5% carbon dioxide. The cell culture reagents were purchased from HyClone, GE Healthcare, and life sciences. The recombinant adenoviruses Ad-Apoptin-hTRETp-E1a (Ad-VT) and Ad-Mock were constructed and preserved in our laboratory (Li et al., 2010).

We purchased the control, AKT, eIF4E, and AMPK siRNAs from RIBOBIO (China). According to the efficacy of their knockdown effect, si-AKT, si-eIF4E, and si-AMPK were used in this study. The sequence of si-eIF4E, si-AKT and si-AMPK were 5′-AAGCAAACCTGCGGCTGATCT-3′, 5′-TTCATCATC GAAGTACCT-3′ and 5′-GAGGAGAGCTATTTGATTA-3′. The cells were transfected with 30 nM siRNA using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

The human MRP1, mTOR, and eIF4E cDNA were purchased form (You Biosciences, Hunan, China) and cloned into the pCDNA 3.1 plasmid. The mTOR, eIF4E plasmids and the corresponding empty vector were transfected into MCF-7/ADR cells using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

Experimental Animals

Female BALB/c nude mice (4–5 weeks old) were purchased from Beijing vital River Laboratory Animal Technology Co., Ltd., and

the animal experiment protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Changchun University of Chinese Medicine. All surgeries were performed under anesthesia using sodium pentobarbital, and all efforts were made to minimize animal suffering. After the experiment, the remaining mice were euthanized. The applied method of euthanasia was an intraperitoneal injection of three times the anesthetic dose of sodium pentobarbital (150 mg/kg) and for 2–3 min. The euthanasia method was performed following the AVMA Guidelines for the Euthanasia of Animals.

Colony Formation Assay

The cells were seeded in a 12-well plate and cultured for 24 h. Ad-VT and different reagents were added according to the different groups. After 48 h, the cell supernatants were discarded, and the cells washed 3 times with PBS. A total of 600 μL 0.4% crystal violet staining solution was added to each well. After 10 min incubation at room temperature, the staining solution was discarded, and the cells washed 3 times with PBS and placed in a dry environment prior taking photos for analysis the formation of cell colonies. (Jabir et al., 2020).

Annexin V-FITC/PI Flow Detection Assay

The cells were seeded in a 12-well plate and cultured for 24 h. Ad-VT and different reagents were added according to the different groups. After 48 h, the cells were collected and resuspended in 500 ul staining solution (containing 5 μL FITC and 5 μL PI). The samples were stained in the dark for 20 min, at room temperature then transferred to the flow tube and properly labeled before flow cytometry (Ibrahim et al., 2021).

CCK-8 Assay

The cells were seeded into 96-well plates and cultured for 24 h. Ad-VT and different reagents were added according to the different groups. After 48 h, each medium in the wells was replaced by a 100 μL culture solution containing the CCK-8 staining solution that was prepared at a ratio of 1:9 in a dark environment. The plate was then incubated at 37°C in a 5% CO_2 incubator for 2 h. After incubation, a microplate reader was used to detect the OD value in each well at a wavelength of 450 nm (Hao et al., 2021). The detection of the inhibition rate of breast cancer cells was performed according to the following calculation formula:

$$\begin{aligned} Cell\ inhibition\ rate = {} & (OD(average\ of\ the\ control\ group) \\ & - OD(average\ of\ the\ experimental\ group)) \\ & / OD(average\ of\ the\ control\ group) \times 100\% \end{aligned}$$

Western Blot

The whole cell protein extract was prepared using RIPA cell lysate containing protease inhibitors. The same amount of protein samples was separated on a 10% SDS polyacrylamide gel and transferred to a PVDF membrane. The membranes were blocked with 5% skim milk for 1–2 h, then incubated overnight at 4°C with primary antibodies followed by incubations with 2 h incubations with secondary antibodies at room temperature. Finally, enzyme-linked chemiluminescence (ECL) was used for the detection. The results were quantitatively analyzed with chemiluminescence and fluorescence imaging systems. The detailed steps were performed as previously described (Chen et al., 2019).

Immunofluorescence

The cells were seeded in a 12-well plate (with sterile cell slides) and cultures for 24 h. Ad-VT and different reagents were added according to the different groups. After 48 h, the wells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100, blocked with 1% bovine serum albumin (BSA) for 2 h, incubated with the primary antibody overnight at 4°C, and wash 3 times with PBS. Secondary antibodies labeled with FITC or CY3 were incubated for 2 h, and the cell slides analyzed by Zeiss confocal microscope (Jabir et al., 2021).

Animal Assay

The MCF-7/ADR cells (3×10^6) were inoculated into the chest subcutaneously of 5-week-old BALB/c nude mice to establish a tumor model (n = 6). The tumors' size was measured, and survival checked every week. After 28 days of treatment, the animals were euthanized, and each tumor was fixed with formalin and subjected to immunohistochemical staining. The growth curve was drawn, and the tumor volume calculated as follows:

$$\begin{aligned} Tumor\ volume(mm^3) = {} & (long\ diameter\ of\ tumor \\ & \times short\ diameter\ of\ tumor^2)/2 \end{aligned}$$

The inhibition rate was calculated using the formula:

$$\begin{aligned} Tumor\ inhibition\ rate = {} & (1 - treatment\ group\ tumor\ volume/ \\ & control\ tumor\ volume) \times 100\% \end{aligned}$$

Immunochemistry

The tissue sections were deparaffinized, rehydrated, and incubated in 3% H_2O_2 methanol for 15 min to eliminate endogenous peroxidase activity. The slides were put in 0.01M sodium citrate buffer (pH 6.0) and incubated at 95°C for 20 min to perform antigen retrieval. Following this step, the slides were incubated with the primary antibody overnight at 4°C. After the incubation, another incubation was performed with the secondary antibody for 1 h at room temperature, followed by DAB staining, and the sections were counterstained with hematoxylin. The mean density analysis method was used for the evaluation of the immunostaining: The Image-Pro Plus 6.0 software was used to select the same yellow brown as a unified standard for judging positivity of the immunostaining in all photos, and analyze each photo to obtain the cumulative light of each photo, the density value (IOD) and the pixel area of the tissue (AREA). This step was followed by the calculation of the average optical density value IOD/AREA (Mean Density).

TABLE 1 | The resistance index (RI) of adriamycin in MCF-7/ADR cells (mean ± SD, n = 3).

Compounds	IC50(nM)		RI
	MCF-7	MCF-7/ADR	
Adriamycin	347 ± 68.5	5644 ± 341.8	16

Statistical Analysis

The data were presented as mean ± standard error of the mean (SEM). GraphPad Prism 6.0 software was used to perform the statistical analyses of unpaired two-tailed Student's t-tests or analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

Gemcitabine and Taxol Sensitivity

MCF-7 and MCF-7/ADR cell lines were treated with different concentrations of adriamycin, respectively. The results showed that the IC_{50} of MCF-7 and MCF-7/ADR cells to adriamycin was 347 ± 68.5 and 5644 ± 341.8 nM (**Table 1**). The resistance index of MCF-7/ADR cells to adriamycin was 16. It is suggested that MCF-7/ADR is not sensitive to adriamycin.

Ad-VT Induces Cell Death in MCF-7 Cells and MCF-7/ADR Cells

Ad-VT can specifically replicate and express apoptin protein in tumor cells. It can induce specific apoptosis of tumor cells. In this experiment, colony formation assay, and the CCK-8 assay showed that Ad-VT significantly indues cell death in MCF-7 and MCF-7/ADR cells, and this effect was higher in MCF-7/ADR cells compared to that in MCF-7 cells (**Figures 1A–D**). Ad-VT had also a stronger effect with a MOI of 40, reaching approximatively 40% in MCF-7/ADR cells and 30% in MCF-7 cells. These results demonstrate that Ad-VT induces a significant cell death in MCF-7 cells, which is not associated with MCF-7 cells' resistance to Adriamycin.

Ad-VT Reduces the Resistance of MCF-7/ADR Cells to Adriamycin

Our results indicated that MCF-7/ADR cells do not respond to Adriamycin when used at concentrations of 500–2000 nM and in combination with Ad-VT (**Figures 1E–H**). To further investigate a potential relationship between Ad-VT and Adriamycin resistance, the ad-VT treatment dose was increased, and we found that after increasing the dose to 60 MOI, the treatment concentration of 500 nM Adriamycin could induce cell death in MCF-7/ADR cells (**Figure 1I**). Therefore, it is suggested that 60

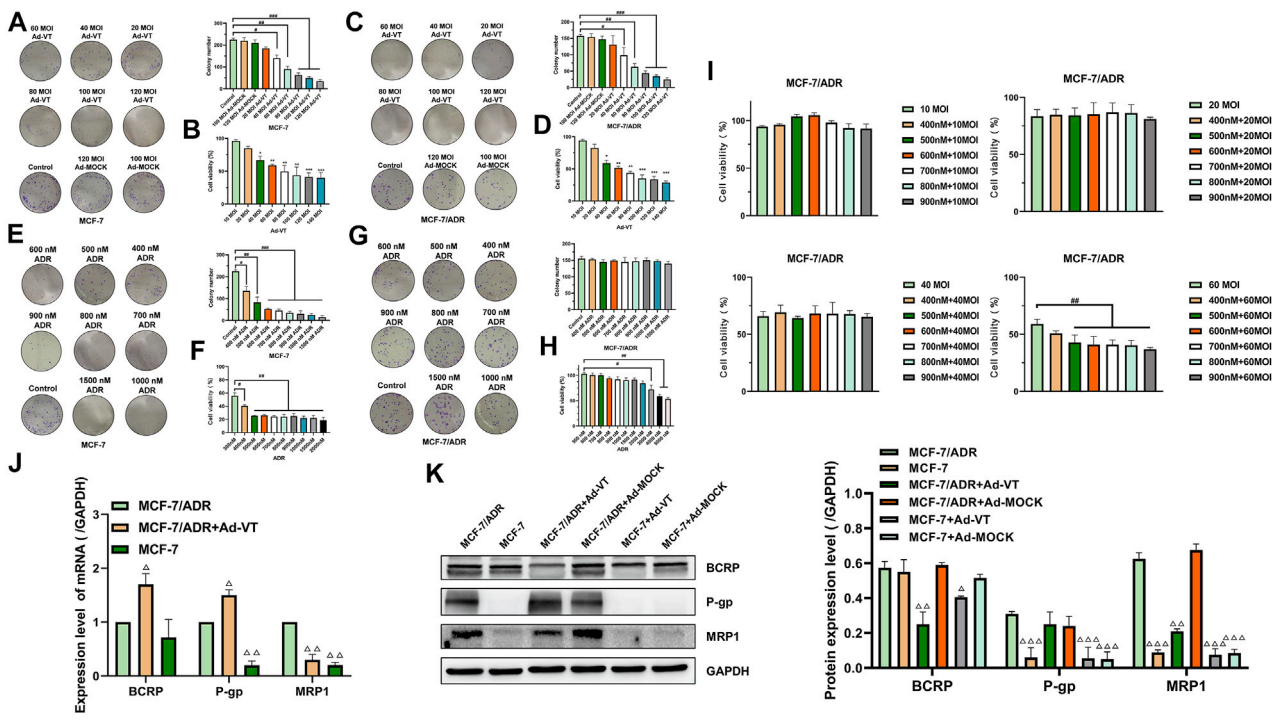

FIGURE 1 | Synergistic effect of Ad-VT and ADR. The colony formation and CCK-8 assay analyzes the cell viability of MCF-7 **(A,B)** and MCF-7/ADR **(C,D)** cells after infection with Ad-VT. The colony formation and CCK-8 assay analyzes the cell viability of MCF-7 **(E,F)** and MCF-7/ADR **(G,H)** cells after adding with adriamycin (ADR). **(I–K)** CCK-8 and western blot assay analyzes the effect of Ad-VT on ADR resistance in MCF-7/ADR cells. Data were representative of three independent experiments (n = 3). The unpaired Student's test-test was used. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) when compared with 10 MOI Ad-VT. ($^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$, $^{\triangle\triangle\triangle}p < 0.001$) when compared with MCF-7/ADR.

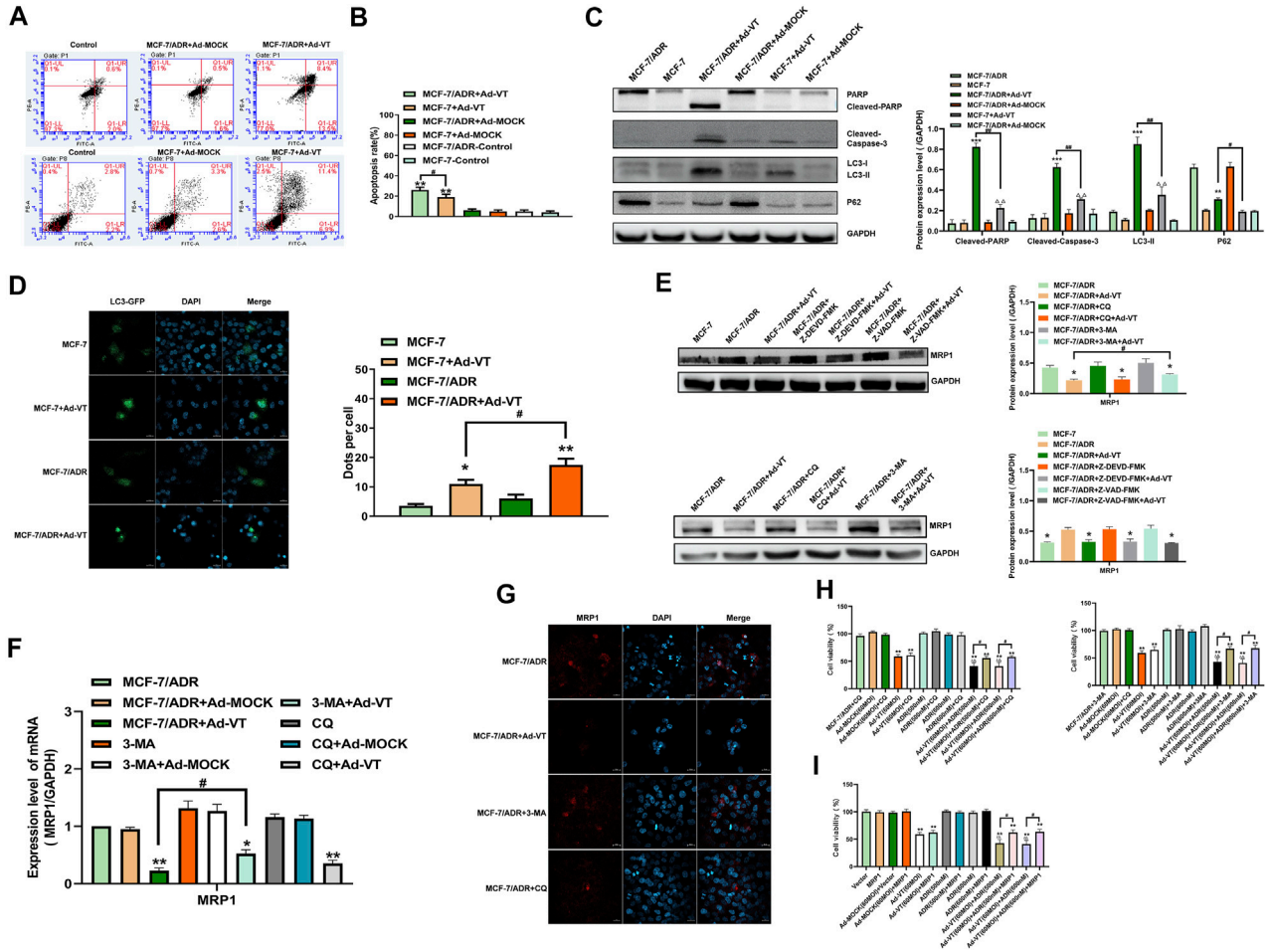

FIGURE 2 | Detection of the levels of Ad-VT-induced autophagy and apoptosis in MCF-7/ADR cells. **(A,B)** The apoptotic levels of MCF-7 and MCF-7/ADR cells were detected by flow cytometry after Annexin V-FITC/PI staining. **(C)** Western blot analyzes of the protein expression levels of autophagy and apoptosis-related proteins. **(D)** Analyze of changes in autophagic flux using a LC3-GFP plasmid. **(E,F)** Analyze of MRP expression level using inhibitors of apoptosis and autophagy. **(G)** Analysis of autophagy effects on the resistance of Ad-VT-treated MCF-7/ADR cells to ADR by immunofluorescence staining. **(H)** Using autophagy inhibitors to analyze whether autophagy affects the resistance of Ad-VT-treated MCF-7/ADR cells to ADR. **(I)** Analysis of the change in the resistance of Ad-VT-treated MCF-7/ADR cells to ADR after MRP1 overexpression in MCF-7/ADR cells. The scale bar equals 20 μm. Data were representative of three independent experiments (n = 3). The unpaired Student's test-test was used. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. ($^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001) when compared with MCF-7/ADR.

MOI Ad-VT can reduce the resistance of MCF-7/ADR cells to Adriamycin.

We also analyzed the expression of multidrug resistance-related proteins in MCF-7 and MCF-7/ADR cells and found that MRP1 and P-gp were not expressed in MCF-7 but were highly expressed in MCF-7/ADR cells. After adding Ad-VT, we found that Ad-VT significantly reduces the expression of MRP1 in MCF-7/ADR cells. A decrease in the level of P-gp protein was also observed; however, the difference was not significant (**Figures 1J,K**). Similar results were obtained at the transcriptional level, where the addition of Ad-VT significantly reduced the MRP1 gene expression level. These results indicate that Ad-VT can reduce the drug resistance of MCF7/ADR cells to Adriamycin, which may be caused by the reduction of MRP 1 protein.

Ad-VT Induced Apoptosis and Autophagy in MCF-7/ADR and MCF7 Cells

In our previous studies, we found that Ad-VT induces apoptosis of tumor cells and also causes autophagy (Chen et al., 2019). In this study, we performed an Annexin V experiment and show that treatment with 60 MOI Ad-VT induces more apoptosis in MCF-7/ADR cells compared to that in MCF-7 cells (**Figures 2A,B**). After detection of the protein expression levels of PARP and caspase-3, we found that the cleavage levels of the two

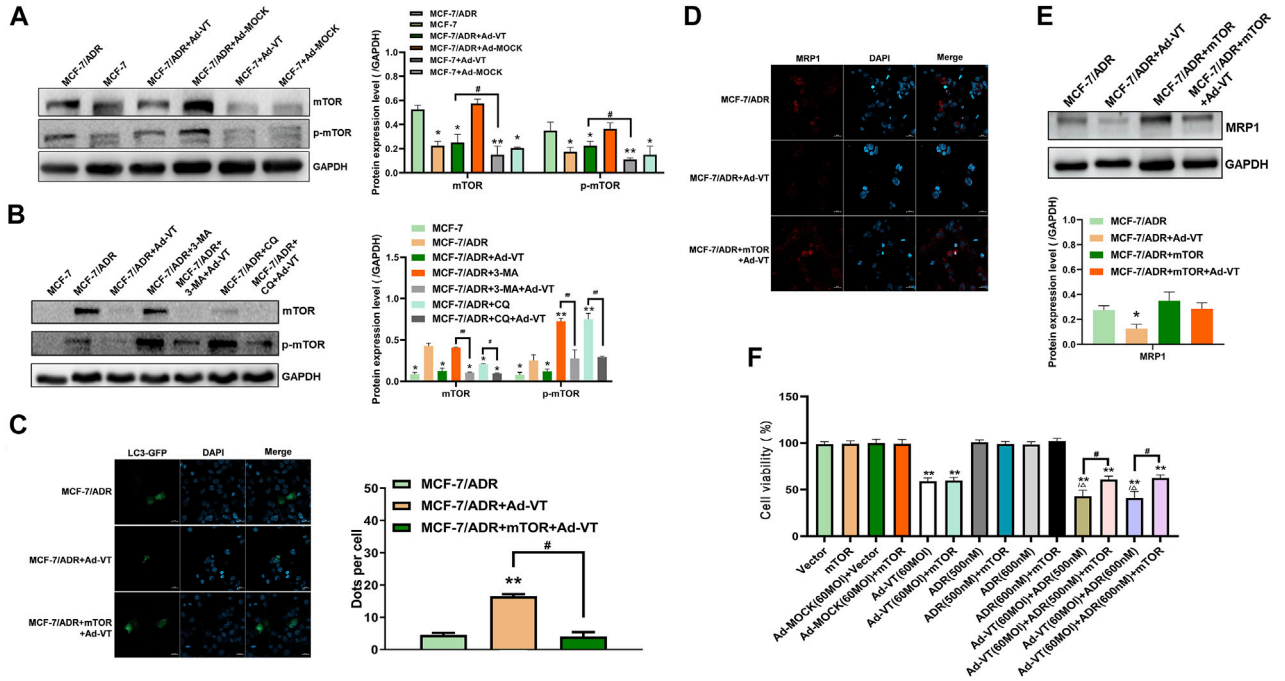

FIGURE 3 | Detection of mTOR role in ADR resistance. **(A)** Western blot analyzes of the changes of mTOR protein level in MCF-7 and MCF-7/ADR cells after infection with Ad-VT. **(B)** Western blot analysis of the changes in mTOR protein level in MCF-7/ADR cells after inhibiting autophagy. **(C)** Analysis of the changes in autophagic flux with LC3-GFP plasmid after mTOR overexpression. **(D,E)** Western blot and immunofluorescence staining analyses of the changes in MRP1 protein level after mTOR overexpression. **(F)** Analysis of the change in the resistance of Ad-VT-treated MCF-7/ADR cells to ADR after mTOR overexpression in MCF-7/ADR cells. The scale bar equals 20 μm. Data were representative of three independent experiments ($n = 3$). The unpaired Student's test-test was used. $^{\#}p < 0.05$. ($^{*}p < 0.05$, $^{**}p < 0.01$) when compared with MCF-7/ADR. ($^{\triangle}p < 0.05$) when compared with MCF-7/ADR + Ad-VT.

proteins in MCF-7/ADR cells were higher than that in MCF-7 cells after treatment with Ad-VT (**Figure 2C**).

Then we analyzed the expression levels of autophagy-related proteins and found that the expression of LC3-II in MCF-7/ADR cells that were infected with 60 MOI Ad-VT was significantly higher than that in MCF-7 cells (**Figure 2C**). We also carried out an GFP-LC3 transfection test and found that the number of MCF-7/ADR cells with an LC3 green fluorescence was significantly higher than that in MCF-7 cells (**Figure 2D**). These results suggest that Ad-VT can induce strong apoptosis and autophagy in MCF-7/ADR cells, which may be related to an Ad-VT-mediated reduction of drug resistance in MCF-7/ADR cells.

Ad-VT Causes Changes in the Resistance of MCF-7/ADR Cells to Adriamycin Through Changes in Autophagy

As Ad-VT induces autophagy and apoptosis, we analyzed whether these two types of programmed cell death are related to drug resistance. Therefore, we analyzed the expression of drug resistant proteins in MCF-7/ADR cells that were treated with 60 MOI Ad-VT in the presence of different inhibitors of autophagy and apoptosis. The results showed that the inhibition of autophagy in these cells increases MRP1 level of expression and that the inhibitory effect of 3-MA was higher than that of CQ. However, the inhibition of apoptosis had no effect on MRP1 level of expression (**Figures 2E–G**). Next, a CCK-8 test was performed on MCF-7/ADR cells that were treated with 60 MOI Ad-VT, transfected with MRP1 expression plasmid, and treated with autophagy inhibitors. The results showed that the killing effect of Ad-VT-induced adriamycin on MCF-7/ADR cells was inhibited after inhibiting autophagy and MRP1 overexpression, and the effect of 3-MA was higher than that of CQ (**Figures 2H,I**). These results indicate that Ad-VT influences the resistance of MCF-7/ADR cells to Adriamycin by inducing autophagy.

Ad-VT Causes Changes in the Resistance of MCF-7/ADR Cells to Adriamycin Through Changes of mTOR

mTOR activation is frequently reported in many human cancers, including lung, pancreatic, gastric, and breast cancers. In addition, mTOR seems to play an important role in cancer occurrence, development, and chemotherapy. Therefore, we studied whether the activation of mTOR was related to the change in Ad-VT induction of MCF-7/ADR cells' resistance to Adriamycin. The results of western blot showed that mTOR is activated in MCF-7/ADR cells, but not in MCF-7 cells, and that the activity of mTOR in MCF-7/ADR cells is also inhibited after adding 60 MOI Ad-VT (**Figure 3A**). It is suggested that Ad-VT

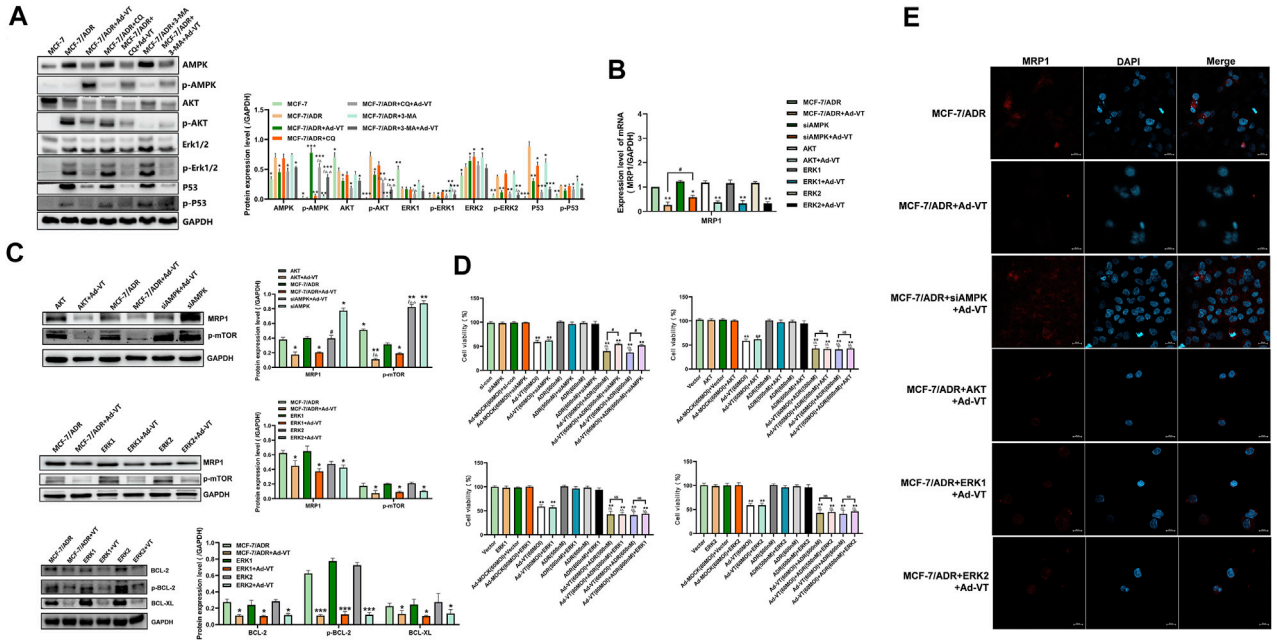

FIGURE 4 | Detection of the role of proteins acting upstream of mTOR in ADR resistance. **(A)** Western blot analysis of the changes in expression levels of proteins acting upstream of mTOR in MCF-7 and MCF-7/ADR cells after adding Ad-VT. **(B,C)** Western blot and qPCR analyses of the changes in MRP1 protein levels after AKT/ERK1/2 overexpression and AMPK silencing. **(D)** Analyze of the change in the resistance of Ad-VT-treated MCF-7/ADR cells to ADR after AKT/ERK1/2 overexpression and AMPK silencing in MCF-7/ADR cells. **(E)** Immunofluorescence staining analysis of the changes in MRP1 protein level after AKT/ERK1/2 overexpression and AMPK silencing. The scale bar equals 20 μm. Data were representative of three independent experiments ($n = 3$). The unpaired Student's test-test was used. $^{\#}p < 0.05$. ($^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001) when compared with MCF-7/ADR. ($^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$) when compared with MCF-7/ADR + Ad-VT.

may induce a change in the resistance of MCF-7/ADR cells to Adriamycin through inhibiting the activation of mTOR. We also analyzed mTOR protein expression after adding an autophagy inhibitor and found that the inhibition of autophagy significantly increases the activation of mTOR, and that treatment of these cells with 60 MOI Ad-VT treatment reverses the effects caused by the autophagy inhibitors (**Figure 3B**). Then we overexpressed mTOR in these cells and found mTOR increased the expression of MRP1 and also decreased the level of the autophagy (**Figures 3C–E**). The results of CCK-8 also showed that killing effect of Adriamycin on MCF-7/ADR cells is inhibited after mTOR overexpression (**Figure 3F**). The combination of the above results with the results of autophagy inhibition shows that Ad-VT causes a change in MCF-7/ADR cells' resistance to Adriamycin through mTOR.

Ad-VT Changes the Resistance of MCF-7/ADR Cells to Adriamycin *via* AMPK Pathway

After analyzing the role of mTOR in drug resistance, we analyzed the upstream protein of mTOR. Through the detection of protein level, it was found that Ad-VT activated AMPK and inhibited AKT, ERK1/2 and p53 in MCF-7/ADR cells. Studies showed that the activation of AMPK and the inhibition of AKT and ERK 1/2 could inhibit the activation of mTOR (**Figure 4A**). So we silenced and overexpressed these three proteins. The results showed that the expression of MRP1 after silencing AMPK with the addition of 60 MOI Ad-VT was higher than that after overexpression of AKT and ERK1/2, which may indicate that AMPK is the key protein that causes Ad-VT to reduce adriamycin resistance (**Figures 4B,C,E**). This result was also confirmed in the subsequent analysis of transcription level. We also conducted the CCK-8 test, and found that the killing effect of Ad-VT-induced adriamycin on MCF-7/ADR cells was significantly inhibited after AMPK was silenced, but there was no significant change after overexpression of AKT and ERK1/2 (**Figure 4D**). Subsequently, we also analyzed the reticulum-related proteins Bcl-2 and Bcl-XL downstream of ERK1/2. It was found that overexpression of ERK1/2 did not significantly affect the downstream BCL-XL and BCL-2 expression levels (**Figure 4C**). The above results suggest that the change of Ad-VT mainly leads to in mTOR expression through a change in AMPK expression, which leads to a change in MCF-7/ADR cells' Adriamycin resistance.

Ad-VT Causes Changes in MCF-7/ADR Cells' Resistance to Adriamycin Through the AMPK-mTOR-eIF4F Signaling Axis

Following the analysis of the upstream signaling pathway of mTOR, we also investigated downstream mTOR proteins. Among the reported proteins involved in mTOR-induced drug resistance, S6K and eIF4E play the most important roles (Tee and Blenis, 2005). The results showed that in MCF-7 and MCF-7/

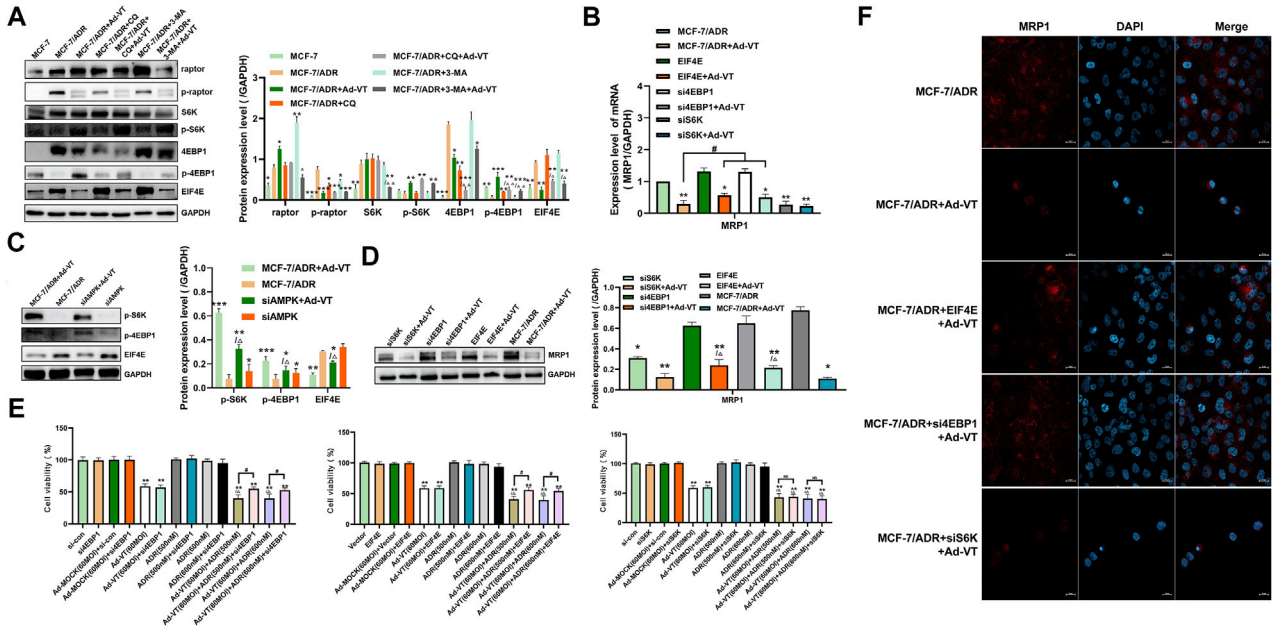

FIGURE 5 | Detection of the role of proteins acting downstream of mTOR in ADR resistance. **(A)** Western blot assay analysis of the changes in the expression of proteins acting downstream of mTOR in MCF-7 and MCF-7/ADR cells after infection with Ad-VT. **(B,D)** Western blot and qPCR analyses of the changes in MRP1 protein level after eIF4E overexpression and S6K/4EBP1 silencing. **(C)** Western blot analysis of the changes of proteins acting downstream of mTOR in MCF-7/ADR cells after AMPK silencing. **(E)** Analysis of the change in the resistance of Ad-VT-treated MCF-7/ADR cells to ADR after eIF4E overexpression and S6K/4EBP1 silencing in MCF-7/ADR cells. **(F)** Immunofluorescence staining analysis of the changes in MRP1 protein level after eIF4E overexpression and S6K/4EBP1 silencing. The scale bar equals 20 μm. Data were representative of three independent experiments ($n = 3$). The unpaired Student's t-test was used. $^{\#}p < 0.05$. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) when compared with MCF-7/ADR. ($^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$) when compared with MCF-7/ADR + Ad-VT.

ADR cells, S6K was not activated, but it was activated after adding 60 MOI Ad-VT (**Figure 5A**). However, previous reports showed that the activation of S6K leads to an increase in drug resistance; thus, we silenced the S6K in MCF-7/ADR cells found that its silencing reduces MRP1 expression level and Adriamycin resistance in MCF-7/ADR cells. However, we also found that treatment of the cells with 60 MOI Ad-VT activates S6K. These results were contradictory and suggest that Ad-VT does not cause a change in Adriamycin resistance in MCF-7/ADR cells through in S6K expression (**Figures 5B,F**). Then, we overexpressed eIF4E in MCF-7/ADR cells that were also treated with 60 MOI Ad-VT and found that MRP1 level increased significantly in these cells, indicating that eIF4E is a key protein involved in Ad-VT-mediated reduction in Adriamycin resistance (**Figures 5B,F**). This result was also confirmed in the subsequent transcriptional analysis. The CCK-8 test showed that the apoptotic effect of Ad-VT on eIF4E expressing cells was decreased, and that the apoptotic effect of Adriamycin on MCF-7/ADR cells was suppressed. We also analyzed eIF4E protein expression following AMPK silencing and found that its level was significantly decreased; however, after treatment of cells with 60 MOI Ad-VT, its level significantly increased (**Figures 5C,D**). It was reported that mTOR regulates the eIF4E signal axis downstream of 4EBP1, leading to a change in eIF4E expression (Sarbassov et al., 2005; Hsieh et al., 2012). Therefore, we also analyzed 4EBP1 protein expression and found that its expression level was opposite to that of eIF4E protein expression. After silencing 4EBP1 in MCF-7/ADR cells, the expression of eIF4E and MRP1 increased, and the resistance of MCF-7/ADR cells to Adriamycin after Ad-VT infection increased (**Figures 5E,F**). The above results suggest that Ad-VT can change the resistance of MCF-7/ADR cells to Adriamycin through the AMPK-mTOR-eIF4F signal axis.

Ad-VT Induces the Changes of Adriamycin Resistance in MCF-7/ADR Cells *In-vivo*

We constructed a subcutaneous tumor-bearing model of MCF-7/ADR cells in nude mice. The results showed no obvious change in the tumor volume in ADR group compared with that in the control group, but the tumors' volume significantly decreased after adding Ad-VT, and there was a significant difference compared with that in the Ad-VT group. After adding an autophagy inhibitor, the tumors' volume reduction in the ADR + Ad-VT group was significantly reduced (**Figures 6A–C**). On the contrary, after the addition of the autophagy promoter, rapamycin (RAPA), the reduction of the tumors' volume in the ADR + Ad-VT group was the most significant (**Figures 6A–C**). We also found that the addition of an autophagy inhibitor increases the toxicity of Adriamycin, resulting in the death of mice, but no death was found after the addition of autophagy enhancers (**Figures 6D,E**). The immunohistochemistry results showed that adding Ad-VT can inhibit the expression of MRP1, eIF4E and p-mTOR, and increase

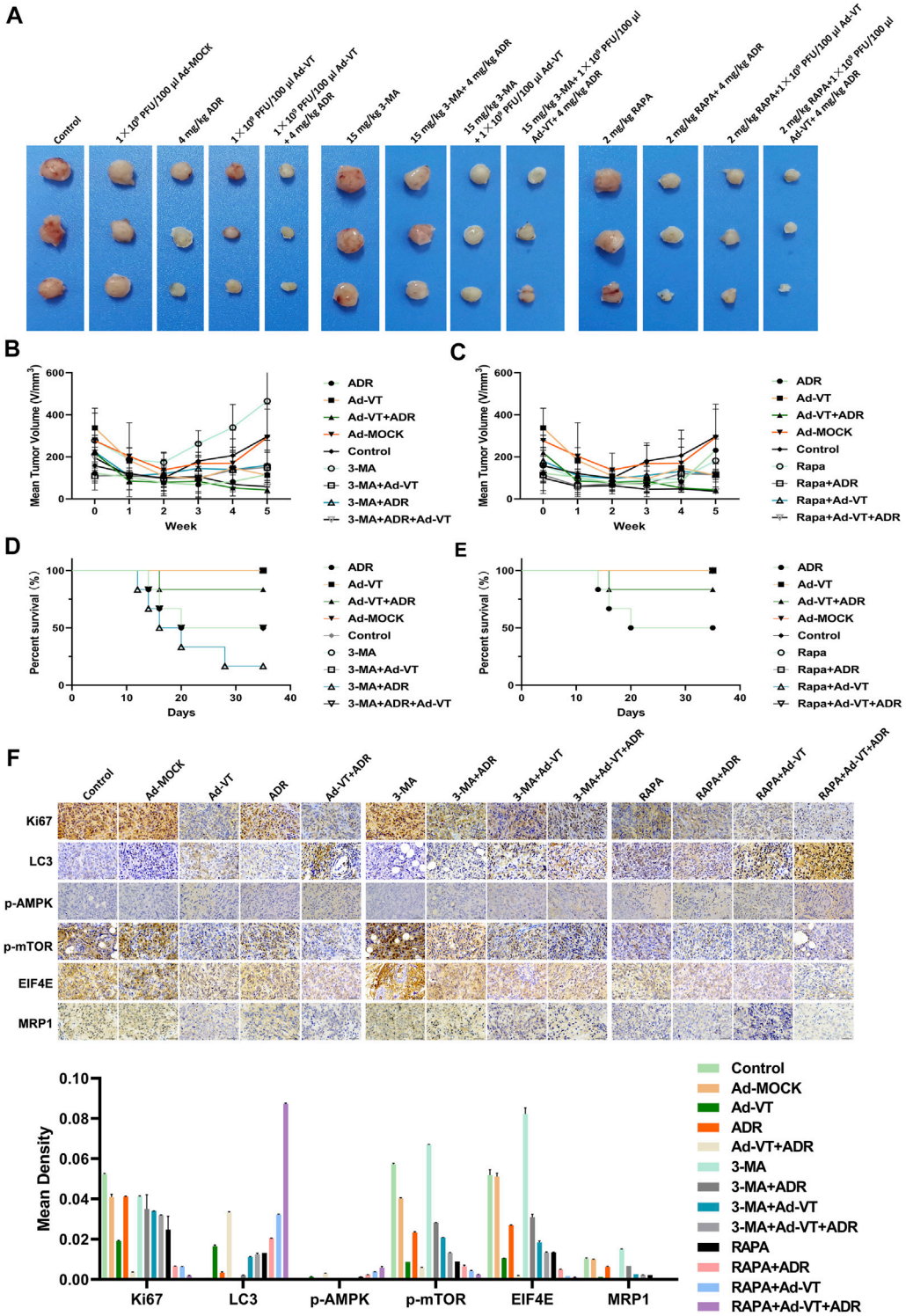

FIGURE 6 | Detection of the effect of Ad-VT on Adriamycin resistance *in vivo*. **(A,B,C)** Length and width of xenograft tumors that were weekly measured for 5 weeks using Vernier calipers. The average tumor inhibition was calculated using the formula: (1 − treatment group tumor volume/control tumor volume) × 100%. **(D,E)** The survival rate of tumor-bearing mice was daily recorded for 5 weeks. **(F)** Expression of LC3, p-AMPK, p-mTOR, eIF4E, MRP1, and Ki67 in the xenograft tumors tissues were detected by IHC. The scale bar equals 50 μm. Data were representative of three independent experiments (*n* = 3).

the expression of p-AMPK (**Figure 6F**). Opposite results were obtained by adding an autophagy inhibitor. These results are consistent with the results obtained *in vitro*. These results indicate that Ad-VT can also cause changes in the resistance of MCF-7/ADR cells to adriamycin *in vivo*, and that the regulation of autophagy can significantly affect the effect of Ad-VT.

DISCUSSION

The ATP binding cassette family of transporters (ATP-binding cassette transporter, ABC) is a large group of ATP driven pumps, which consists of two transmembrane domains and two cytoplasmic ATP binding domains. According to the homology of amino acid sequence and domain sequence of the conserved region, it was found that there are 49 members of human ABC superfamily belonging to 7 subfamilies of ABCA-ABCG (Higgins, 1992; Sarbassov et al., 2005). Under physiological conditions, ABC transporters are widely distributed in various tissues and organs of the human body, where they transport ions, amino acids, nucleic acid, polysaccharides, polydermis, proteins, drugs, and other substances, and participate in the absorption, distribution, and excretion of these substances in the human body. Therefore, they play an important role in stabilizing the internal environment. Recent studies have found that some ABC transporters are abnormally expressed in human malignant tumors, which correlate with the occurrence and development of tumors, the emergence of chemotherapy multidrug resistance, and poor prognosis in cancer patients (Szakacs et al., 2006).

To investigate whether Ad-VT induces drug resistance to Adriamycin in the breast cancer cell line, MCF-7/ADR cells, we performed an apoptotic test following treatment of the cells with a combination of Ad-VT and Adriamycin at different concentrations. The results showed that increasing the dose of Ad-VT to 60 MOI can induce Adriamycin-mediated apoptosis (500 nM) of MCF-7/ADR cells, through reducing their drug resistance to Adriamycin.

In breast cancer cells, there are many types of drug-resistant proteins such as BCRP, P-gp, and MRP1. MDR1 is one of the earliest and most widely researched multidrug resistance gene. It was first identified in Chinese hamster ovary cells and is a member of the ABC transporter superfamily, also known as ABCB1. The membrane glycoprotein that is encoded by ABCB1 is named P-glycoprotein (P-gp). Its structure is mainly composed of two transmembrane regions and two nucleotide binding regions (Locher, 2009; Iakusheva et al., 2014). It has been found that P-gp can utilize the energy obtained from ATP hydrolysis to discharge toxic metabolites or exogenous substances out of the cells appositively to concentration gradient; thus, protecting the body cells from toxic substances (Iakusheva et al., 2014). MRP1 is a drug-resistant protein that was identified in the Adriamycin-resistant small cell lung cancer cell line, H69/AR, in 1992. It also belongs to the superfamily of ABC transporters and is called ABCC1. MRP1 is mainly located on the plasma membrane of cytoplasmic organelles, such as endoplasmic reticulum, Golgi apparatus and vesicles that are involved in cytoplasmic transport. It is also expressed on the cell membrane of normal cells and on the cell membranes and in the cytoplasm of tumor cells. Under physiological conditions, MRP 1 is expressed at a low level in most human tissues, and is involved in oxidative stress, detoxification and defense against exogenous poisons (Cole et al., 1992; Cole, 2014). According to previous reports, the MRP1-mediated drug resistance in tumor cells is mainly due to the isolation of chemotherapy drugs in cytoplasm vesicles, which makes chemotherapy drugs unable to reach nuclear targets, resulting in drug resistance. Apart from its inability to transport paclitaxel, the drug resistance spectrum of MRP1 is similar to that of MDR1 (Cole, 2014). BCRP (ABCG 2) is also a member of the ABC transporter superfamily and the first Adriamycin-resistant protein that was identified in the breast cancer resistant cell line, MCF-7/AdrVP, and therefore, was named as breast cancer resistant protein (Oostendorp et al., 2009; Drozdzik et al., 2014). Like MDR 1 and MRP 1, it can transport substances out of cells using the energy obtained from ATP hydrolysis, but the monomer ABCG 2 has no transport function, and requires the formation of a homodimer or homopolymer to complete substrates' transport.

We detected these 3 types of drug-resistant proteins and found that the addition of Ad-VT significantly reduces the expression of MRP1, but there was no decrease in the expression of BCRP and P-gp. At the transcription level, we also found that the addition of Ad-VT only reduces the copy number of MRP1 but had no effect on BCRP and P-gp. This suggests that the Ad-VT-mediated decrease in drug resistance to Adriamycin was caused by the decrease in MRP1 protein expression.

It was reported that autophagy is closely related to drug resistance and that it regulates the survival and death of cancer cells (Hanahan and Weinberg, 2011). Traditionally, the relationship between autophagy and drug resistance has been divided into two distinct mechanisms and their related effects: One is associated with its protective mechanism against tumor drug resistance, and the other is related to autophagy-induced cell death, which increases tumor sensitivity to apoptosis (Li et al., 2012; Schwartz-Roberts et al., 2013; Li et al., 2017).

Ad-VT has been shown to promote the apoptosis and autophagy of MCF-7 cells (Chen et al., 2019), and therefore, we first detected the changes in apoptosis and autophagy in MCF-7 and MCF-7/ADR cells by investigating the expression of related key proteins. We found that autophagy and apoptosis in MCF-7/ADR cells were higher compared to those in MCF-7 cells following infection with Ad-VT. Therefore, we speculated that the aggravation of autophagy and apoptosis by the Ad-VT infection may have caused the change in cell death resistance. Then we analyzed different inhibitors of apoptosis and autophagy and found that the inhibition of apoptosis does not cause changes in the expression of drug-resistant proteins after adding Ad-VT, while the inhibition of autophagy increases their expression levels. These results indicate that our speculation may be correct and suggest that autophagy plays a key role in causing the change in drug resistance. They also indicate that autophagy my be mediating the Ad-VT induced cell death in MCF 7/ADR cells. The addition of an autophagy inhibitor and the CCK-8 test confirmed these observations.

When using autophagy inhibitors to analyze the changes of drug-resistant proteins, we found that the effect of 3-MA was higher than that of CQ, and thus, we anchored the research direction to the early stage of autophagy, in which the mTOR protein plays an important role in various cellular activities and in drug resistance. Then, we analyzed whether the activation of mTOR was related to the change in MCF-7/ADR cells' resistance to Adriamycin that is induced by Ad-VT. The results showed that mTOR was activated in MCF-7/ADR cells, but not in MCF-7 cells, and that the activity of mTOR in MCF-7/ADR cells was inhibited infection with Ad-VT. The overexpression of MTOR also increased the expression of MRP 1, which inhibited the apoptotic effect of Adriamycin on MCF-7/ADR cells. These results indicate that Ad-VT causes a change MCF-7/ADR cells' drug resistance to Adriamycin through mTOR.

After analyzing the role of mTOR in drug resistance, we analyzed the expression of mTOR upstream proteins. The results showed that after silencing AMPK and the addition of Ad-VT to the cells, the expression of MRP1was higher than that of cells with AKT and ERK1/2 overexpression, indicating that AMPK protein is the key protein that leads Ad-VT infected MCF-7/ADR cells to decrease their resistance to Adriamycin. The CCK-8 test also showed that the cell death effect of Ad-VT on AMPK knockdown MCF-7/ADR cells was lower than that of MCF-7/ADR cells overexpressing AKT and ERK 1/2.

After analyzing the expression of mTOR upstream proteins, we also analyzed the expression of mTOR downstream proteins. The activation of S6K and eIF4E can increase the drug resistance of tumor cells to chemotherapy drugs (Tee and Blenis, 2005). Therefore, we have analyzed their protein expression levels in this experimental setting. The results showed that S6K is not activated in MCF-7 and MCF-7/ADR cells; however, Ad-VT infection of the cells activated its expression. Subsequently, the silencing of S6K did reduce the expression level of MRP1 and Adriamycin resistance in MCF-7/ADR cells, but the infection with Ad-VT activated S6K. These results were contradictory and suggested that Ad-VT did not cause the change of Adriamycin resistance in MCF-7/ADR cells through S6K.

However, the expression of eIF4E was significantly decreased after infection with Ad-VT, and the level of MRP1 significantly increased after eIF4E overexpression and infection of the cells with Ad-VT. In the subsequent CCK-8 test, we found that in the cells overexpressing eIF4E, the cell death effect of Ad-VT decreases and the apoptotic effect of Adriamycin on MCF-7/ADR cells was inhibited. After addition of an autophagy inhibitor and the silencing AMPK, we found that the level of eIF4E protein, which was significantly decreased after adding Ad-VT, significantly increased. Studies have shown that mTOR regulates the eIF4E signaling axis downstream 4EBP1, which leads to a change in eIF4E expression (Sarbassov et al., 2005; Hsieh et al., 2012), and therefore, we also analyzed the expression of 4EBP1. Indeed, its expression level was opposite to that of the eIF4E protein, and the expression of eIF4E and MRP1 increased after 4EBP1 silencing. The resistance of MCF-7/ADR cells to adriamycin after Ad-VT infection was also increased.

Although extensive experiments were performed in this study to reveal the potential of Ad-VT in MCF-7/ADR cells in reducing the resistance of cells to adriamycin, there are still limitations here. In this study, we only analyzed the drug resistance of MCF-7 cells. In future studies, different types of breast cancer cells should be added, and other tumor cells should be added to perform a more in-depth study on the role of Ad-VT in reducing drug resistance.

In summary, Ad-VT can not only induce the cell death of MCF-7/ADR cells, but also reduce drug resistance to Adriamycin by increasing autophagy, which is caused by the AMPK-mTOR-4EBP1-eIF4F signaling axis. We suggest that the oncolytic adenovirus Ad-VT plays an important role in the combination of chemotherapy drugs and could be used as a drug to cell death in breast cancer cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Changchun University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LS, XL, and YQL. Performed the experiments: YRL, YZ, JH, JF, WL, SL, ZX, XY, and NJ. Analyzed the data: YRL, XL, LS, and YQL. Contributed reagents/materials/analysis tools: YZ, SL, and ZX. Wrote the paper: YQL and YRL. All authors read and approved the final manuscript.

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REVIEW
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Genetic Modifications That Expand Oncolytic Virus Potency

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Oncolytic viruses (OVs) are a promising type of cancer therapy since they selectively replicate in tumor cells without damaging healthy cells. Many oncolytic viruses have progressed to human clinical trials, however, their performance as monotherapy has not been as successful as expected. Importantly, recent literature suggests that the oncolytic potential of these viruses can be further increased by genetically modifying the viruses. In this review, we describe genetic modifications to OVs that improve their ability to kill tumor cells directly, to dismantle the tumor microenvironment, or to alter tumor cell signaling and enhance anti-tumor immunity. These advances are particularly important to increase virus spread and reduce metastasis, as demonstrated in animal models. Since metastasis is the principal cause of mortality in cancer patients, having OVs designed to target metastases could transform cancer therapy. The genetic alterations reported to date are only the beginning of all possible improvements to OVs. Modifications described here could be combined together, targeting multiple processes, or with other non-viral therapies with potential to provide a strong and lasting anti-tumor response in cancer patients.

Keywords: oncolytic virus, cancer therapy, genetic modifications, oncolytic potency, metastasis

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INTRODUCTION

Since first observing that viruses can induce tumor regressions a century ago (Bierman et al., 1953; Bluming and Ziegler, 1971), the possibility of using viruses as a cancer therapy has maintained the interest of many scientists. Oncolytic viruses (OVs) selectively target tumor cells, leaving healthy cells unharmed. Their mechanisms are multi-dimensional; OVs infect and lyse tumor cells, generating local tumor burden reduction (Kooti et al., 2021; Santos Apolonio et al., 2021). Some OVs also infect and kill tumor-supporting cells in the tumor microenvironment such as endothelial cells and fibroblasts (Pikor et al., 2015), helping dismantle the environment that supports the tumor. Finally, many OVs induce cytokine secretion and expose tumor-associated antigens, which favours an anti-tumor immune response (Lichty et al., 2014). The struggle faced by scientists is to enhance the potency of these three activities to a point where the cancer is fully eliminated. Several OVs have progressed to human trials and even achieved FDA approval for specialized use in patients (Fukuhara et al., 2016; Cook and Chauhan, 2020; Macedo et al., 2020). For example, the herpes viruses T-VEC, G207 and G47Δ (Rider et al., 2019; Uche et al., 2021); the adenovirus DNX-2401 or Tasadenoturev (Ene et al., 2021) and Oncorine (Liang, 2018); the reovirus pelareopep (Müller et al., 2020); the vaccinia virus Olvi-Vec (Manyam et al., 2021); and the coxsackievirus CAVATAK (Annels et al., 2019). However, many articles and reviews have reiterated that OVs developed thus far are

insufficient as a monotherapy. Accordingly, two major strategies are being explored to boost the cancer therapeutic potency of OVs; one is to combine OVs with other cancer therapies, and the second is to modify OVs further genetically to increase their potency (Zainutdinov et al., 2019; Santos Apolonio et al., 2021). This review will focus on the latter; genetic modifications to OVs that produce improvement in their ability to kill tumor cells directly, dismantle the tumor microenvironment, or promote anti-tumor immunity. In the future, combining genetically modified OVs with other OVs and/or other non-viral therapies, may present a feasible path to using viruses in cancer therapy.

While this review focuses on genetic strategies to make OVs more potent, it is important to recognize that OVs must first exhibit strong tumor selectivity and safety. OVs include RNA viruses such as reovirus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), measles virus (MV), poliovirus and coxsackievirus; and DNA viruses such as parvovirus, adenovirus, vaccinia virus and herpes simplex virus (HSV). Some of these OVs are inherently more infectious towards transformed cells, such as reovirus (Strong et al., 1998; Norman et al., 2004; Shmulevitz et al., 2005) and parvovirus (Nüesch et al., 2012; Geiss et al., 2017). These viruses are naturally cleared by healthy tissues, but features of tumors such as specific cell receptors, intracellular enzymes, or reduced antiviral response, favour replication of these viruses in tumor cells. Other viruses need to be genetically modified to be more selective towards tumor cells, such as adenovirus, vaccinia virus or HSV. For example, a common strategy to make adenoviruses selective to tumor cells is genetic manipulation of the essential adenovirus E1A protein (Niemann and Kühnel, 2017). E1A is necessary for adenoviruses to replicate in non-transformed cells because one of its functions is to inactivate cellular retinoblastoma protein and activate transcription factor E2F-induced cell cycle genes (Niemann and Kühnel, 2017; Sohn and Hearing, 2019). The retinoblastoma binding activity of E1A is dispensable for replication in tumor cells that already harbor dysregulated cell cycle and compensate for the absence of this E1A activity (Fueyo et al., 2000). A different approach is to selectively transcribe indispensable viral proteins under the control of specific transcription factors that are upregulated in tumors; for example, placing E1A gene expression under the control of the hTERT promoter (Shay and Bacchetti, 1997; Wirth et al., 2003). Additional modes of selectivity will be described for specific OVs throughout the review, as a prelude to novel genetic modification strategies that increase *potency*.

Development of secondary tumors at sites distal to the primary tumor (metastasis) is one of the main challenges in cancer therapy and the principal cause of mortality (Fares et al., 2020). Metastasis is a complex process that involves a cascade of steps, starting with activation of invasion and metastatic phenotype (Hanahan and Weinberg, 2011). Epithelial cells undergo a process of *trans*-differentiation known as epithelial-mesenchymal transition (EMT) to acquire the ability to migrate, invade, resist stress, and disseminate (Lambert et al., 2017). Cancer cells are then able to spread from the local tumor environment to intravasate blood and lymphatic vessels. Cancer cells travel through the lymphatic and blood systems as single cells or in clusters. Ultimately, these cells arrest and extravasate through endothelial cells to colonize secondary sites, where they can proliferate immediately or stay in a dormant state for even years depending on environmental factors (Hanahan and Weinberg, 2011; Lambert et al., 2017; Fares et al., 2020). OVs with their tumor selectivity and the potential to be delivered systemically are a promising therapy against metastasis. This review will therefore describe genetic modifications that increase OV potency in general, but also focus on strategies that directly aim to enhance potency against metastases.

ENGINEERING OVS TO ENHANCE VIRUS REPLICATION AND KILLING IN THE PRIMARY TUMOR AND METASTATIC SITES

One of the ways by which OVs exert their oncolytic activities is by directly infecting and killing cancer cells. One strategy to increase oncolytic potency is therefore to increase the OV's replication or tumor killing abilities. Enhanced OV replication in tumors would also amplify secondary effects, such as increases in tumor antigen presentation, anti-tumor immune cell recruitment, and virus dose for dissemination to distal sites of metastasis. Keeping in mind that no known natural virus requires tumors as host, but rather that researchers have harnessed viruses for this task, it is not surprising that viruses need to be genetically modified or selected to be optimally infectious and lethal towards cancer cells. The question becomes, what specific changes are needed to make a given virus thrive better in targeted cancers? Below are examples of diverse and sometimes unpredictable changes to OVs that promote direct infection and killing activities. These are summarized in **Table 1**.

Herpes Simplex Virus

Herpes simplex virus, type 1 (HSV-1) is an enveloped, double-stranded linear DNA virus (Watanabe and Goshima, 2018). HSV-1 has a large genome, encoding at least 83 genes that function to mediate virus replication, modulate the host cell, and subvert the immune response. As is commonly known, HSV-1 replication in mucosa causes cold sores, but HSV-1 can also persist latently in trigeminal ganglia, sometimes causing encephalitis upon reactivation. When transforming HSV-1 into an OV, it is important to eliminate neuro-invasive abilities to reduce the risk of encephalitis (Kanai et al., 2012). Two virus genes commonly deleted from HSV-1 to destroy neural tropism are UL56 and γ34.5. The UL56 protein associates with host kinesin motor protein KIF1A to facilitate neuroinvasion (Koshizuka et al., 2005). The γ34.5-encoded ICP34.5 protein blocks cellular protein translation and anti-viral responses and is necessary for virus replication in neurons. For example, G207 is a second generation genetically modified HSV-1 with deletions in γ34.5 and an inactivating insertion of *LacZ* in the *ICP6* gene. The latter encodes the large subunit of the viral ribonucleotide reductase, a key enzyme for DNA synthesis. The combination

TABLE 1 | Oncolytic viruses with enhanced virus replication and killing ability.

Virus backbone	New virus	Modification	Reason	Result	Reference
HSV[a]	Synco-2D	Addition of hyperfusogenic glycoprotein of gibbon ape leukemia virus into Baco1	Increase fusogenic ability	Increased survival and reduced metastasis in mouse ovarian, prostate and breast cancer	(Nakamori et al., 2003, 2004a, 2004b)
HSV	OncSyn	gBsyn3 syncytial mutation incorporated into an already attenuated HSV-1 virus (NV1020)	Increase fusogenic ability	Reduced mouse tumor growth and metastasis breast cancer model	(Israyelyan et al., 2007, 2008)
HSV	OncdSyn	A second syncytia-enhancing mutation introduced into viral glyco-protein K of OncSyn	Increase fusogenic ability	Reduced and/or inhibited mouse breast tumor metastases	Israyelyan et al. (2008)
HSV	ΔN146	Truncation of the viral protein $\gamma_1$34.5 (to aa 147–263) rather than deletion as in most oncolytic HSV	Keep some anti-viral subversion functions	Reduced mouse breast tumor growth and lung metastases	Liu and He, (2019)
Adenovirus	Ad5-Δ24RGD	Addition of RGD (Arg-Gly-Asp) to fiber	Expand receptor recognition	Prolonged survival in a mouse metastatic breast tumor model	Ranki et al. (2007)
Adenovirus	ColoAd1 or Enadenotuvirev	Directed evolution	Enhance killing ability	Reduced mouse colon cancer metastasis to liver	Kuhn et al. (2008)
Adenovirus	TelomeKiller	Addition of the red fluorescent protein KillerRed with E1A and E1B driven by the hTERT promoter	Generation of reactive oxygen species (ROS) upon green light irradiation	Reduced lymph node metastases size in a mouse rectal tumor model	Takehara et al. (2016)
VSV[b]	rVSV-NDV/FL (L289A))	Addition of a Newcastle disease virus fusion protein	Increase fusogenic ability	Prolonged survival in a multifocal liver metastases rat model	(Ebert et al., 2004; Yamaki et al., 2013)
VSV	VSV-p14	Addition of p14 fusion protein from reptilian repovirus	Increase fusogenic ability	Reduced tumor growth and increased survival in mouse breast and colon cancer models	Le Boeuf et al. (2017)
VSV	VSV-GP	Addition of the lymphocytic choriomeningitis virus (LCMV) glycoprotein	Increase safety and reduce neuro-toxicity	Prolonged survival and reduced tumor growth in a mouse melanoma model	(Muik et al., 2011, 2014; Kimpel et al., 2018)
Reovirus	T3v1, T3v2	Directed evolution	Increase replication	Prolonged survival in a mouse melanoma model	(Shmulevitz et al., 2012; Mohamed et al., 2015a)
Adenovirus		Overexpression of adenovirus death protein (ADP)	Early cell death	Tumor growth reduction	Doronin et al. (2000)

[a]*HSV, herpes simplex virus.*
[b]*VSV, vesicular stomatitis virus.*

of both deletions permits replication in tumor cells while preventing a productive infection in normal tissue (Mineta et al., 1995; Uche et al., 2021). Patient-derived xenograft studies in nude mice showed that pediatric brain tumors are particularly sensitive to G207 (Friedman et al., 2018). Accordingly, G207 was recently tested in a phase 1 trial in pediatric malignant high-grade glioma. Patients showed an increase in tumor infiltrating $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes and no serious adverse effects related to G207 administration. Median overall survival was 12.2 months, in contrast with the 5.6 median overall survival usually observed in this setting (Friedman et al., 2021). G47Δ is a third generation HSV-1 based on G207 with an additional deletion in the *α47* gene, involved in antigen presentation. G47Δ is more effective than G207 at preventing tumor growth in animal models, while showing a similar safety level (Todo et al., 2001). G47Δ received the Orphan Drug Designation and the conditional approval for the treatment of malignant glioma in Japan.

Another important HSV-1 for oncolytic therapy is Talimogene Laherparepvec (T-VEC). This virus has deletions in the γ34.5 gene as well as the gene encoding ICP47 involved in suppressing antigen presentation. Furthermore, the human granulocyte macrophage colony-stimulating factor (GM-CSF) cDNA was incorporated into T-VEC to increase recruitment and activation of antigen-presenting cells to tumors (Conry et al., 2018). The combination of safety and immunomodulation has made T-VEC an effective therapy against melanoma (Liu et al., 2003). In 2015, after successful phase I, II and III clinical trials, T-VEC was approved by the U.S. Food and Drug Administration (FDA) and European Medicines Agency for clinical use (Andtbacka et al., 2015; Mondal et al., 2020). The approval of this engineered oncolytic virus, T-VEC, is a great advancement to the use of OVs in patients (Mondal et al., 2020).

Although T-VEC improved median overall survival from 18.9 to 23.2 months in clinical trials of 436 patients with unresectable stage IIIB to IV melanoma (Andtbacka et al., 2015), there remains interest to further augment T-VEC potency towards melanoma patients. Moreover, research is still necessary to enhance potency of HSV-derived OVs towards an assortment of other cancer

types. Given that HSV-1 depends on membrane fusion for entry, assembly and cell-cell spread (Weed and Nicola, 2017), two independent research groups focused on increasing potency by augmenting the fusogenic ability of HSV-derived OVs. The Zhang group designed a new version of a fusogenic HSV (called Synco-2D), in which the hyperfusogenic glycoprotein of gibbon ape leukemia virus was incorporated into the already fusogenic "Baco 1" mutant HSV (Nakamori et al., 2003). In a xenograft mouse model of peritoneal metastatic ovarian cancer established by Hey-8 cells, Synco-2D exhibited a dramatic effect on tumor growth and mouse survival (8/8 survivors) relative to Baco-1 virus (5/8 survivors) and PBS (0/8 survivors). The same group demonstrated that intravenous injection of Synco-2D, reduced metastases in both the PC-3M-Pro4 prostate cancer xenograft model and the 4T1 cell metastatic syngeneic breast tumor model (Nakamori et al., 2004a; Nakamori et al., 2004b). Interestingly, they found that Synco-2D caused increased $CD8^+$ T cell activation and antitumor immunity in the 4T1 model. Also, since results suggest that Synco-2D is a promising oncolytic virus that targets metastasis in three different animal tumor models, it would be important to compare Synco-2D directly to T-VEC and other competing HSV-based OVs in clinical testing.

The Kousoulas group (Israyelyan et al., 2007) also created a new fusogenic HSV-based OV called "OncSyn." OncSyn was built upon the 'NV1020' attenuated HSV-1 containing modifications that eliminate UL56, α0, γ34.5, and α4 to confer safety and tumor selectivity. Into NV1020, the authors introduced a single amino acid change (the gBsyn3 mutation) in the surface viral fusion protein, glycoprotein B, resulting in bigger syncytial plaques and increased virus replication. OncSyn was efficient *in vivo* at reducing tumor growth in a xenograft mouse model system using MDA-MB-435S-lux human breast cancer cells. Later, a second syncytia-enhancing mutation was introduced into the viral transmembrane glycoprotein K, creating the "OncdSyn" OV (Israyelyan et al., 2008), enhancing fusion of otherwise resistant cells. Both OncSyn and OncdSyn were tested in the highly metastatic interscapular 4T1 breast tumor model. Both viruses reduced the number of mice with metastases in internal organs such as liver, spleen, and kidneys, with OncdSyn exhibiting slightly better response at early timepoints than OncSyn. However, OncSyn and OncdSyn were not compared with the parental virus NV1020 in the *in vivo* setting, and hence it is not possible to conclusively attribute an advantage to the fusogenic mutants.

Enhancing HSV-derived OVs replication in tumors can also be achieved by fine-tuning the precise modification of HSV-1 genes such as $\gamma_1$34.5. As mentioned, the $\gamma_1$34.5 HSV-1 gene blocks cellular IFN-mediated anti-viral response, but also activates protein translation by inhibiting phosphorylation of translation initiation factor eIF2α. The $\gamma_1$34.5 gene is deleted from most HSV-based OVs such as T-VEC, which contributes to specificity of these OVs towards cancer cells that often harbor compromised IFN signalling. The He group (Liu and He, 2019) questioned whether removing the N-terminal domain of γ34.5 responsible for IFN-mediated antiviral subversion was sufficient for specificity, and whether keeping the remaining domains of γ34.5 that facilitate activation of protein translation could promote OV replication in tumors. In comparison to the γ34.5 null mutant (Δ γ34.5), an N-terminal truncation mutant of γ34.5 (ΔN146) achieved higher viral replication in tumor cells *in vitro* and was more resistant to IFN- α exposure. In the metastatic 4T1 syngeneic breast tumor mouse model, ΔN146 treatment significantly reduced lung metastases relative to Δ γ34.5 and PBS treatments (10 vs. 15 vs. 25 nodules/lung). These studies suggest that the ΔN146 mutant, which maintains ability to stimulate virus protein synthesis, may provide a better oncolytic virus *in vivo* to reduce metastasis.

Adenovirus

Adenovirus has a broad tissue tropism, so for safety reasons oncolytic adenoviruses must be modified to selectively replicate in tumor cells. Once specificity is achieved, adenovirus can be further modified to encode exogenous proteins that favour virus replication/production in tumors. The natural receptor of adenovirus 5, Coxsackievirus–adenovirus receptor, is not abundant in cancers; so, one approach to increase the infectivity of this virus in cancers is to expand receptor recognition. In Ranki et al. (2007), the RGD (Arg-Gly-Asp) domain was added to the fiber protein encoded by a recombinant adenovirus with a truncated E1A gene (Ad5-Δ24), creating the Ad5-Δ24RGD. RGD is a ligand for αβ integrin receptors which are abundantly expressed in malignant cancer cells (Wu et al., 2019). Ad5-Δ24RGD significantly prolonged survival when compared with an Ad lacking the RGD domain (Ad5Δ24E3) in the M4A4-LM3 xenograft metastatic breast cancer model. Strangely, no differences in the presence of primary tumors, as monitored by fluorescence emission of GFP positive M4A4-LM3 cells, were observed in mice treated with the two viruses. More detailed animal studies are required to understand how binding to αβ integrins promoted survival in this metastatic model. Interestingly, the Ad5-Δ24RGD virus has been tested and improved during the last years, creating what is currently commercially known as DNX-2401 or Tasadenoturev. This modified adenovirus received an Orphan Drug Designation by the FDA for recurrent glioblastoma (Philbrick and Adamson, 2019; Ene et al., 2021).

As a complement to rationally engineering OVs to thrive in cancer cells as described above, directed evolution has provided a worthwhile strategy to enhance OV replication in cancer cells. In Kuhn et al. (2008), pools of adenovirus serotypes were passaged on human tumor cell lines to promote recombination and emergence of more-potent viral variants. The ColoAd1 variant, also called Enadenotucirev, was exhibited higher oncolytic activities in colon cancer cell cultures. ColoAd1 is derived from the Ad11p serotype, which is less prevalent in the human population than Ad5. Since most patients are seronegative for Ad11p, ColoAd1 may be less-quickly neutralized by host antibodies providing an advantage in systemic administration for metastatic cancers, although an advantage of host seroprevalence for ColoAd1 has yet to be empirically demonstrated. When evaluated in a metastatic colon cancer xenograft mouse model, ColoAd1 reduced

growth of liver metastases relative to the parental Ad11p virus. ColoAd1 also replicated better in tumor biopsies than the parental virus.

Both directed evolution and rational design approaches have demonstrated that adenovirus OV potency can be enhanced by intensifying the cell killing activities of this virus. OVs kill the tumor cells they infect during the late stages of their replication cycle. Despite that adenovirus is ultimately lytic towards tumor cells, studies found that inducing earlier cell death can promote oncolytic activity. For example, Doronin et al. (2000) found that an E1A-modified adenovirus reengineered to overexpress the adenovirus death protein (ADP) exhibited increased replication and cell-cell spread in human A549 lung carcinoma cells and reduced the size of A549-derived tumors in nude mice, relative to their parental strain. Remarkably, Uil et al. (2011) also discovered that increased expression of ADP enhances oncolytic activity of adenovirus but using an unbiased directed evolution approach. Specifically, Ad5 expressing an error-prone polymerase was used to select mutants with improved replication in SKOV-3 ovarian carcinoma cells. The variant "F421Y" was found to carry a mutation at the splice acceptor site of an ADP-encoding exon that enhanced ADP expression levels. F421Y increased cell killing of SKOV-3, human breast (SKBR-3) and prostate (PC-3) carcinoma cell lines. To our knowledge, however, these viruses have not yet been compared in immune competent animal models, nor evaluated for their activity towards metastases.

Finally, Takehara et al. (2016) took a very innovative approach to enhance cell killing by adenovirus. The authors developed TelomeKiller, a tumor-specific replicating adenovirus that expresses the red fluorescent protein KillerRed under the control of the CMV promoter inserted into E3 gene of adenovirus. KillerRed is a photosensitizer that generates reactive oxygen species (ROS) upon green light irradiation/photodynamic therapy (PDT). TelomeKiller was evaluated in an intraperitoneal lymph node metastasis model, where HCT116-GFP human colorectal cells were implanted into the submucosal layer of the rectum. Virus was directly injected into the rectal tumors. Three days later, GFP-expressing metastases were subjected to PDT. Twenty-one days after virus injection, the authors observed that the GFP signal had decreased in metastatic lymph nodes in all PDT + TelomeKiller-treated mice, whereas the signal increased in control mice or mice treated with virus but not PDT. These results suggest that TelomeKiller in combination with PDT efficiently targets lymph node metastases and that adenovirus replication alone is not enough to shrink metastases.

Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is best known to farmers since it causes mild fever and blisters in cattle (Buijs et al., 2015). It is an enveloped single-stranded negative sense RNA virus in the family *Rhabdoviridae*. In humans, VSV selectively replicates in cancer cells which tend to have defective or reduced type I IFN responses. VSV has some advantages as an OV, including its rapid replication, lack of pre-existing immunity in humans, broad tropism, and easily manipulated genome (Simovic et al., 2015). However, in some animal studies, wild-type VSV treatment has presented neurological symptoms. Because of this, current VSV mutants are generated with mutations in their matrix protein (M) or membrane fusion protein (G) to eliminate neurotropism (Buijs et al., 2015).

Several attempts have been made to enhance VSV OV potency by increasing membrane fusion, similar to above-described modifications to HSV-based OVs. In Yamaki et al. (2013), a previously designed recombinant VSV virus (rVSV-NDV/FL (L289A)) expressing a Newcastle disease virus fusion protein was tested in two metastatic models of colorectal cancer (Ebert et al., 2004). First, when RCN-H4 colorectal cancer cells (CRC) are injected into the liver, they produce lesions in the liver. In this CRC liver metastasis model, rVSV-NDV/FL (L289A) significantly increased long-term survival. In the second model, CRCs are instead injected systemically *via* venous infusion; herein, rats develop CRC metastatic lesions in their lungs. In the systemically-administered CRC lung metastasis model, the efficiency of rVSV-NDV/FL (L289A) was less impressive, significantly prolonging survival but not generating long-term surviving rats. While survival data suggested a promising improvement to OV potency, it would have been informative to also assess the metastatic burden directly in the animal experiments. More recently, Le Boeuf et al. (2017) pseudotyped VSV with the p14 fusion protein of fusogenic reptilian reovirus and found significant improvement of oncolytic potency in several animal models. While the VSV G protein only induces cell fusion at low pH in lysosomes, the p14 reovirus fusion protein induces membrane fusion at neutral pH. Accordingly, the VSV-p14 displayed syncytia at normal pH, and promoted higher virus yields and dissemination in cancer cell cultures and spheroids. VSV-p14 resulted in smaller tumor volumes and increased survival in the 4T1 orthotopic metastatic breast tumor model, without altering biodistribution and safety of the virus. Two additional mouse models were applied to assess if p14 incorporation into VSV improves protection against metastatic disease. First, 4T1 mammary tumors were allowed to metastasize prior to excision of the primary tumor and OV administration through the tail vein. In this model, VSV-p14 extended survival significantly more than the control VSV-GFP virus. Second, mice were intravenously administered CT26 colon cancer cells expressing lacZ then systemically treated by the OVs. In this experiment, VSV-p14 significantly reduced lacZ + metastatic nodules relative to VSV-GFP or untreated mice. Moreover, VSV-p14 seemed to increase tumor immunity; for example, increasing the number of activated $CD4^+$ and $CD8^+$ T cells in the spleen, draining lymph nodes, and tumors, relative to controls. With multiple independent researchers finding a benefit for syncytium formation in oncolytic potency of VSV, this seems a promising avenue to continue building upon.

Lastly, Muik et al. (2014) and Kimpel et al. (2018) found that VSV pseudo-typed with the less-immunogenic lymphocytic choriomeningitis virus (LCMV) glycoprotein (VSV/LCMV-GP) lacked VSV's neurotoxicity, induced fewer neutralizing antibodies, and reduced lung metastasis in a syngeneic B16-OVA melanoma model (Muik et al., 2011). Specifically, mice injected with B16-OVA cells intravenously were treated with tail vein injections of VSV/LCMV-GP or left

untreated. After 14 days, the number of lung metastases was significantly reduced in VSV/LCMV-GP treated mice compared to untreated mice, and the remaining metastases were smaller. While the efficiency of VSV/LCMV-GP was not compared to VSV in the B16 melanoma model to demonstrate the direct advantage of adding the LCMV-GP into VSV, previous comparisons in non-metastatic glioma xenograft models found an advantage of VSV/LCMV-GP over VSV. Regardless, the idea of using a surface glycoprotein that is less immunogenic and therefore enables multi-dosing of OVs with reduced virus neutralization is worthy of note for future developments.

Reovirus

Mammalian orthoreovirus (reovirus) naturally circulates among humans and other mammals through the fecal-oral route without causing disease. Remarkably, unmodified serotype 3 reovirus (T3wt) was also found to infect, disseminate amongst, and kill tumor cells. Healthy untransformed cells do not support rampant replication and spread of reovirus because they have fewer enzymes that support reovirus uncoating during entry, they do not efficiently undergo cell death to release progeny virions, and because untransformed cells mount a strong interferon antiviral response. Several clinical trials have demonstrated the safety of T3wt (also commercially known as pelareopep by Oncolytics Biotech Inc.) in cancer patients, and some trials have demonstrated a moderate but improvable oncolytic effect (Clements et al., 2014). Indeed, FDA granted Orphan Drug Designation to T3wt for breast, ovarian, pancreatic, peritoneal and gastric cancers (Müller et al., 2020). In the Shmulevitz laboratory, we have isolated reovirus mutants that replicate better than T3wt in a panel of tumor cells while retaining limited replication in untransformed cells. Two of these mutants, T3v1 and T3v2, have an advantage in virus disassembly which leads to increased virus replication and larger plaque size (Mohamed et al., 2015a). In a syngeneic mouse B16 metastatic melanoma model, flank tumors were injected intratumorally with these mutants at days 14, 16 and 18 post-injection of B16 cells. T3v1 and T3v2 increased survival relative to T3wt in this metastatic model (Shmulevitz et al., 2012). We also found that genetic variations in wild-type reovirus strains impact tremendously their replication ability in different tumor cell lines such as mouse ID8 ovarian cancer, human Huh 7.5 hepatocarcinoma, human H1299 non-small cell lung carcinoma and mouse B16-F10 melanoma cell lines (Mohamed et al., 2020a). The oncolytic effects *in vitro* translated into a reduction in melanoma tumor growth in the B16 animal tumor model (Mohamed et al., 2020b). This evidence suggests that genetic modifications can improve reovirus potency in pre-clinical models which is auspicious for clinical trials. Several additional reovirus mutants have been found to promote binding, uncoating or antiviral response *in vitro* (Mohamed et al., 2015b), some of which exhibit different cell receptor tropisms (van den Wollenberg et al., 2012). Unfortunately, these mutants have not been tested in tumor models, so their oncolytic potential remains to be characterized.

OVERCOMING THE EXTRACELLULAR MATRIX BARRIER TO VIRUS DISSEMINATION TO METASTATIC SITES

Metastases present a large challenge when treating late-stage cancer patients. OVs offer potential to target metastases directly, or indirectly through OV-induced anti-tumor responses. To improve direct targeting of metastases, specific strategies that promote OV dissemination have been investigated (summarized in **Table 2**). One such strategy involves altering the extracellular matrix to improve virus dissemination out of the local tumor and into secondary tumor sites.

The tumor microenvironment consists of cells embedded in a non-cellular component, mainly extracellular matrix (ECM). The cellular component includes cancer cells, immune cells, fibroblasts, pericytes, endothelial cells, adipocytes, and mesenchymal stem cells. The spaces between the cells are composed of interstitial fluid, cell-free DNA, exosomes, as well as ECM (Baghban et al., 2020). While the composition of the tumor ECM depends on the type of tumor, the most common molecules expressed by solid tumors are fibrillar collagens, fibronectin, elastin, and laminins (Henke et al., 2019). These molecules are produced either by the cancer cells or other cells of the tumor microenvironment such as fibroblasts (Naba et al., 2012). Cancer-associated fibroblasts (CAFs) are described as great secretors of collagen, which is linked with resistance to therapies and poor prognosis (Provenzano et al., 2008; Mammoto et al., 2013). ECM in the tumor does not have the same characteristics as that in normal tissues. In the tumor, ECM is more rigid, abundant and dense (Henke et al., 2019). Because of this, tumor ECM acts as a barrier for therapeutic agents such as OVs. At the same time, the barrier impairs the oxygen and nutrients supply, activating apoptosis and senescence. ECM interactions also can lead to activation of signalling pathways in tumor cells that promote survival and avoid cell cycle arrest. In addition, tumor ECM has an important role regulating EMT and metastasis (Henke et al., 2019). Thus, the ECM is a candidate cancer therapeutic target.

At the onset of metastasis, during the invasion process, remodeling of the ECM is mainly done by metalloproteases (MMPs). MMPs are proteolytic enzymes that degrade most ECM molecules and regulate the activity of other important proteins in the tumor microenvironment such as growth factors, cytokines, chemokines, proteinases and cell receptors (Egeblad and Werb, 2002). MMPs are secreted by different cells within the tumor including cancer cells, CAFs, and neutrophils (Kessenbrock et al., 2010). MMPs are overexpressed in most cancers and are indicative of increased tumor aggressiveness and shortened patient survival (Egeblad and Werb, 2002; Hadler-Olsen et al., 2013). Given the prevalence of MMPs in tumor ECM, both adenovirus- and vaccinia- based OVs have been genetically modified to exploit the natural functions of MMPs and enhance virus dissemination.

In addition to E1A-deleted adenoviruses described in previous sections, adenoviruses lacking E1B proteins (Ad-ΔE1B) show specificity towards transformed cells and have extensively been evaluated for cancer therapy. The E1B proteins normally block

TABLE 2 | Oncolytic viruses that dismantle the tumor microenvironment to improve virus dissemination.

Virus backbone	New virus	Modification	Reason	Result	Reference
Adenovirus	Ad-ΔE1B-RLX	Addition of relaxin to Ad-ΔE1B	Improve virus distribution in the tumor	Reduced lung metastasis in a mouse melanoma model	Kim et al. (2006)
Adenovirus	OV-RLX-5T35H	Addition of relaxin to Ad5/35 adenovirus	Improve virus distribution in the tumor	Reduced metastasis and improved survival in a mouse pancreatic tumor model	Ganesh et al. (2007)
VV[a]	GLV-1h255	Addition of MMP-9 to GLV-1H68	Improve virus distribution in the tumor	Did not alter metastasis in a mouse prostate cancer model	Schäfer et al. (2012)
Adenovirus	VCN-01	Addition of hyaluronidase (PH20) into fiber-modified AdΔ24E1A	Improve virus distribution in the tumor	Reduced metastasis in a metastatic osteosarcoma mouse model	Martínez-Vélez et al. (2016)
Adenovirus	EnAdDNAse	Addition of exonuclease DNAse I into ColoAd1 Enadenotuvirev	Improve virus distribution in the tumor	Reduced tumor growth and improved virus spread	Tedcastle et al. (2016)
Adenovirus	EnAdPH20	Addition of hyaluronidase into ColoAd1 Enadenotuvirev	Improve virus distribution in the tumor	Reduced tumor growth and improved virus spread	Tedcastle et al. (2016)
Reovirus	S1-T241I	Mutation of viral binding protein sigma1 to impede cleavage by tumor proteases	Improve virus distribution in the tumor	Improved virus distribution in primary tumors	Fernandes et al. (2019)

[a]*VV, vaccinia virus.*

p53 tumor suppressor activities and promote nuclear export of viral mRNAs (O'Shea et al., 2004; O'Shea et al., 2005); Ad-ΔE1B therefore depends on transformed cells to overcome the absence of E1B functions. To improve the distribution of E1B-deleted adenovirus, the Yun group created an adenovirus expressing relaxin (Ad-ΔE1B-RLX). Relaxin is a 6 kDa protein hormone that upregulates matrix metalloproteinases (MMPs) (Unemori et al., 1996), which in turn help degrade the ECM (Stamenkovic, 2003). The authors hypothesized that ECM impedes virus spread, and therefore that removing the ECM with relaxin would promote virus dissemination and broaden activity to metastatic sites. In Kim et al. (2006), the intratumor injection of Ad-ΔE1B-RLX in a murine syngeneic B16 metastatic melanoma model reduced tumor metastasis in lungs significantly relative to the adenovirus without relaxin (Ad-ΔE1B). While clearly the Ad-ΔE1B-RLX provided advantage over Ad-ΔE1B, the precise mechanism was not confirmed by quantifying the levels of MMPs and extent of ECM degradation. In addition, relaxin has pleiotropic activities that extend from cell signaling activation and nitric oxide production to expression of MMPs, stromal cell-derived factor (SDF)1-α and vascular endothelial growth factor (VEGF) that impact vasculogenesis (Ng et al., 2018). Each of these functional consequences could affect activity and dissemination of an oncolytic virus. Interestingly, even in ECM-devoid cell cultures, Kim et al. (2006) observed that Ad-ΔE1B-RLX has advantage over Ad-ΔE1B in establishing virus plaques more rapidly and inducing apoptosis of tumor cells. Therefore, it is likely that effects of relaxin are multifaceted, and it would be interesting to know which effects are most critical for enhancing activities of oncolytic adenovirus.

In further support for potential benefits of modifying oncolytic viruses to encode relaxin, the Jooss group also observed an advantage of adding relaxin to adenovirus; they used the Ad5/35 chimeric fiber-encoding adenovirus which exhibits tumor specificity by requiring CD46 receptors abundant on tumor cells for attachment. In the PC-3luc prostate metastatic xenograft model, the Ad5/35 chimeric adenovirus expressing relaxin (OV-RLX-5T35H) increased virus titers in the primary tumors and reduced collagen staining compared with tumors treated with the virus without relaxin (OV-5T35H) (Ganesh et al., 2007). The reduced collagen staining adds direct evidence for the effects of relaxin on the ECM. When metastases in lymph nodes and lungs were analyzed in this same tumor model, they observed that the percentage of mice with metastases was reduced to zero in the OV-RLX-5T35H-treated group from 27% in the OV-5T35H-treated group and 80% in the PBS-treated group. The reduction in metastasis correlated with increased animal survival, supporting that an engineered adenovirus expressing relaxin increases infectivity in the primary tumor, reduces metastases and improves survival.

To directly address the impact of MMPs on oncolytic virus activities, Schäfer et al. (2012) incorporated matrix metalloproteinase 9 (MMP-9) into the oncolytic vaccinia virus strain GLV-1h68, creating the new strain GLV-1h255. Tumor specificity of the original GLV-1H68 virus, also known as GL-ONC1, is conferred by removal of viral genes (specifically, F14.5L, thymidine kinase J2R and hemagglutinin A56R) and consequential dependence on tumor associated cellular processes. The addition of MMP-9 to GLV-1H68 did not change virus infectivity *in vitro* but improved tumor

regression and virus titers in tumors in the PC-3 xenograft tumor model of prostate cancer. MMP-9 expression and collagen reduction were validated in primary PC-3 tumors. Intriguingly, when volumes of lumbar and renal lymph node metastases were evaluated, there were no differences between the GLV-1h68- and GLV-1H255-treated mice, suggesting that the addition of MMP-9 did not alter the metastasis-reducing effect of GLV-1h68 or, alternatively, that the increased virus mobilization was counterbalanced by the increased mobility of the tumor cells. It would be interesting to see the effects of GLV-1H255 relative to GLV-1h68 in a tumor model that differs in ECM composition or response to MMP-9. Moreover, as with all viruses discussed in this review, it would be highly informative to compare relaxin to MMP-9 in the same oncolytic virus and model system and establish if these genetic modifications produce overlapping or distinct contributions to oncolytic mechanisms.

As an alternative strategy to increase tissue permeability, Martínez-Vélez et al. (2016) introduced hyaluronidase into an oncolytic adenovirus with intentions to hydrolyze the ECM constituent hyaluronan. This adenovirus strain VCN-01 contains modifications to the viral genome that produce a more specific and powerful virus (E2F-binding motif in the E1A promoter, a modified fiber and a 24-bp deletion in the E1A gene). Moreover, VCN-01 also encodes the human PH20 gene that encodes soluble hyaluronidase and a modified fiber protein designed to increase virus half-life in blood. When administered systemically, VCN-01 reduced lung metastases by 20% in a lung metastatic osteosarcoma xenograft model (human 531 MII osteosarcoma cells injected through tail vein) relative to PBS control. It would have been worthwhile however to evaluate the contribution of each individual change in VCN-01 on reducing metastases, by comparing adenoviruses with these single and combined genomic modifications. Such studies would help suggest which of the four modifications are worth inclusion in both adenovirus and other virus-based oncolytic therapies.

Non-apoptotic cell death produced within tumors releases large fragments of DNA to the ECM (Kroemer et al., 2013; Tedcastle et al., 2016), which could also impede OV dissemination. In Tedcastle et al. (Tedcastle et al., 2016), the authors inserted the gene encoding the exonuclease DNAse I into the oncolytic adenovirus Enadenotucirev [EnAd, previously described in section A1 (Kuhn et al., 2008)], to eliminate free DNA and enhance virus spread. They also included a EnAd-based virus armed with hyaluronidase as a control (EnAdPH20). Viruses were intratumorally injected at a relative low dose [1×10^9 viral particles (vp)/tumor] to be able to observe the increase in virus spread. In the DLD human colon carcinoma xenograft model, EnAdDNAse and EnAdPH20 viruses significantly inhibited tumor growth relative to PBS or unmodified virus. Virus replication and spread in the tumors 32 days post infection was higher with EnAdDNAse than with EnAd or EnAdPH20. Also, at 32 days post infection, tumors conserved enzymatic activity suggesting a persistent expression of the virus-encoded enzymes. This research exemplifies the value of comparing two different engineering approaches to increase virus spread in the same platform, since it clearly suggests the advantage of DNAse as an additional modification.

With respect to genetically modifying OVs to express ECM-degrading enzymes as a strategy to increase OV dissemination, one should consider the impact of such enzymes on tumor progression as well. ECM and MMPs play many roles in promoting cancer development; they regulate tumor growth, apoptosis, angiogenesis, invasion, metastasis as well as the anti-tumor immune response (Egeblad and Werb, 2002). In fact, inhibition of MMPs is the basis of several anticancer therapies (Winer et al., 2018). Accordingly, it is critical to ask whether expressing MMPs in OVs as a strategy to enhance OV dissemination would also come with negative consequences to cancer development. The balance between increasing virus dissemination versus inadvertently increasing cancer progression can be very difficult to achieve, and we look forward to future studies that also evaluate cancer parameters such as angiogenesis and cancer cell invasion as possible unwanted consequences of expressing ECM-degrading enzymes.

While the studies described above focused mostly on the ECM as a physical barrier to virus dissemination, our laboratory wondered if ECM might also directly impact the infectious activity of an oncolytic virus. As described in section A1, reovirus (T3wt) shows inherent specificity towards tumors with limited replication in healthy tissues (Duncan et al., 1978; Coffey et al., 1998; Norman et al., 2004). Reovirus naturally infects through the enteric tract, but infections are rapidly cleared by the immune system with little-to-no symptom (Organ and Rubin, 1998). In the enteric tract, reovirus exploits gut proteases to augment infection, so in Fernandes et al. (2019), we wondered what effect, if any, breast tumor proteases could have on reovirus infectivity. We discovered that breast tumor extracts decreased reovirus infectivity by 100-fold by cleaving reovirus cell attachment proteins and decreasing attachment of reovirus particles to breast tumor cells. Specifically, a zinc-dependent metalloprotease released by breast cancer cells was responsible for the inactivation of reovirus. To overcome this restriction, we created a reovirus with a single mutation in the protease cleavage site of the reovirus cell-attachment protein σ1 (T249I); this mutant retained attachment to breast tumor cells despite MMP presence. Future studies are necessary to determine if the T249I mutation in reovirus, by overcoming negative effects of MMPs, also promotes oncolytic activities in models of cancer metastasis. Importantly however, in contrast to the strategies described above for adenovirus- and vaccinia- based OVs that increase MMP activities to promote OV dissemination, our findings with reovirus beckon a consideration for decreasing MMP activities as a strategy to increase activities of OVs that are negatively impacted by such host enzymes. In other words, a precise understanding of the direct relationship between a specific OV and ECM-modifying enzymes in tumors seems necessary to make the most beneficial genetic modifications to OVs.

ENGINEERING OVS TO REDUCE TUMOR BURDEN BY ALTERING ANGIOGENESIS

The genetic modifications described in section A focus on enhancing the replication, killing, and dissemination of OVs,

TABLE 3 | Oncolytic viruses that inhibit angiogenesis and alter tumor signaling.

Virus backbone	New virus	Modification	Reason	Result	Reference
VV[a]	OVV-CXCR4-A-mFc	Addition of the N terminal region of CXCL2 that functions as CXCR4 antagonist	To block CXCR4 and stop cancer development	Reduced metastasis in mouse breast and ovarian tumor models	(Gil et al., 2013, 2014)
HSV[b]	34.5ENVE	Addition of Vasculostatin-120	To inhibit tumor vascular-ization	Prolonged survival and reduced metastasis in ovarian and breast tumor mouse models	(Bolyard et al., 2014; Meisen et al., 2015)
Sendai virus	rSeV/dMFct14 (uPA2) or BioKnife	Fusion protein modified to be cleaved by uPA and not trypsin	Selective killing in uPA-expressing cells	Reduced tumor burden in a mesothelioma mouse model cancer and reduced secondary tumor growth in a head and neck carcinoma model	(Morodomi et al., 2012; Tanaka et al., 2019)
Adenovirus	Ad.sTβRFc	Addition of a soluble form of TGF-β receptor II fused with human immunoglobulin Fc fragment	Inhibition of TGF-β signaling	Decreased bone metastasis and prolonged survival in mouse and prostate breast cancer bone metastatic tumor models	(Hu et al., 2010, 2011, 2012; Zhang et al., 2012)
Adenovirus	Ad.dcn	Addition of human decorin	Activation of anti-tumorigenic signaling pathways	Reduced tumor progression, prolonged survival and decreased bone and lung metastasis in breast cancer bone metastatic models	(Xu et al., 2015; Yang et al., 2015; Zhao et al., 2019)
Adenovirus	rAd.DCN.GM	Addition of human decorin and granulocyte macrophage colony stimulating factor (GM-CSF)	Activation of anti-tumorigenic signaling pathways and immune system (natural killer cells, macrophages and dendritic cells)	Reduced tumor growth and pulmonary metastasis in a mouse colorectal cancer	Liu et al. (2017)
Adenovirus	Ad5/3-D24-hTNFa	Addition of human TNFα	Activation of apoptosis	Reduced tumor growth in a xenograft mouse model of prostate cancer and a metastatic mouse melanoma model	Hirvinen et al. (2015)
Adenovirus	Ad5/3-E2F-d24-hTNFa-IRES-hIL2	Addition of human TNFα and human IL-2	Activation of apoptosis and induction of anti-tumor immunity	Reduced tumor growth in a Syrian hamster model	Havunen et al. (2017)
Adenovirus	Ad.IR-E1A/TRAIL	Addition of TNF-related apoptosis-inducing ligand (TRAIL)	Activation of apoptosis	Reduced colorectal metastases in the liver in a mouse model	Sova et al. (2004)
Adenovirus	P55-HTERT-HRE-TRAIL	Addition of TRAIL to virus with E1A controlled by the hTERT promoter and E1B controlled by a hypoxia response element	Increase tumor specificity of virus replication and apoptosis activation	Prolonged survival and decreased metastasis in a mouse metastatic breast tumor model	Zhu et al. (2013)
Adenovirus	Ad/TRAIL-E1	Addition of TRAIL, with TRAIL and E1A under the control of the hTERT promoter	Increase tumor specificity of virus replication and apoptosis activation	Reduced metastasis in a peritoneal dissemination mouse tumor model; increased apoptosis in the metastases	Zhou et al. (2017)
Adenovirus	M4	Addition of a fragment of antisense STAT3 to the backbone adenovirus Ad5/dE1A	Silencing of transcription factor STAT3	Decreased tumor growth, invasiveness, and peritoneal dissemination in an orthotopic mouse model of gastric cancer	Han et al. (2009)
Adenovirus	ZD55-SATB1	Addition of SATB1 shRNA	Silencing of transcription factor SATB1	Decreased primary tumor growth and inhibited pulmonary metastasis in a metastatic prostate cancer model	Mao et al. (2015)
VV[a]	OVV-BECN1	Addition of Beclin-1	Activation of autophagy	Reduced tumor growth in xenograft murine models of leukemia and non-Hodgkin lymphoma	(Lei et al., 2020; Xie et al., 2021)
NDV[c]	rNDV-18HL	Addition of an antibody against CD147	Blocking of CD147	Reduced liver metastasis and prolonged survival in an orthotopic mouse hepatoma model	Wei et al. (2015)
Adenovirus	Ad.wnt-E1A (delta24bp)-TSLC1	Addition of TLSC1; Viral protein E1A expression under the control of Wnt promoter	Cancer stem cell specificity of virus replication and increasing expression of TLSC1	Reduced liver metastasis in a hepatocellular carcinoma mouse model	Zhang et al. (2017)

[a]*VV, vaccinia virus.*
[b]*HSV, herpes simplex virus.*
[c]*NDV, newcastle disease virus.*

so that OVs might exhibit increased direct oncolytic activities in tumors and secondary sites of metastasis. But during their habitation in tumors, OVs have the potential to also deliver exogenous genes that indirectly contribute to cancer treatment. In this section, we will discuss OVs (summarized in **Table 3**) genetically modified to express factors that modulate angiogenesis.

Endothelial cells (ECs) are main components of blood vessels and important elements of the tumor microenvironment because they supply the nutrients and oxygen requirements of growing tumors. Angiogenesis, the process of creating new blood vessels, is therefore fundamental for cancer development and a common target for cancer therapy (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003; Potente et al., 2011; Mander and Finnie, 2018). Angiogenesis is regulated by soluble factors such as the C-X-C chemokine ligand 12 (CXCL12) and its receptor type 4 (CXCR4) (Guo et al., 2016; Najafi et al., 2019). While CXCL12 is mainly secreted by cells associated with the tumor microenvironment, CXCR4 is expressed by ECs, cancer cells and cancer stem cells (Cornelison et al., 2018; Yi et al., 2019). CXCL12/CXCR4 signaling promotes an immunosuppressive environment, ECM remodeling, reprogramming of tumor cells, tumor angiogenesis, and metastasis (Mortezaee, 2020). In particular, the role of CXCL12/CXCR4 in angiogenesis is well described. ECs in the tumor microenvironment overexpress CXCR4 in response to hypoxia (Schioppa et al., 2003) and the secretion of CXCL12 by tumor cells and cells in the tumor microenvironment, recruits ECs into the tumor (Salcedo and Oppenheim, 2003). CXCL12 secretion also influences the transformation of tumor cells to mimic blood vessels (Yang et al., 2016). Importantly, the blocking of CXCL12/CXCR4 axis inhibits tumor growth and impairs metastasis (Sun et al., 2013; Zhou et al., 2019a).

CXCR4 blocking has been evaluated in several clinical trials as a strategy to reduce cancer development, but since this chemokine is abundantly expressed at both tumor and non-tumor sites, CXCR4 blockade specifically in tumors can be difficult. To resolve the specificity issue, the Kozbor group in Gil et al. (2013) designed a tumor-selective vaccinia virus expressing the N terminal region of CXCL12 that functions as a CXCR4 antagonist (OVV-CXCR4-A-mFc). Specifically, they used the vaccinia Western Reserve strain with thymidine kinase (TK) and vaccinia growth factor (VGF) genes interrupted to make the virus tumor specific. They incorporated either EGFP or CXCR4-A-mFc into the TK locus. The efficacy of the viruses to target metastasis was then evaluated in the syngeneic mouse 4T1 breast tumor model. 4T1 cells were orthotopically implanted and when cells were disseminated to the lungs, virus was injected intravenously. Histologic analysis showed that the control group had an average of 20 metastatic nodules in the lungs, whereas OVV-EGFP- and OVV-CXCR4-A-mFc-treated animals had 6.6 and 2.6 metastatic colonies, respectively. They also evaluated the efficacy of the OVV-CXCR4-A-mFc when it was administered before or after excision of the primary tumor. For the pre-operative setting, mice were injected with 4T1 cells then 10 days later, virus was injected. Primary tumors were resected 8 days after virus injection. The OVV-CXCR4-A-mFc-treated group showed higher survival compared with control and OVV-EGFP. In the post-operative setting, tumors were resected 18 days after cell injection and then viruses were injected. In this experiment, OVV-CXCR4-A-mFc-treated mice survived longer than control and OVV-EGFP-treated mice. More importantly, survival for OVV-CXCR4-A-mFc group was longer in the post-operative setting than the pre-operative setting (42% vs. 20% disease-free after 110 days). These studies suggest that injecting viruses after tumor resection was more efficient at targeting metastases, and that the addition of the CXCR4 agonist promoted the oncolytic activities of vaccinia virus. The Kozbor group observed similar results in a syngeneic metastatic model of ovarian cancer (ID8-T cells, which are derived from ascites of ID8 tumor-bearing mice) (Gil et al., 2014). They associated the increased survival following OVV-CXCR4-A-mFc treatment with a reduction of CXCL12 and VEGF as well as cancer-initiating, endothelial, myeloid and plasmacytoid dendritic cells in the tumor microenvironment. They also detected increased activated T cell infiltration and anti-tumor immune response.

In addition to chemokines, there are many factors that control vascularization of specific tissues. Brain-specific angiogenesis inhibitor 1 (BAI1) is an orphan G protein-coupled receptor that is cleaved extracellularly to release a 120 kDa fragment called Vasculostatin-120, which inhibits endothelial cell migration, proliferation, and tube formation (Kaur et al., 2005). In Bolyard et al. (2014), the authors created an oncolytic HSV that expressed Vasculostatin-120, called 34.5ENVE. When injected intraperitoneally in a murine xenograft model of disseminated peritoneal ovarian cancer, 34.5 ENVE prolonged survival from 49 to 63 days relative to the virus control, and reduced tumor burden as measured by bioluminescence imaging. Likewise, the presence of intraperitoneal metastases and ascites at time of death was diminished with the 34.5 ENVE treatment to 25% (2/8 mice with metastasis) from 50% (4/8) with the virus control and 100% with PBS (8/8). In Meisen et al. (2015), 34.5 ENVE was then tested in the breast cancer brain metastasis (BCBM) model, where breast tumor cells Met-1 or DB-7 were injected into the brains of mice. Intratumoral injection of 34.5 ENVE decreased tumor growth and improved survival compared to untreated controls in both models. However, unlike the Bolyard et al. (2014) study, 34.5 ENVE was not compared with a control oncolytic virus in the BCBM models, and therefore it remains to be seen if expression of Vasculostatin-120 provided an important improvement to oncolytic potency.

Anti-angiogenic therapies have been promising since their discovery because angiogenesis is practically absent in normal tissues (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003), so a therapy targeting it can be very specific for cancer. However, their use has not shown the expected success (Roukos et al., 2009; Ferrara and Adamis, 2016). For example, anti-angiogenic drugs such as anti-CXCL12 or Vstat120, can increase tumor hypoxia and necrosis which stimulates the secretion of pro-angiogenic factors and therefore promotes tumor growth (Potente et al., 2011). Furthermore, the abundance of pro-angiogenic factors in the tumor

microenvironment favours resistance to anti-angiogenic drugs. In both cases, the dose and administration frequency will be very important (Mander and Finnie, 2018). It will be important to see if OVs that modulate angiogenic factors also come with undesirable consequences. Moreover, since CXCL12 has several functions promoting tumor growth and metastasis besides its pro-angiogenic role (Mortezaee, 2020), it is possible that inhibiting CXCL12 comes with secondary benefits for tumor reduction beyond angiogenesis reduction, and may serve as a better target.

OV MODIFICATIONS THAT ALTER TUMOR CELL SIGNALING

There are several factors that modulate tumor growth, invasion and metastasis, such as adhesive signals from the ECM, mechanical pressures from the ECM, cell to cell interactions, microbiome as well as soluble signals (growth factors and cytokines) (Fares et al., 2020). In the following section, we will describe different cellular signaling pathways that have been modified or exploited by OVs (summarized in **Table 3**) with the objective of reducing tumor growth and metastasis.

Urokinase Plasminogen Activator and its Receptor

One of the protease systems that participates in the ECM disassembly process to promote invasion, migration and metastasis is the urokinase plasminogen activator-urokinase plasminogen activator receptor (uPA-uPAR) system (Pillay et al., 2007). uPA is a serine protease that converts plasminogen to plasmin, which participates in the degradation of fibrin, blood clotting factors and ECM (Mahmood et al., 2018). The uPA-uPAR system is overexpressed in several cancers, and its inhibition leads to tumor regression and metastasis reduction in animal models (Pillay et al., 2007). Transgenes that exploit the uPA-uPAR system have also been explored as potential boosters of oncolytic virus activity.

The authors in Morodomi et al. (2012) took advantage of the increased expression of uPA in cancer cells in designing a novel recombinant Sendai virus that had uPA-specific cell-cell fusion killing activity [rSeV/dMFct14 (uPA2) or BioKnife]. One of the modifications in this virus is that the trypsin-dependent cleavage site of the fusion (F) gene is manipulated to be susceptible to uPA and not trypsin, so that killing would be specific to uPA-expressing cells. They established an orthotopic xenograft model of human malignant mesothelioma by injecting H226-luc cells into the right thoracic cavity of nude mice. Seven days after tumor cell injection, they intrapleurally injected BioKnife-GFP or the control virus rSeV/dM-GFP. *In vivo* bioluminescence imaging demonstrated that BioKnife-GFP significantly reduced tumor burden at 7 and 14 days relative to control virus. They detected virus by GFP expression in the tumor at 7 days post-infection that correlated with increased apoptosis in the BioKnife-GFP-treated group. In Tanaka et al. (2019), BioKnife was further evaluated in a murine orthotopic head and neck squamous cell carcinoma syngeneic model where the head and neck squamous cell carcinoma (HNSCC) cell line SCCVII were injected into the floor of the mouth at day 0. Virus treatments were administered intratumorally at days 1, 2, 3 and 4. At day 4, HNSCC cells were inoculated into the subcutaneous region of the left flank to simulate metastasis. While BioKnife-GFP did not have notable effects on the primary tumor, this OV considerably reduced secondary tumor growth relative to virus control and increased $CD8^+$ lymphocyte infiltration in the secondary tumor. However, more experiments are needed to totally dilucidated the role of immune cell activation in BioKnife's mechanism.

Transforming Growth Factor β

In the complexity of the tumor microenvironment, many molecules that participate actively in the invasion, migration and metastatic processes display complex regulatory circuits. For example, uPA/uPAR and MMPs activate the latent form of transforming factor β (TGF-β), while TGF-β regulates the expression of uPA and MMPs in cancer cells (Annes et al., 2003; Santibanez et al., 2018). TGF-β is a key cytokine in all stages of cancer development. At the beginning, it acts as tumor suppressor promoting growth arrest and apoptosis of malignant cells. Later, it functions as a tumor promoter activating cell growth, angiogenesis, EMT, metastasis, anti-tumor immune evasion and chemotherapy resistance (Hao et al., 2019). TGF-β binds and elicits its effects through TGF-β type I and type II receptors (TβRI and TβRII) that possess serine/threonine kinase activity. Several signaling pathways are activated via TGF-β, including the canonical SMAD, MAPK, RHO-like GTPase and PI3K/AKT pathway (Hao et al., 2019).

The Seth group combined the oncolytic power of adenovirus with inhibition of TGF-β signaling to generate Ad. sTβRFc, a replicating adenovirus in which the cytomegalovirus immediate early (CMV) promoter drives expression of a soluble form of TGF-β receptor II fused with human immunoglobulin Fc fragment (sTGFβRIIFc). Hu et al. (2010) tested this recombinant adenovirus in a bone metastatic xenograft tumor model established by intracardiac injection of human MDA-MB-231 breast cancer cells. Virus was injected *via* tail vein on days 4 and 7. The authors observed a significant decrease in bone metastases evaluated by X-ray and immunohistochemistry in mice treated with Ad. sTβRFc relative to virus control (Ad.luc2). Analysis of calcium levels in blood revealed reduced hypercalcemia with Ad. sTβRFc compared to virus control, indicating that the virus inhibited bone metastases and osteolytic bone destruction. Further studies (Hu et al., 2011) using *in vivo* imaging confirmed that Ad. sTβRFc decreased metastasis and prolonged survival relative to virus control. Ad. sTβRFc also reduced metastatic tumor burden relative to control virus in the immune competent 4T1-luc2 bone metastatic breast cancer model (Zhang et al., 2012). The efficacy of treating metastatic prostate cancer with this modified adenovirus was also tested by the Seth group (Hu et al., 2012). Bone metastases were established by intracardiac injection of nude mice with PC-3-luc cells prior to intravenous injection with Ad. sTβRFc or control virus. By whole-body bioluminescence imaging they

found that Ad. sTβRFc reduced tumor growth most efficiently than virus control. Similar to results with the human breast tumor model, Ad. sTβRFc inhibited hypercalcemia and growth of prostate cancer metastases in the bone. Taking the Seth group's publications together, inhibition of TGF-β signaling by Ad. sTβRFc seems to provide advantage in treating prostate and breast cancers that metastasize to the bone.

Decorin

Although pro-tumorigenic signals are expected in the tumor environment, some anti-tumorigenic molecules such as decorin can be detected. Decorin belongs to the small leucine-rich proteoglycan family of proteins and is a component of the ECM (Sofeu Feugaing et al., 2013). In the matrix, decorin acts as anti-tumorigenic agent by repressing signal transduction pathways such as cell proliferation, angiogenesis, and migration (Neill et al., 2012; Sofeu Feugaing et al., 2013; Zhang et al., 2018).

The Seth group, with the focus on manipulating signaling pathways involved in cancer progression, created an adenovirus expressing human decorin (Ad.dcn) (Xu et al., 2015). The authors evaluated the activity of Ad. dcn in the same xenograft metastatic PC-3-luc prostate cancer mouse model used in Hu et al. (2012) using *in vivo* bioluminescence, X-ray, and micro-computed tomography to monitor tumor burden. They observed that Ad. dcn significantly inhibited tumor progression, decreased bone destruction and prolonged survival relative to the virus control without decorin (Ad.luc). Similarly, Yang et al. (2015) showed that Ad. dcn inhibited growth of bone metastases in a xenograft MDA-MB-231 breast cancer mouse model. More recently, Zhao et al. (2019) explored the ability of Ad. dcn to inhibit pulmonary metastasis in the highly aggressive syngeneic 4T1-luc orthotopic mouse model. When mammary tumors were palpable (~7 days) and at day 10, viruses were injected either intratumorally or intravenously. Lung metastases were analyzed at day 25 by histopathological assays. They determined that intratumoral or intravenous deliveries of Ad. dcn reduced tumor growth and pulmonary metastases, increasing the frequency of lung metastasis-free-mice relative to virus control (Ad.Null). However, intratumoral injections were more effective at reducing primary tumor growth and expressing the transgene, whereas intravenous delivery was more successful at preventing lung metastases. Decorin target genes were downregulated in the tumor as well as in the metastases, indicating a direct activity of virus-derived decorin on cell signalling. It would be interesting to establish if decorin activities at metastases are from direct virus replication at metastatic sites or via circulation. Should decorin (or any virus-derived cytokine) be found in circulation, it would be necessary to ensure that the levels do not negatively affect healthy tissues. Furthermore, Ad. dcn treatment, systemically or intratumorally, induced an upregulation of $CD8^+$ T cells in peripheral blood.

To boost the innate immune response, decorin was combined with granulocyte macrophage colony stimulating factor (GM-CSF), an immune stimulator of natural killer cells, macrophages, and dendritic cells in an oncolytic adenovirus (Liu et al., 2017). In this virus (rAd.DCN.GM), decorin was expressed under control of the CMV immediate early promoter, while GM-CSF expression was driven by the E1B promoter. Cancer-specific virus replication was controlled by placing the TERT promoter upstream of E1A. Using the CT26 xenograft model of colorectal cancer, the authors demonstrated that intratumoral injection of virus (rAd.DCN, rAd.GM, or rAd.DCN.GM) significantly decreased tumor volume relative to treatment with rAd.Null and mock. When pulmonary metastases were analyzed, 5/6 mice were tumor-free in the rAd.DCN.GM group, 4/6 mice were tumor-free in the rAd.GM and rAd.DCN groups, while only 2/6 mice were tumor-free in the rAd.Null-treated group. rAd.DCN.GM increased $CD8^+$ T cells in spleen and peripheral blood, reduced TGF-β expression and augmented dendritic cells in the spleen, suggesting that both decorin and GM-CSF contribute to rAd.DCN.GM mechanisms of action.

TNF-α/TRAIL

Programmed cell death or apoptosis can be beneficial for oncolytic therapy because it does not only kill the tumor cell but also releases tumor antigens that stimulate the anti-tumor immune response (Zhou et al., 2019b). Tumor necrosis factor alpha (TNFα) is a cytokine produced by immune cells such as macrophages and monocytes that, besides its role inducing apoptosis and necrosis, can regulate inflammation, growth, and proliferation of normal and transformed tissues (Atzeni and Sarzi-Puttini, 2013; Fitzgerald et al., 2013). The localized production of this cytokine by oncolytic viruses can be very beneficial by preventing systemic toxicity. Consequently, TNFα coding sequences have been added to an oncolytic adenovirus with an Ad5/3 chimeric capsid and a 24 bp deletion in the constant region 2 of E1A to make virus replication selective for tumor cells with a defective retinoblastoma/p16 pathway. Armed virus Ad5/3-D24-hTNFa produced TNFα in tumors, reduced tumor growth and improved survival relative to control virus in a PC-3 MM2 xenograft murine model of prostate cancer. It also reduced tumor growth and increased tumor specific CD8 T cells in a metastatic B16-OVA immunocompetent murine model of melanoma (Hirvinen et al., 2015), although it should be noted that human adenovirus does not replicate in murine cells. Later, the same group tested the armed virus in combination with another anti-tumor inflammatory cytokine IL-2 (Havunen et al., 2017). The new armed virus Ad5/3-E2F-d24-hTNFa-IRES-hIL2 (or OAd.TNFa-IL-2) with the transgenes incorporated into the E3 region, showed a significant reduction in tumor growth in an HapT1 immunocompetent Syrian hamster model relative to control unarmed virus (OAd). OAd.TNFa-IL-2 virus also increased CD4/CD8 T cell infiltration in the tumor microenvironment (Havunen et al., 2017).

Three research groups have introduced TNF-related apoptosis-inducing ligand (TRAIL) to adenovirus OVs to increase apoptosis of tumoral cells. In its native form TRAIL is a transmembrane protein that binds to death receptors DR4 and DR5 to induce extrinsic apoptosis (Yuan et al., 2018), although most therapeutic agents incorporating TRAIL use an engineered soluble form of the protein. Soluble TRAIL has the advantage of acting on uninfected tumor cells near the site of

injection. In Sova et al. (2004), the authors created an Ad5/35 fiber-substituted oncolytic adenovirus that infects cells independently of the coxsackievirus and adenovirus receptor (CAR) and instead enters via CD46 which is highly expressed in malignant tumor cells. The TRAIL transgene was inserted into this virus, creating the oncolytic vector Ad. IR-E1A/TRAIL. The viruses were tested in the xenograft model of liver metastasis generated by infusing human LoVo colorectal carcinoma cells via the portal vein into immunodeficient CB17 mice. Two weeks after two sequential intravenous injections of virus, mice were euthanized and evaluated for liver metastases. The authors found that administration of Ad. IR-E1A/TRAIL reduced tumor burden 10-fold relative to untreated mice and approximately 2-fold relative to the virus control Ad. IR-E1A/AP (without TRAIL), without causing toxicity to the liver.

In an independent effort to apply TRAIL towards enhancing adenovirus-based OV potency, Zhu et al. (2013) combined two modifications of the E1 region of adenovirus to increase the specificity of virus replication to tumor cells: placing adenoviral genes E1A and E1B under the control of hTERT promoter and HRE (hypoxia response element) respectively. Into this viral backbone they incorporated a TRAIL expression cassette driven by the CMV promoter, creating P55-HTERT-HRE-TRAIL. This TRAIL-expressing virus decreased tumor growth in a mouse xenograft orthotopic model of breast cancer (MDA-MB-213 cells). Higher levels of apoptosis measured as TUNEL staining were found in P55-HTERT-HRE-TRAIL treated tumors versus virus control (P55-HTERT-HRE). When the virus activity was evaluated in a simulated model of metastasis (MDA-MB-231-luc injected into the left heart ventricle), P55-HTERT-HRE-TRAIL-treated group showed 60% survival at day 60 whereas the virus control group (P55-HTERT-HRE) showed only 20% survival. The reduction of metastases was confirmed with *in vivo* imaging every 7 days.

Lastly, Zhou et al. (2017) developed an adenovirus expressing TRAIL and viral E1A under control of the tumor-specific hTERT promoter. They evaluated the effect of intraperitoneal injection of Ad/TRAIL-E1 on metastasis in an *in vivo* MKN45 cell peritoneal carcinomatosis xenograft mouse model. They found that Ad/TRAIL-E1 significantly reduced the number of mesentery tumors (22.8 ± 10.3) relative to the virus control (Ad/GFP-E1, 65.3 ± 34.4). Ad/TRAIL-E1 also reduced tumor weight and increased survival relative to Ad/GFP-E1 although the difference was not statistically significant. The expression of TRAIL and the level of apoptosis in the disseminated tumors was higher in the Ad/TRAIL-E1-treated group than in the virus control group.

Gene Expression Regulators: STAT3 and SATB1

The examples of genetic modifications of OVs discussed above focus on blocking ligand-receptor interactions. Other groups have modified OVs to targeted molecules that are downstream of receptors in various signaling pathways. One such target is the transcription factor STAT3 which is downstream of cytokine and growth factor receptors. This factor is involved in the regulation of autonomous properties of tumor cells such as proliferation as well as communication with other cells in the tumor microenvironment, resulting in increased vascularization, migration, invasion, and immunosuppression (Groner et al., 2008). Han et al. (2009) modified an oncolytic adenovirus to inhibit STAT3 by inserting a 770 bp antisense fragment of STAT3 into the ADP locus of Ad5/dE1A, previously generated with a deletion of amino acids 121–129 in E1A. They evaluated the resulting virus, M4, for its ability to inhibit metastasis in an orthotopic model of gastric cancer established using explanted MKN-45 xenograft tumor fragments (Huang et al., 2008). Viruses were injected into the tail vein for five consecutive days, then 6 weeks later mice were assessed for tumor growth and metastases. The authors observed that M4 prolonged survival and decreased tumor growth, invasion of the liver, and peritoneal dissemination compared to control virus without the STAT3 antisense sequence. Importantly, M4 also decreased STAT3 expression in tumors. As STAT3 is involved in immunosuppression, it would be interesting to examine the activity of this virus in an immune competent mouse model as well.

Another key regulator of tumor progression and metastasis is the transcription factor SATB1. SATB1 belongs to the SATB (Special AT-rich Binding protein) family. These proteins are high-order chromatin organizers, and histone and post-translational modifiers (Naik and Galande, 2019). STAB1 is highly expressed in numerous malignancies, including breast, prostate, liver, and bladder cancers. In addition, SATB1 promotes a highly aggressive phenotype due to its role activating the EMT process that leads to metastasis and invasion (Glatzel-Plucinska et al., 2019). In order to silence this important tumorigenic factor, Mao et al. (2015) constructed the virus ZD55-SATB1, in which the E1B-55K sequence was replaced with a SATB1-targeted shRNA expression cassette. The authors evaluated ZD55-SATB1 in the subcutaneous DU145 prostate cancer model. ZD55-SATB1 inhibited growth of primary tumors and lung micrometastases. Histopathological analyses of tumors revealed that ZD55-SATB1 inhibited expression of SATB1 and induced a higher level of apoptosis than the virus control (ZD55-EGFP).

Beclin-1

Cell death can be the result not only of apoptosis, but also of other processes such as autophagy. The induction of autophagy has been explored by some groups introducing Beclin-1 to VV. Beclin-1's phosphorylation regulates the initiation of autophagy, facilitating the recruitment of autophagic proteins and autophagosome biogenesis (Menon and Dhamija, 2018). The OVV-BECN1 was created in a VV backbone with a TK viral gene deletion for tumor selectivity. OVV-BECN1 induced cell death through autophagy and not apoptosis in hematologic malignant cells *in vitro*. On the other side, OVV-BECN1 reduced tumor growth and increased survival significantly in a K62-luciferase cells xenograft murine model of leukemia. Presence of Beclin-1 and autophagic vacuoles were found in the OVV-BECN1 treated tumors by IHQ and electron microscopy respectively (Lei et al., 2020). OVV-BECN1 also decreased tumor growth in a murine non-Hodgkin lymphoma xenograft model (Xie et al., 2021).

CD147

Various molecules are upregulated on the surface of tumor cells to support cancer progression, including CD147, a glycoprotein involved in regulation of the tumor microenvironment and tumor growth. CD147 induces the expression of MMPs and the uPA/uPAR system promoting invasion and metastasis. In addition, CD147 regulates tumor cell adhesion and angiogenesis (Iacono et al., 2007; Landras et al., 2019). Strategies have been developed to block CD147 activity because of its important role in cancer progression (Iacono et al., 2007; Landras et al., 2019). Wei et al. (2015) used reverse genetics to construct a recombinant Newcastle disease virus (NDV) expressing an antibody against CD147 (rNDV-18HL). They tested rNDV-18HL in the SMMC-7721 orthotopic hepatoma model. Starting 1 week after implantation, viruses were intravenously injected twice weekly for 3 weeks. Virus replication and anti-CD147 antibody were detected at the tumor site by immunohistochemistry. Furthermore, mice treated with rNDV-18HL showed a significantly reduced number of intrahepatic metastases and prolonged survival relative to virus control (NDV Italien). Future studies may demonstrate the utility of this novel approach using oncolytic viruses delivering therapeutic antibodies to the tumor site.

Tumor Suppressor TSLC1

Adhesion proteins and other molecules are important to maintain tissue structure and organization. In some cases, downregulation of these molecules in the tumor microenvironment can promote invasion and metastasis. This is the case of the tumor suppressor lung 1 (TSLC1) protein, a cell-cell adhesion protein that also functions intracellularly by interacting with several signaling proteins involved in tumorigenesis, supressing EMT and inducing apoptosis (Liang et al., 2011). The Wang group (Zhang et al., 2017) investigated whether oncolytic adenovirus delivery of TSLC1 specifically to cancer stem cells (CSCs) of hepatocellular carcinoma (HCC) could impact tumor progression. To do this, they created an adenovirus that encodes TSLC1 and placed expression of the viral delta-24 E1A protein (unable to bind Rb) under the control of Wnt promoter (Ad.wnt-E1A (Δ24bp)-TSLC1). Wnt signaling is highly activated in CSC supporting self-renewal ability and multi-differentiation potential (de Sousa E Melo and Vermeulen, 2016). To test Ad. wnt-E1A (delta24bp)-TSLC1 and its efficacy targeting CSCs *in vivo*, they established a tumor model by injecting subcutaneously MHCC-97H-luc spheres. When tumors reached 100 mm^3, test and control viruses were injected intratumorally. Tumor growth was monitored *in vivo* by bioluminescence imaging and showed a significant reduction in tumor burden in mice treated with Ad. wnt-E1A (Δ24bp)-TSLC1 compared to mice treated with the control (Ad.wnt-E1A (Δ24bp)-EGFP). In addition, the number of metastatic nodules were significantly reduced in Ad. wnt-E1A (Δ24bp)-TSLC1-treated mice. This study demonstrated that CSCs can be effectively targeted by oncolytic adenovirus, and that overexpression of the tumor suppressor TSLC1 may reduce metastasis.

Overall, this section describes that many of the signalling pathways involved in tumor invasion and metastasis processes are possible candidates for manipulation through oncolytic viruses that deliver exogenous genes to the tumors. So far, OV researchers have focused on manipulating signalling pathways that modulate the ECM and tumor microenvironment. Studies to date have concentrated on evaluating the overall change to primary tumor and metastatic burden, without in-depth analysis of relative virus burden and spread, or specific changes to tumor and tumor-supporting cells or immune cell populations. In future, approaches such as single-cell sequencing of tumor and metastatic samples could contribute immensely towards establishing the best ways to apply these modified OVs. For example, while blocking CD147 lead to reduced metastasis, it also reduced MMP expression which may inadvertently dampen dissemination of the OV as described in section B. Determining What then is the best balance of inhibiting CD147 versus encouraging MMP activity? It will be interesting to test the effect of re-introducing MMPs to CD147-inhibited conditions, to establish if virus dissemination is restored and survival further enhanced.

CONCLUSION

We have summarized and contextualized many approaches to genetically modify OVs to either support improved virus replication and spread, or to help dismantle the tumor microenvironment. The modifications described in this review were all able to improve oncolytic therapy, either by reducing primary tumor growth or metastasis. We have broadly categorized the advancements into those that (A) promote virus replication in tumors and/or death of tumor cells, (B) overcome the ECM barrier to virus dissemination within tumors or to metastatic sites, (C) reduce angiogenesis, and (D) stimulate cell signalling pathways to dismantle the tumor microenvironment or promote cell death (**Figure 1**). There have also been many genetic modifications to OVs aimed specifically at enhancing anti-tumor immunity, but these are already aptly described in complementary reviews (de Graaf et al., 2018; Jamieson et al., 2020; Zhang et al., 2020).

Remaining Challenges: While writing this review, we have noticed several general limitations that if overcome, could help further advance oncolytic viruses. First, most modifications to OVs have focused on DNA viruses such as adenovirus, HSV and vaccinia virus. The focus on DNA viruses is likely because these viruses have a large genome size and are more-easily manipulated. However, given that reverse genetics approaches for RNA viruses are rapidly advancing, it would be interesting to test some of the modifications described in this review in RNA viruses that possess oncolytic activity but lack sufficient oncolytic potency. For example, VSV, measles virus, NDV, coxsackieviruses and polioviruses are all being develop into oncolytic viruses and may benefit from some of the genetic modifications summarized in this review. Second, unfortunately some publications did not compare the modified virus with the control unmodified virus, making it difficult to determine the benefit of the specific genetic alteration. Third, each publication uses its own animal model, and therefore it is challenging to compare between

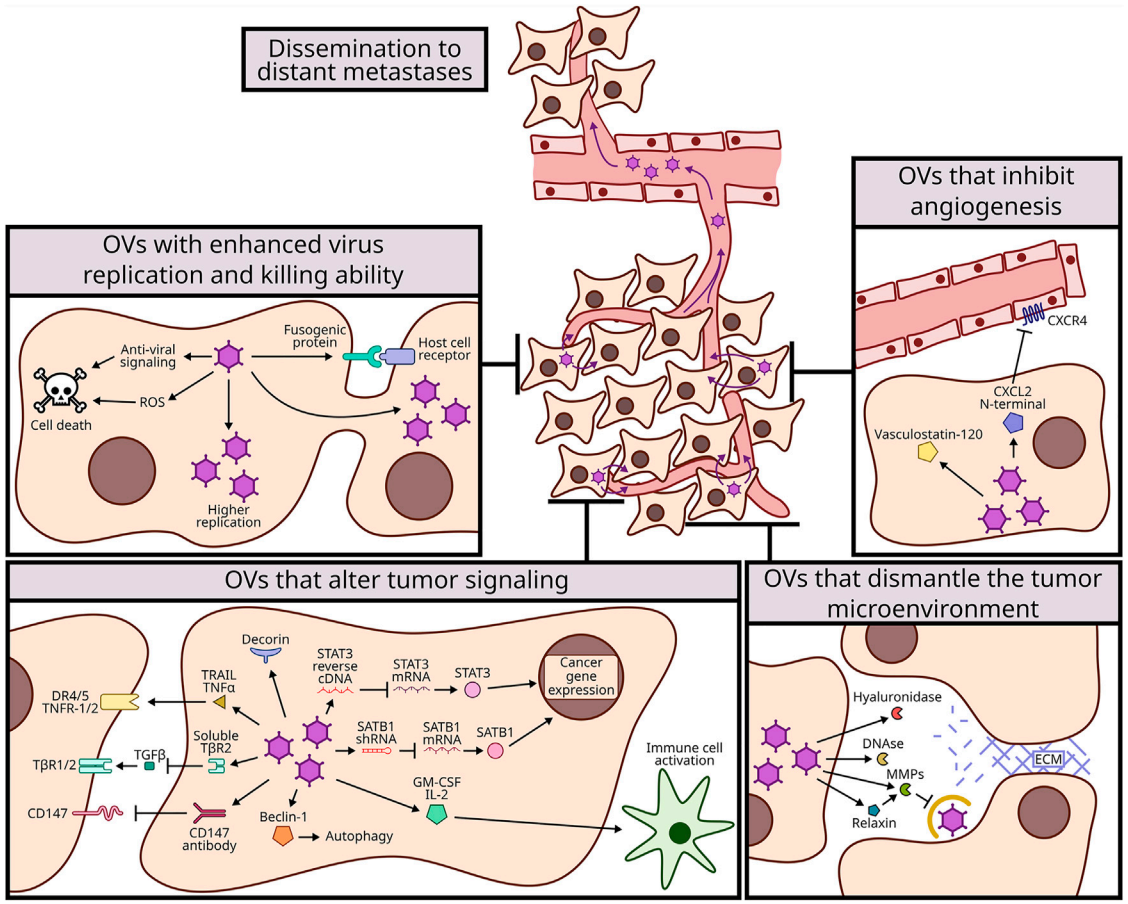

FIGURE 1 | Mechanisms of genetic modifications to improve oncolytic viruses (OVs) dissemination to distant metastases. OVs have been modified to expand their oncolytic potency and with that to spread more efficiently to metastatic sites. These modifications were categorized in 4 mechanisms: enhancing virus replication and killing ability (top left, section A and **Table 1**); dismantling the tumor microenvironment (bottom right, section B and **Table 2**); inhibiting angiogenesis (top right, section C and **Table 3**); and altering tumor signaling (bottom left, section D and **Table 3**).

models, OVs, and other standard therapies. It would be worthy to standardize and compare the best therapies in the same models with uniform protocols. Fourth, most studies focused on measurements of tumor size and metastatic burden, leaving many molecular insights unknown. For example, it was not always clear if the genetic modification of the virus functioned as anticipated to manipulate the desired molecular pathway or process. It was also not always clear the effect of modifications on virus amplification, tumor cell death, or anti-tumor immunity. In future, delineating the molecular details of the oncolytic viruses will allow best advancements to overcome remaining deficiencies in activities. Lastly, clinical testing is needed to fully evaluate the OVs described in this review, since responses of mice do not always predict responses in humans.

Hope for future: Although we have categorized the OV genetic modifications according to their dominant activity, the modifications are probably interconnected; for example, a modification that makes the OV more efficient at tumor cell killing is likely also to expose more tumor antigens and increase the anti-tumor immune response. As another example, modifying the CXCL12/CXCR4 signaling pathway to alter angiogenesis will also likely attract more immune cells that respond to this chemokine. As methods such as single-cell sequencing become more affordable, it will be very exciting to achieve a more wholistic view of the effects of each genetic modification to OVs.

When considering that each individual change described in this review made at least an incremental improvement to the activity of the oncolytic virus, it is very likely that combination of modifications could achieve the potency needed for durable cancer therapy. The trick will be to fully understand the mechanisms of each approach and the impact on virus, tumor, and immunity, so that combinations of genetic modifications have additive or ideally synergistic effects. If then considering that most modifications improved T cell infiltration, addition of checkpoint inhibitors to overcome immune suppression could further promote tumor-specific immunity. Ultimately, the optimal combination of genetically modified OVs, other cancer-targeting drugs, and tumor immunity-stimulating therapies will be achieved.

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FC: conception and design, literature research, writing, figure, review, and revisions. TG: figure, review, and revisions. MH: conception and design, review, and revisions. MS: conception and design, review, and revisions.

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Utility of a Recombinant HSV-1 Vaccine Vector for Personalized Cancer Vaccines

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Current approaches to cancer immunotherapy include immune checkpoint inhibitors, cancer vaccines, and adoptive cellular therapy. These therapies have produced significant clinical success for specific cancers, but their efficacy has been limited. Oncolytic virotherapy (OVT) has emerged as a promising immunotherapy for a variety of cancers. Furthermore, the unique characteristics of OVs make them a good choice for delivering tumor peptides/antigens to induce enhanced tumor-specific immune responses. The first oncolytic virus (OV) approved for human use is the attenuated herpes simplex virus type 1 (HSV-1), Talimogene laherparepvec (T-VEC) which has been FDA approved for the treatment of melanoma in humans. In this study, we engineered the recombinant oncolytic HSV-1 (oHSV) VC2-OVA expressing a fragment of ovalbumin (OVA) as a fusion protein with VP26 virion capsid protein. We tested the ability of VC2-OVA to act as a vector capable of stimulating strong, specific antitumor immunity in a syngeneic murine melanoma model. Therapeutic vaccination with VC2-OVA led to a significant reduction in colonization of tumor cells in the lungs of mice intravenously challenged B16cOVA cells. In addition, VC2-OVA induced a potent prophylactic antitumor response and extended survival of mice that were intradermally engrafted with B16cOVA tumors compared with mice immunized with control virus.

Keywords: HSV-1, VC2, oncolytic virotherapy, herpes, cancer, personalized vaccine

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INTRODUCTION

It is currently understood that cancers result from individual cellular transformation events resulting in genetically and phenotypically unique tumors even within the same tissue environment (Al-Hajj and Clarke, 2004). This is problematic for the development of therapeutic or prevention strategies that seek to treat patient populations based on common features of tumors such as their tissue of origin. It is not surprising therefore, that current drugs for treating cancer only work for a small number of patients with a given cancer type (Chiriva-Internati and Bot, 2015). Thus, a personalized medicine approach is needed to tailor immunotherapies that are based on identifiable characteristics of patient-specific tumors.

Current molecular diagnostics, including genomic and proteomic tools, allow us to employ greater precision in the design and delivery of anti-cancer treatments and therapies (Krzyszczyk et al., 2018; Nassar et al., 2020; Rodriguez et al., 2021). These tools avail physicians and scientists with

incredible amounts of information regarding mutations that are unique to a particular patient. Examples include the identification of druggable pathways that result from such mutations, or the targeting novel kinase fusions in various cancer types (Stransky et al., 2014; Krzyszczyk et al., 2018). Additionally, these tools can be used to identify so-called tumor associated antigens (TAAs) (Hu et al., 2018). TAAs are the protein products of mutated genes that are not found in the proteome of healthy, non-transformed cells. TAAs result from genetic mutations and are unique to specific patients. As the immune system has evolved to discriminate self from non-self and eliminate non-self, TAAs can be used to target host immune responses to cells that bear these TAAs (Hu et al., 2018). This approach results in a "personalized" therapy.

Personalized therapies include CAR-T-cells, bispecific antibodies, and several approaches to induce *de novo* TAA specific immune responses via mRNA and vaccines, peptide vaccines and viral vectored TAAs (Slingluff et al., 2007; Kantoff et al., 2010; Rittig et al., 2011; Hu et al., 2018; Krzyszczyk et al., 2018). While there are currently no FDA-approved TAA vaccines, many groups have reported successes in clinical and pre-clinical work, and there is a great deal of interest and activity in this area (Goldman and DeFrancesco, 2009).

Regarding viral vectored TAA vaccines, there are several approaches currently being pursued (Holay et al., 2017). Viral vectors must possess both safety as well as immunogenicity. There are several attributes of human herpesviruses that inform their use as vaccine vectors: 1) they can infect humans in the presence of a significant anti-viral host response, 2) their relative safety, 3) their large size allowing the insertion of multiple transgenes within their viral genomes without compromising viral replication and infectivity, 4) the ease of genetic manipulation allowing the rapid and efficient generation of recombinant viruses, 5) the availability of anti-herpes drugs to control potential breakthrough infections, and 6) availability of a significant body of knowledge regarding the molecular biology of human herpesviruses which allows targeted manipulation of the viral genome to avoid downregulation of specific immune responses while augmenting others (Uche et al. 2021).

Our laboratory has developed the HSV-1 vaccine vector strain, VC2 (Stanfield et al., 2014). Specific mutations in VC2 glycoprotein K (gK) and the UL20 membrane protein abrogate its ability to infect neurons and establish latent infection (Jambunathan et al., 2015). The inability to establish latent infection and subsequently reactivate, is a unique safety feature. We have shown in several animal trials, including mouse, guinea pig, and non-human primate studies, that VC2 is a safe and immunogenic vaccine strain (Stanfield et al., 2017,., 2018; Naidu et al., 2020). We have further shown that VC2 confers protection of against lethal HSV genital and ocular infection (Stanfield et al., 2014; Bernstein et al., 2019; Naidu et al., 2020).

Previously, we reported that VC2 induced potent anti-tumor immune responses when administered intratumorally to melanoma tumors in immunocompetent mice (Uche et al., 2021a). Herein, we evaluated the utility of VC2 as a vaccine vector for prophylactic and therapeutic anti-cancer applications. To this end we generated the recombinant virus, VC2-OVA, expressing the immunogenic OVA peptide fused in-frame to the amino-terminus of the VP26 viral capsid protein. This allows maximal expression of the immunogen in infected cells, as well as its incorporation into the virion particle. We evaluated the efficacy of VC2-OVA in a syngeneic mouse model of melanoma. Specifically, we took advantage of widely used experimental mouse models of melanoma that express ovalbumin: B16cOVA (melanoma). Finally, we evaluated the differences between intradermal, subcutaneous and intramuscular routes of vaccination with VC2-OVA. Vaccination with VC2-OVA prevented the growth of engrafted tumors in both prophylactic and therapeutic settings. Importantly, our results show that the specific route of vaccination had a profound impact on the success of prophylactic treatment. Taken together these data demonstrate the potential of the VC2-vectored approach for personalized anti-cancer therapeutics.

MATERIALS AND METHODS

Animals

Four-to five-week-old female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in pathogen-free facilities. Protocols involving animals were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC), and all animal experiments were performed in accordance with the protocols.

Construction of the VC2-OVA Virus

The bacterial artificial chromosome (BAC) plasmid VC2 was used to construct VC2-OVA as previously described (Stanfield et al., 2014). High-efficiency markerless DNA manipulation of VC2 was achieved using two-step red-mediated recombination (Karstentischer et al., 2006). Oligonucleotides used in the construction of the recombinant virus are presented in **Supplementary Table S1**. Recombinant HSV-1 was recovered after BACs were transfected into Vero cells using Lipofectamine according to the manufacturer's protocol. DNA was extracted from viral stocks, and VP26 was sequenced to ensure the presence of the desired mutation. Virus for experimentation was purified as follows: Vero cells were infected and at full cytopathic effect (CPE), cells and supernatant were harvested. The cellular portion was separated from the supernatant by centrifugation at 4,000 RPM for 10 min. The supernatant was removed and the cell pellet was lysed by freezing and thawing of the pellet 3 times. The supernatant was added to the lysed cellular portion followed by a second round of centrifugation at 4,000 RPM for 10 min. The supernatant was aliquoted and titered to perform experiments.

Western Blot Analysis

Vero cells were uninfected or infected at an MOI 1 with either VC2 or VC2-OVA for 24 and 48 h in a six well plate. Adherent cells were washed 3x in PBS followed by lysis in 200 µl of NP40 lysis buffer with protease/phosphatase inhibitors. Twenty

microliters of whole cell lysate were then mixed with Laemmli sample buffer (Bio-Rad) and 1 µl of β-mercaptoethanol to a final 1x concentration. These mixtures were then boiled at 100°C for 10 min and cooled on ice before loading into a 12% Mini-PROTEAN TGX precast gel (Bio-Rad) and separated for 1 h at 100 V in 1x Tris-Glysine-SDS buffer (Bio-Rad). Separated protein was then transferred to a nitrocellulose membrane in 1x Tris-Glysine + 20% methanol (Bio-Rad). The membrane was then blocked for 30 min in 5% BSA in PBS-T. Rabbit anti-VP26 (Kind gift from Prashant Desai, Johns Hopkins), was diluted 1: 1,000 in 5% BSA PBS-T and applied to the membrane and incubated overnight at 4°C while rocking. The next day, the membrane was then washed 3x with PBS-T and secondary goat anti-Rabbit IgG (Abcam: ab6721) diluted 1:1,000 in 5% BSA PBS-T applied to the membrane and incubated at room temperature for 1 h. The membrane was then washed 3x in PBS-T and visualized using ECL Western Blot Substrate (Pierce) and exposure film.

Cell Culture

The ovalbumin-expressing B16 melanoma cell line (B16cOVA) was a kind gift from Dr. Timothy N.J. Bullock (University of Virginia, Charlottesville, Virginia, United States). B16cOVA cells were grown in RRPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% filtered, heat inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY), 100 µg/ml Primocin (Invivogen, San Diego, CA), plus 10 µg/ml Blastocydin (Invitrogen Life Technologies, Grand Island, NY). African green monkey kidney (Vero) cells were cultured in DMEM containing 10% FBS and 100 µg/ml Primocin.

Tumor Engraftment and Treatment Regimens

For prophylactic assessment, mice were not treated; intramuscularly; intradermally; or subcutaneously vaccinated with 1×10^6 pfu of VC2 or 1×10^6 pfu of VC2-OVA in volumes of 100 ul. Fourteen days after prime immunization, booster immunizations were administered. Six days post-boost, mice were engrafted with 5×10^5 B16cOVA cells in 100 µl PBS orthotopically in the dermis of the dorsal left dorsal pinna. Tumors were measured approximately every 2,3 days by using a digital caliper when tumors reached 50 to 100 mm^3. Tumor volumes were calculated by using formula 1/2 (length × $width^2$). Tumor bearing mice were euthanized when tumors reached greater than 1000 mm^3 or when mice were excessively moribund. To assess the therapeutic effect, mice were injected intravenously with 5×10^5 B16cOVA cells in 100 µl PBS, and then intramuscularly; intradermally; or subcutaneously vaccinated the next day for two consecutive days. Mice were sacrificed 3 weeks post engraftment, and lungs were removed and the tumor colonies on the lung surface were counted.

ELISPOT Assays

One day after boost vaccination, mice were sacrificed, and spleens were removed.

Splenocytes (7.5×10^5) were isolated and cultured overnight with either gB peptide (1 µg/ml) or ovalbumin [OVA257–264 (SIINFEKL)] peptide (1 µg/ml). IFN-γ-producing splenocytes were quantified according to the manufacturer's instructions using an Immunospot (Shaker Heights, OH) murine IFN-γ single-color ELISPOT assay.

Statistical Analysis

All statistical analyses were done using GraphPad Prism nine Software (GraphPad Software, Inc., San Diego, CA). Analysis of data between three or more groups was performed by using one-way ANOVA. Survival data were presented using Kaplan–Meier survival curves and differences among groups were analyzed by the log rank test. A p-value of 0.05 or less was considered statistically significant in all analyses herein.

RESULTS

Construction and Characterization of Ovalbumin Expressing Virus

We wished to fully exploit the potential of viruses to deliver antigen and promote strong, broad, and effective anti-immunogen responses in the host. To this end, we fused the immunogenic portion of chicken egg ovalbumin to VP26, the minor capsid protein of HSV-1 (**Figure 1A**). Ovalbumin is a common experimental immunogen with an extensive history of use for studying immunogenicity of novel vaccine approaches (Karandikar et al., 2019). VP26 is present at approximately 900 copies in each virion (Kobayashi et al., 2017). This means that in an inoculum of 10^6 pfu we can deliver nearly 10^9 OVA-VP26 antigens. However, this extrapolation is likely an underestimation due to a particle to pfu ratio for tissue culture-derived HSV-1 reported to be 100:1 (Mahiet et al., 2012). Further, the fusion of an antigen to the viral particle allows access to the exogenous antigen presentation pathway to promote the development of TH2 responses in addition to traditional TH1 responses to viral vectored antigens. Using BAC mutagenesis, a portion of ovalbumin containing the canonic $CD8^+$ peptide (SIINFEKL, $OVA_{257-264}$) was fused to the amino terminus of VP26 to generate VC2-OVA.

To confirm expression of the fusion protein in recovered VC2-OVA, Vero cells were infected at a multiplicity of infection of 1. Twenty-four and 48 hours post-infection, protein lysates were prepared, and a western blot was performed. Using an antibody to detect VP26 we readily observed a protein of the expected size (12kDA (Desai and Person, 1998)) in lysates from cells infected with parental VC2 virus (**Figure 1B**). However, in lysates from cells infected with VC2-OVA we observed a protein at an apparent molecular mass of approximately 25 kDa, the expected molecular weight of the VP26-OVA fusion protein (**Figure 1B**).

To determine any effect of fusing ovalbumin to VP26 on viral replication we performed a multi-step growth curve comparing parental VC2 virus and VC2-OVA. Vero cells were infected at an MOI of .01 and cells were harvested at 0, 4, 12, 24 and 48 h post infection. Standard plaque assays were performed to quantify virus in cell lysates. We were unable to identify any difference in viral replication between parental and VC2-OVA viruses (**Figure 1C**).

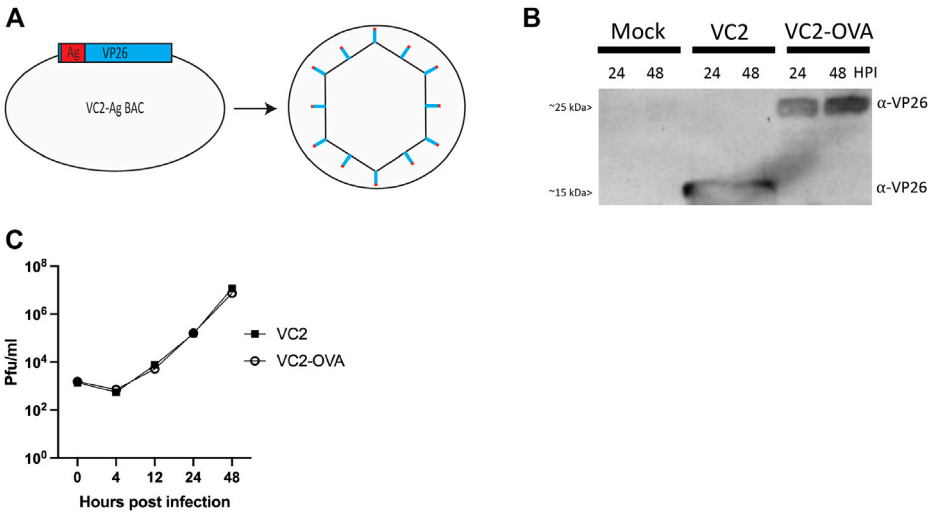

FIGURE 1 | Construction of VC2-OVA virus. **(A)** VC2-OVA. **(B)** Expression of OVA VP26 fusion protein. Vero cells were infected at a multiplicity of infection of 1. Twenty-four and 48 hours post infection, protein lysates were prepared and analyzed by western blot. The blot was stained with antibody to VP26. **(C)** Growth curve of VC2-OVA and parental VC2 viruses. Vero cells were infected at a multiplicity of infection of .01. Supernatants and cell pellets were harvested at indicated times post infection, and plaque assays were performed to determine viral titers.

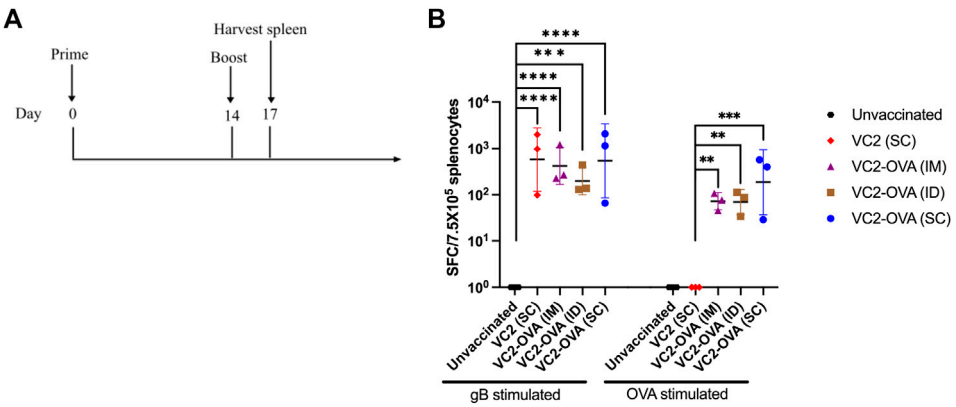

FIGURE 2 | Vaccination with VC2-OVA induces OVA specific T cell response. **(A)** Timeline of treatment regimen. Mice were untreated or prime vaccinated intramuscularly, or intradermally, or subcutaneously with 1×10^6 pfu of VC2-OVA or parental VC2 virus. Fourteen days later, boost immunizations were administered. 72 hours post boost, mice were euthanized, and their spleens were harvested. **(B)** Isolated splenocytes (7.5×10^5) were cultured overnight with either gB peptide (1 μg/ml) or OVA257–264 [SIINFEKL] peptide (1 μg/ml), and IFN-γ producing cells were quantified by ELISPOT assay. N = 3 mice per group. Data were analyzed using one-way ANOVA. **, $p < 0.01$, ***, $p < 0.001$.

Immunogenicity of VC2-OVA in Mice

To test the ability of VC2-OVA to induce OVA-specific immune responses we vaccinated mice with VC2-OVA. After 14 days, mice received a second vaccination (boost) with VC2-OVA or the parental virus. 72 hours post boost vaccination, mice were sacrificed and splenocytes were harvested (**Figure 2A**). Splenocytes were incubated with either HSV-1 glycoprotein B peptide or SIINFEKL peptide and ELISPOT analysis was performed. The gB peptide is a dominant $CD8^+$ T-cell epitope (Treat et al., 2017) and serves as a positive control. We observed that vaccination with VC2 and VC2-OVA, induced high levels of gB specific immune responses (**Figure 2B**). However, only in splenocytes from mice vaccinated with VC2-OVA was an OVA specific T cell response detected (**Figure 2B**). Interestingly, there was no significant difference in these responses induced by the different vaccination routes in those animals.

Efficacy of VC2-OVA in an Experimental Mouse Model of Melanoma

We have previously shown the efficacy of parental VC2 in intratumoral treatment of mice engrafted with modified B16F10 melanoma (Uche et al., 2021a). In those previous experiments, we achieved between 50 and 80% cure rates. To

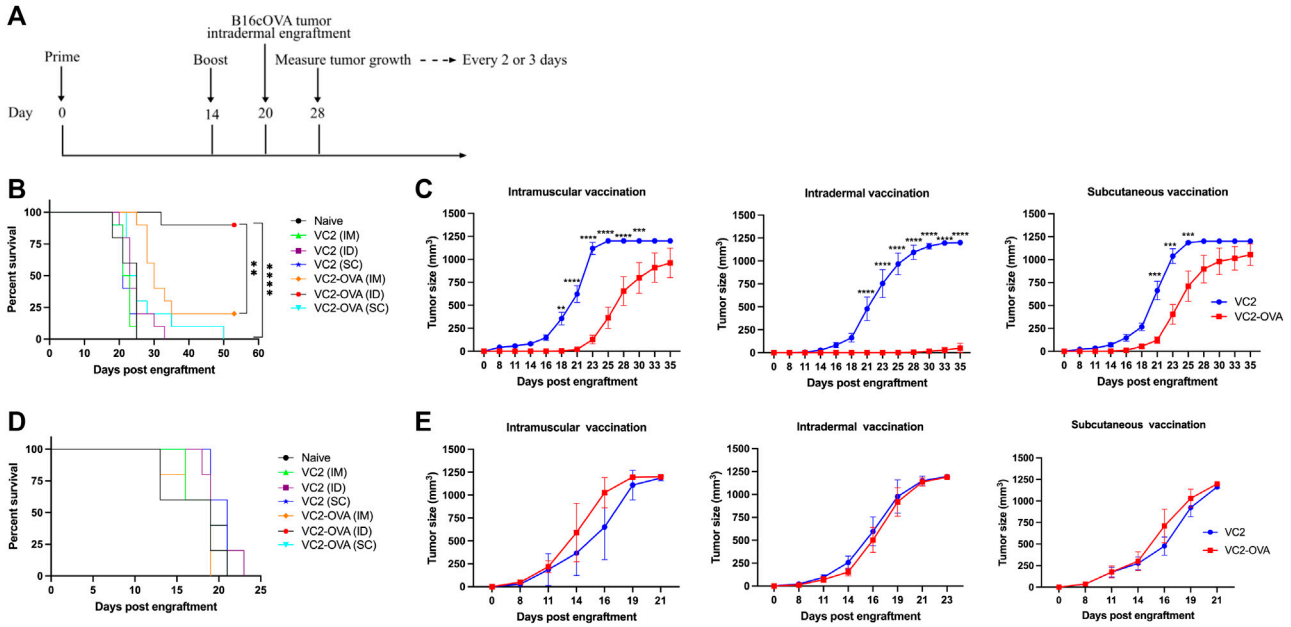

FIGURE 3 | The prophylactic effect of VC2-OVA in B16cOVA tumor model. **(A)** Timeline of treatment regimen. Mice were untreated or prime vaccinated intramuscularly, or intradermally, or subcutaneously with 1×10^6 pfu of VC2-OVA or parent VC2 virus. Fourteen days later, booster immunizations were administered. Six days post vaccination, mice were engrafted with 5×10^5 B16cOVA or B16F10 tumor cells. Mice were observed for tumor growth. Mice were sacrificed when tumors reached greater than 1,000 mm^3 or when the mice became excessively moribund. Kaplan-Meier survival curves **(B, D)**. Tumor volume and growth rates was measured every 2,3 days **(C, E)**. N = 5–10 mice per group. **, $p < 0.001$, ***, $p < 0.001$, ****, $p < 0.0001$.

investigate whether the expression of a tumor-associated surrogate protein can be used to augment anti-tumor immune response, we employed B16F10 cells which express OVA in conjunction with the VC2 OVA expressing virus administered by direct inoculation into engrafted B16cOVA tumors. There were no significant differences between parental VC2 and VC2-OVA (data not shown). We believe that this is due to the very high cure rate with VC2 treatment that could not be significantly augmented by the presence of the OVA antigen. Next, we tested the efficacy of VC2-OVA in preventing tumor growth in mice that had been vaccinated before engraftment of B16cOVA tumors. The relevance of this approach may be seen in a case where surgical resection of a tumor is followed by vaccination against recurrence. In these experiments we compared the efficacy of VC2-OVA using three distinct vaccination routes: intramuscularly (IM), subcutaneously (SC), or intradermally (ID). We chose this approach as recent data suggests that the efficacy of vaccination can be dependent on the route of vaccination (Zhang et al., 2015). Animals were vaccinated twice, 14 days apart, before tumor engraftment 6 days after the second vaccination (**Figure 3A**). Mice vaccinated with VC2 (regardless of route of vaccination) were sacrificed 35 days post engraftment (**Figure 3B**). In contrast to mice vaccinated with parental VC2, all mice vaccinated with VC2-OVA before engraftment had significantly increased median survival times. Interestingly, mice vaccinated with VC2-OVA exhibited survival times that were dependent on route of vaccination. Ninety percent of mice that were ID vaccinated before engraftment arrested tumor growth and survived. Twenty percent of mice that were vaccinated IM survived while none of the mice vaccinated SC survived. Tumor growth rates were consistent with the results of survival with few mice vaccinated intradermally exhibiting tumor growth at all while intramuscular vaccination resulted in slower tumor growth rates than subcutaneous vaccination (**Figure 3C**). For control purposes, we engrafted mice previously vaccinated with either VC2 or VC2-OVA with B16F10 cells which do not express ovalbumin. In these experiments there were no differences in survival times or tumor growth rates, regardless of vaccination with VC2 or VC2-OVA (**Figures 3D,E**).

Next, we investigated the efficacy of VC2-OVA when used in a therapeutic context, where engraftment preceded treatment. In these experiments, B16cOVA cells were inoculated intravenously. The introduction of these cells intravenously leads to colonization of the lungs by the B16F10 cells resulting in tumors that can be enumerated approximately 3 weeks post engraftment. This approach is a commonly used approach to test intervention strategies for metastasis and the development of systemic anti-tumor immunity. B16cOVA cells were administered intravenously, and mice were treated with either VC2 or VC2-OVA IM, SC, or ID 2 days post tumor administration (**Figure 4A**). Twenty-one days post engraftment, mice were sacrificed and colonies of B16cOVA

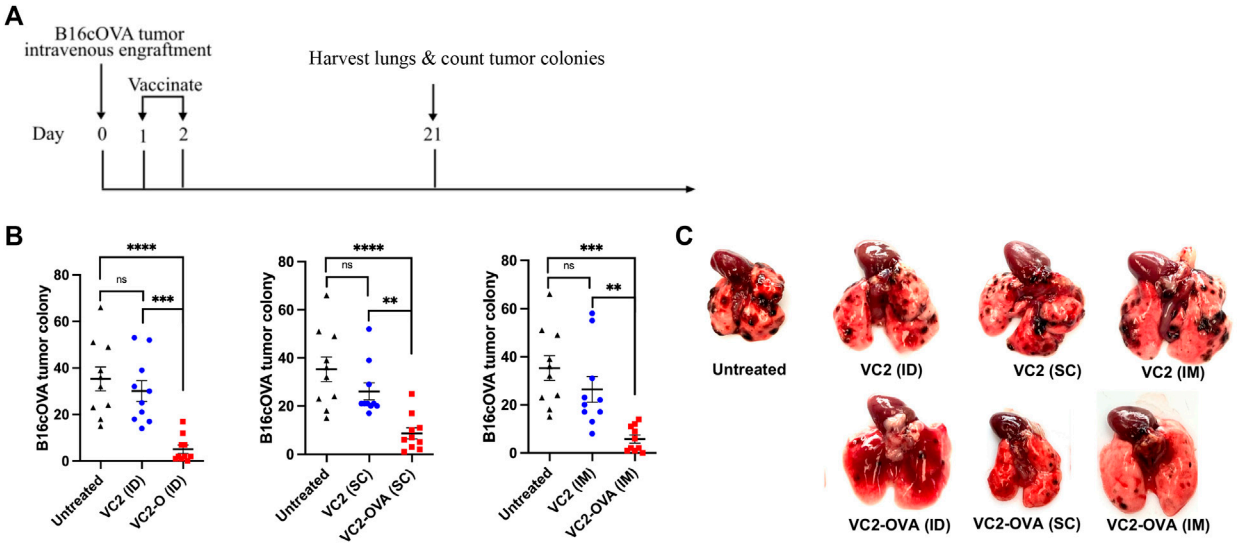

FIGURE 4 | Therapeutic response of VC2-OVA in B16cOVA tumor model. **(A)** Timeline of treatment regimen. Mice were intravenously challenged with 5×10^5 B16cOVA tumor cells. The next 2 days, mice were treated with either VC2-OVA or VC2 through the different vaccination routes. **(B)** Nineteen days post treatment, mice were euthanized and tumor colonies on the lung surface were quantified. **(C)** Representative images of lungs from untreated, VC2, VC2-OVA treated mice 3 weeks postinoculation and quantification of colonization. N = 10 mice per group. Data were analyzed using one-way ANOVA. **, $p < 0.01$,***, $p < 0.001$, ****, $p < 0.0001$, ns = not significant.

cells in the lungs were enumerated (**Figure 4B**). Mice that were left untreated or treated with VC2 had significantly more tumor colonies in their lungs than mice treated with VC2-OVA (**Figures 4B,C**).

DISCUSSION

The identification of safe, and immunogenic vaccine vectors capable of inducing potent immune responses is critical to the development of anti-infectious disease and anti-cancer intervention strategies (Vance et al., 2017). Previously, we demonstrated that the novel HSV-1 (VC2) vaccine vector, can be used to induce potent anti-tumor immune responses in a mouse model of melanoma (Uche et al., 2021b). Herein, we extend our previous findings by demonstrating that VC2 can be readily adapted to promote TAA-specific immune responses capable of extending mouse survival and decreasing tumor growth rates.

Of particular interest is our finding that the route of vaccination was a large factor in the efficacy of treatment. Intradermal route of vaccination proved best in our B16cOVA melanoma model in a prophylactic context. Intramuscular route of vaccination proved to be the least effective in both extending survival and reducing tumor growth rates. It is unclear why intradermal delivery of the virus produced a more efficient vaccination approach. It has been documented that immune responses are affected by the route of vaccination (Belyakov and Ahlers, 2009; Zhang et al., 2015). There are differing reports on whether there is an actual difference in the magnitude or quality of adaptive immune responses generated by differing routes of administration (Ols et al., 2020; Rosenbaum et al., 2021). What makes our study particularly compelling is that we have a functional readout on the route-dependent promotion of anti-tumor responses based on survival and tumor growth rates. Our data strongly suggest that there are significant differences in the outcome of treatment based on the route of administration.

It is important to point out that route of administration is not a one size fits all problem. Likely each route of administration induces specific types of immunity that may be individually suited to protect against different infection and tumor types. Along these lines we note that our studies used two different engraftment sites: intradermal and intravenous. While we saw large differences in route of administration for the intradermally engrafted tumors we didn't find any difference for the route of administration when tumors were engrafted intravenously. These findings suggest that the route of administration may be an important consideration for infections and tumor types at some sites but not others.

In these experiments we have used an experimental immunogen, OVA, to evaluate the utility of HSV-1 in general, and VC2 specifically, as a vector to deliver tumor associated antigens for treatment of cancer. It is important to note that the clinical utility of our approach will depend on the identification of similarly immunogenic tumor associated antigens in human patients. The identification of such antigens in human tumors is an active area of investigation with encouraging results (Buonaguro et al., 2011; Hu et al., 2018). The identification of such antigens is however fruitless without the development of technologies, such as ours, to deliver TAAs to patients. Future experimentation should therefore focus on using highly immunogenic vectors to target tumor specific TAAs.

In summary, we find that there is significant evidence to pursue viral vectored TAA delivery in general and VC2-derived TAA vaccines specifically. VC2 has proven safe and efficacious as an HSV vaccine in a variety of animal models and preparations are ongoing for a pilot in-human trial. As we have shown that VC2 works very well as an oncolytic virotherapy, we are excited about the prospect of using VC2 as a combination OVT and personalized vaccine for the treatment of human and animal cancers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Louisiana State University, School of Veterinary Medicine Institutional Animal Use and Care Committee.

AUTHOR CONTRIBUTIONS

PR conceived, designed study, experimentation, analysis, writing IU carried out experiments, analysis, writing JR and BAS carried out experiments KK writing, analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.832393/full#supplementary-material

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Conflict of Interest: KK has intellectual property rights to the VC2 vaccine, which is licensed from Louisiana State University to Rational Vaccines, Inc. PR and KK are named inventors on patents using VC2 as a vector.

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A Three-Dimensional Organoid Model of Primary Breast Cancer to Investigate the Effects of Oncolytic Virotherapy

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Background: Although several oncolytic viruses have already been tested in early-stage clinical studies of breast cancer, there is still an urgent need to develop patient-derived experimental systems that mimic the response of breast cancer to oncolytic agents in preparation of testing different oncolytic viruses in clinical trials. We addressed this need by developing a protocol to study the effects of oncolytic viruses in stable organoid cell cultures derived from breast cancer tissue.

Methods: We used an established three-dimensional organoid model derived from tissue of 10 patients with primary breast cancer. We developed an experimental protocol for infecting organoid cultures with oncolytic viruses and compared the oncolytic effects of a measles vaccine virus (MeV) and a vaccinia virus (GLV) genetically engineered to express either green fluorescent protein (MeV-GFP) and red fluorescent protein (GLV-0b347), respectively, or a suicide gene encoding a fusion of cytosine deaminase with uracil phosphoribosyltransferase (MeV-SCD and GLV-1h94, respectively), thereby enabling enzymatic conversion of the prodrug 5-fluorocytosine (5-FC) into cytotoxic compounds 5-fluorouracil (5-FU) and 5-fluorouridine monophosphate (5-FUMP).

Results: The method demonstrated that all oncolytic viruses significantly inhibited cell viability in organoid cultures derived from breast cancer tissue. The oncolytic effects of the oncolytic viruses expressing suicide genes (MeV-SCD and GLV-1h94) were further enhanced by virus-triggered conversion of the prodrug 5-FC to toxic 5-FU and toxic 5-FUMP.

Conclusions: We were able to develop a protocol to assess the effects of two different types of oncolytic viruses in stable organoid cell cultures derived from breast cancer tissue. The greatest oncolytic effects were observed when the oncolytic viruses were engineered to express a suicide gene (MeV-SCD and GLV-1h94) in the presence of the prodrug 5-FC. The model therefore provides a promising *in vitro* method to help further testing and engineering of new generations of virotherapeutic vectors for *in vivo* use.

Keywords: oncolytic virus, virotherapy, breast cancer, measles virus, vaccinia virus, organoid cell culture, suicide gene, 5-fluorouracil

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INTRODUCTION

Breast cancer is the most common cause of cancer-associated death in women aged between 20 and 59 years (Siegel et al., 2021). Despite tremendous advances in breast cancer therapy, approximately 20% of all patients experience metastatic recurrence and such metastatic disease still remains incurable. Therefore, there is still an urgent unmet need for new therapeutic options to either prevent and/or treat metastatic disease (Pardoll, 2012).

Oncolytic viruses are emerging as promising agents for the treatment of cancer because they selectively infect and damage cancerous tissues without causing harm to normal tissue (Russell et al., 2012). They offer an attractive combination of tumor-specific cell lysis coupled with immune stimulation through release of tumor antigens and/or other signals to overcome immunosuppression in the tumor microenvironment. Oncolytic viruses achieve tumor-specific lysis in three different ways (Lawler et al., 2017). Firstly, they can enter cells *via* virus-specific, receptor-mediated mechanisms. Secondly, increased viral replication may be supported by rapid cell division in tumor cells. And thirdly, tumor cells support selective virus replication because they often demonstrate deficits in antiviral type I interferon (IFN) signaling (Lawler et al., 2017).

Typically, viruses exhibit a specific cellular tropism that determines which tissues and/or hosts are preferentially infected, and viruses have evolved mechanisms of host cell selectivity by natural selection to improve penetration into host cells. Research conducted with oncolytic viruses led to the differentiation of oncolytic viruses into two groups based on their ability to infect tumor cells. The first group includes oncolytic viruses with natural or intrinsic anti-neoplastic characteristics, and the second group contains ones that have been genetically modified to enhance tumor-selectivity (Hartkopf et al., 2011).

Measles viruses belong to the family of paramyxoviruses (Udem and Cook, 1984). Oncolytic measles viruses are based on attenuated strains which have been used for vaccine purposes for many years and have an excellent safety profile (Aref et al., 2016). Furthermore, they can be genetically engineered with yeast-derived suicide genes that encode for a fusion gene encoding both cytosine deaminase and uracil phosphoribosyltransferase [called FCU1 (Erbs et al., 2000) or SCD (Lampe et al., 2013)] which expresses a chimeric protein that converts the non-toxic prodrug 5-fluorocytosine (5-FC) into highly cytotoxic compound 5-fluorouracil (5-FU) and subsequently into 5-fluorouridine monophosphate (5-FUMP), thereby bypassing an important mechanism of chemoresistance for 5-FU (Hartkopf et al., 2013). 5-FU is a cytotoxic agent that is used for the treatment of breast cancer, and 5-FUMP is the activated form of 5-FU (Slos and Erbs, 2004; Dias et al., 2010). Cytosine deaminase catalyzes the conversion to 5-FU and uracil phosphoribosyltransferase (UPRT) catalyzes the subsequent conversion of 5-FU to 5-FUMP. Therefore, UPRT has the potential to sensitize chemoresistant cancer cells to 5-FU (Hartkopf et al., 2013). An anti-tumor effect of SCD on cancer cells has already been demonstrated in an adenovirus model (Graepler et al., 2005). Oncolytic measles vaccine virus MeV-SCD has previously demonstrated tumor-specific replication in experiments in human hepatoma and ovarian cancer cells (Hartkopf et al., 2013; Lampe et al., 2013). Several oncolytic measles viruses are undergoing clinical development in cancer patients for a variety of malignant diseases, e.g., ovarian or breast cancer (Galanis et al., 2010; Lech and Russell, 2010).

Vaccinia viruses belong to the poxvirus family. Their oncolytic properties have already been demonstrated in clinical trials, while causing only mild flu-like symptoms (Hunter-Craig et al., 1970; Arakawa et al., 1987; Gomella et al., 2001). Similar to oncolytic measles virus MeV-SCD, oncolytic vaccinia virus GLV-1h94 also, encodes the FCU1 suicide fusion gene enabling enzymatic conversion of 5-FC to 5-FU and 5-FUMP (Slos and Erbs, 2004).

Extensive attempts to develop oncolytic viruses for breast cancer with the previously established methods have not been successful so far. Hence, oncolytic viruses have not yet been approved for the treatment of breast cancer. Previously established models used for experimental cancer research, including two-dimensional cultures of immortalized cell lines, patient-derived xenograft models and transgenic mice, fail to mimic adequately the complex tumor microenvironment of human cancer (Yuki et al., 2020). These models have major disadvantages and may only insufficiently represent the patterns of the original cancer patient tumor tissues (Bosma and Carroll, 1991; Kamb, 2005). In particular, many patient-derived xenograft models of breast cancer do not recapitulate the tumor microenvironment of their tumor origin, have low success rates of tumor transplantation, and are relatively expensive because of the need for immune-deficient mice (Murayama and Gotoh, 2019). A three-dimensional organoid model based on patient-derived tumor samples may offer a better way forward. This model should be able to mimic the tumor-immune interactions and mutational status of the original tumor (Bar-Ephraim et al., 2020; Yuki et al., 2020). Additionally, it offers the future prospect of integrating the individual immune system into the model, thereby increasing the reliability of research to enhance the transition of new therapies from bench to beside (Bar-Ephraim et al., 2020). Currently, the addition of the immune system to patient-derived breast cancer organoid cultures is under investigation by several groups. For example, autologous peripheral blood monocytes derived from patient blood samples have been successfully added into the corresponding colon and lung cancer organoid setup (Dijkstra et al., 2018). Tumor-on-a-chip technology may also enable a better understanding of the role of the immune system and its incorporation into an organoid model setup (Moccia and Haase, 2021).

Accordingly, there is an urgent need to develop patient-derived experimental systems that recreate the different aspects of breast cancer *in vitro* to investigate hallmark parameters such as efficiencies of virotherapeutic infections, kinetics of intratumoral viral replication and immune-mediated oncolysis. Recent advances in three-dimensional cell culture technology enable culture of embryonic and adult mammalian stem cells in a way that allows them to exhibit their self-organizing properties. The resulting organoids mimic important structural and functional properties of different organs

TABLE 1 | Tumor characteristics of patients providing breast cancer tissues for the establishment of tumor organoid cultures. ER = estrogen receptor; ER-IRS = estrogen receptor immunoreactive score; PR = progesterone receptor; PR-IRS = progesterone receptor immunoreactive score, Her2 = human epidermal growth factor 2, Her2-IHC-Score = human epidermal growth factor 2-ImmunoHistoChemistry score.

	Age	Diagnosis	Grading	ER		PR		Her2	Her2-IHC-score	Ki67
BC-ORG 1	37	Invasive ductal carcinoma	G1	Pos.	ER-IRS:12 90% ER-staining	Pos.	PR-IRS:12 90%	Neg.	1+	10%
BC-ORG 2	49	Invasive lobular carcinoma	G2	Pos.	ER-IRS:12 90% ER-staining	Pos.	PR-IRS:4 90%	Neg.	1+	5%
BC-ORG 3	52	Invasive ductal carcinoma	G3	Neg.	ER-IRS:0 0% ER staining	Neg.	PR-IRS:0 2%	Neg.	1+	60%
BC-ORG 4	42	Mucinous with associated ductal carcinoma *in situ*	G2	Pos.	ER-IRS:9 80% ER staining	Pos.	PR-IRS:6 40%	Pos.	2+ (FISH pos.)	15%
BC-ORG 5	59	Invasive lobular carcinoma with associated lobular carcinoma *in situ*	G2	Pos.	ER-IRS:12 100% ER staining	Pos.	PR-IRS:1 1–9%	Neg.	1+	10%
BC-ORG 6	67	Invasive lobular carcinoma	G2	Pos.	ER-IRS:12 100% ER staining	Pos.	PR-IRS: 6 n.d.	Neg.	0	10–15%
BC-ORG 7	56	Invasive ductal carcinoma	G2	Pos.	ER-IRS:12 100% ER staining	Pos.	PR-IRS:12 100%	Neg.	1+	5%
BC-ORG 8	52	Tubular carcinoma	G1	Pos.	ER-IRS:12 90% ER-staining	Pos.	PR-IRS:6 60%	Neg.	1+	5%
BC-ORG 9	51	Invasive ductal carcinoma	G3	Pos.	ER-IRS:12 100% ER-staining	Pos.	PR-IRS:1 1%	Pos.	3+	10–15%
BC-ORG 10	62	Invasive ductal carcinoma	G2	Pos.	ER-IRS:12 100% ER-staining	Pos.	PR-IRS:12 100%	Neg.	1+	10–15%

such as kidney, lung, intestine, brain and retina and are currently under investigation as models for predicting drug response especially with regard to personalized cancer treatment (Clevers, 2016; Drost and Clevers, 2018; Rosenbluth et al., 2020). For example, an organoid model of pancreatic cancer and healthy pancreatic tissue was used to determine the effects of oncolytic adenoviruses, and the authors concluded that the response of the pancreatic organoid model to oncolytic adenoviruses might be indicative of in-patient responses of primary pancreatic tumors and metastases (Raimondi et al., 2020). The culture conditions for human mammary epithelial organoids have already been established that create organoids which exhibit the histological and genetic features of the original tumors (Sachs et al., 2018).

In this study we set out to answer the question whether three-dimensional cell cultures are suitable for testing oncolytic virotherapy. We addressed this topic by developing a protocol in a stable three-dimensional organoid model derived from patients with primary breast cancer to determine the oncolytic effects of genetically engineered oncolytic viruses, encoding either marker genes for GFP (oncolytic measles virus MeV-GFP) and for red fluorescent protein (oncolytic vaccinia virus GLV-0b347), or the SCD/FCU1 suicide gene (oncolytic measles virus MeV-SCD, oncolytic vaccinia virus GLV-1h94) on breast cancer organoid cultures.

METHODS

Breast Cancer Patients and Tumor Tissues

Tissue was obtained within a period of 4 months in 2019 from ten female patients aged between 30 and 70 years, who had been diagnosed with primary invasive breast cancer at the obstetrics and gynecology department of the University Hospital Tuebingen (**Table 1**) and who had not received systemic chemotherapy or radiation. Consequently, patients diagnosed with recurrent breast cancer or metastases were excluded from the study. Infiltration of tumor cells in lymph nodes was not defined as an exclusion criterion.

All of the original tumor specimen included in this study were at least 1 cm^3 in size to enable full histopathological analysis, yet still allowing the harvest of sufficient numbers of tumor cells for cultivation. All patients provided written informed consent and the study was approved by the local ethics committees (210/2019BO2).

Processing Breast Cancer Patient Tissue for Establishing Organoid Cell Cultures

Figure 1 illustrates the overall process for preparing breast cancer patient-derived organoid cultures. Tumor tissues derived from each patient were cut into 1 mm^3 sized pieces and digested with a 1:1 mix of advDMEM/F12 +/+/+ (Gibco Advanced Dulbecco's

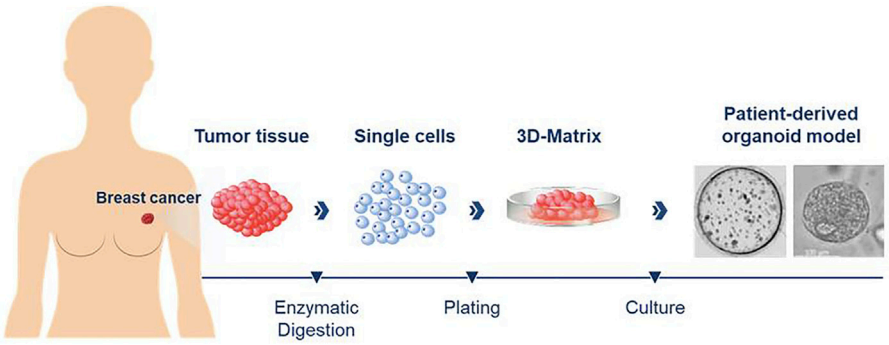

FIGURE 1 | Diagram illustrating the process for preparing patient-derived organoid cultures from breast cancer tissues. Enzymatic digestion of fresh tumor tissues was used to generate singe cells that were then plated in a three-dimensional matrix for culture of the organoids.

Modified Eagle Medium/F-12 with the addition of 1% GlutaMAX, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 1% penicillin-streptomycin (all reagents from Thermo Fisher Scientific, Waltham, MA), collagenase (type IV 5 mg/ml, Sigma-Aldrich, Munich, Germany) and 10 µM Y-27632 (Hoelzel Diagnostika, Cologne, Germany) until sufficient digestion was achieved (after 1–3 h, indicated by the onset of clouding of the solution). The suspension was then transferred into a 15 ml tube containing 10 ml of advDMEM/F12$^{+/+}$/+ and centrifuged at 478 x g for 10 min. The supernatant was removed and the pellet resuspended in 1 ml of TrypLE Express (Thermo Fisher Scientific) and incubated for another 15–30 min. The solution was filtered through a 100 µm filter into a 50 ml tube and washed with additional 10 ml advDMEM F12$^{+/+}$/+. The suspension was centrifuged for 478 x g for 10 min and the supernatant carefully removed. Depending on the size of the remaining cell pellet, it was resuspended in 60–500 µL of advDMEM/F12 +/+/+. For an organoid setup (6 wells in a 48-well plate) an aliquot of 60 µL cell suspension was mixed with 70 µL of Matrigel (Corning, NY, USA). Aliquots of 20 µL were pipetted into each well of a 48-well plate. Afterwards, the culture plate was placed upside down in an incubator at 37°C and 5% CO_2. After 30 min, 280 µL of breast cancer culture medium were added to each well. The medium was changed every 3–4 days. The residual cell suspension not used for plating was resuspended in 700 µL Gibco Recovery Cell Culture Medium (Thermo Fisher Scientific) per vial and frozen. The vials were then transferred to −80°C in cell coolers. For long-term storage the vials were transferred to containers containing liquid nitrogen.

Passaging of Organoid Cultures

The organoid lines were passaged and split based on the confluency of the culture (organoids in the center of the Matrigel drop shedding debris or becoming darker in appearance and/or becoming larger than 300 µm in diameter) ranging from 5–20 days. After removal of the culture medium the wells were washed with 1 ml of PBS. Afterwards the Matrigel domes were mechanically scraped off the bottom of the culture plate with a pipette tip and collected in TrypLE Express (1 ml for 6 wells). The solution was incubated for 5 min at 37°C. After the addition of 10 ml of advDMEM/F12 +/+/+ the solution was centrifuged for 10 min at 478 x g. The cell pellet was resuspended in advDMEM/F12 +/+/+ and organoids were plated and cryopreserved as described earlier.

Breast Cancer Tissue Organoid Culture Medium

The medium used for culturing the breast cancer organoid cultures contained the following ingredients: 50% conditioned medium from L-WRN cells (ATCC #CRL-3276) (Miyoshi and Stappenbeck, 2013) (containing Wnt3a, R-spondin 3, and Noggin), Heregulin 5 nmol/L (Peprotech, NJ, USA), fibroblast growth factor 7 (FGF7) 5 ng/ml (Peprotech), fibroblast growth factor 10 (FGF10) 20 ng/ml (Peprotech), epidermal growth factor (EGF) 5 ng/ml (Peprotech), A83-01 500 nmol/L (Tocris, Wiesbaden, Germany), Y27632 5 µmol/L (Hölzel), SB202190 (Sigma-Aldrich), Gibco B27 Supplement 2% (Thermo Fisher Scientific), N-acetyl-cysteine 1.25 mmol/L (Sigma-Aldrich), nicotinamide 5 mmol/L (Sigma-Aldrich), Primocin 50 µg/ml (InvivoGen, Toulouse, France), Gibco advDMEM/F12 50% (Thermo Fisher Scientific).

Infecting Breast Cancer Organoid Cultures With Oncolytic Viruses

Protocol 1–Infection With Oncolytic Viruses 24 h After Passaging

Organoids were passaged according to the standard method described above. After centrifugation and removal of the supernatant the cell pellet was resuspended in 260 µL of breast cancer culture medium. Aliquots of 60 µL were taken for plating out in 60% Matrigel according to the standard passaging protocol for further cultivation. For the remaining 200 µL of organoid suspension, 2,500 µL of breast cancer culture medium and 300 µL of Matrigel were added. This suspension was plated into 12 wells of an untreated 48-well cell culture plate with a 250 µL drop size and an estimated density of 2.5×10^4 cells per well. After 24 h the amount of virus calculated for a defined viral concentration was

suspended in breast cancer culture medium and 50 µL per well were added. This results in two wells for each concentration point. The plate was then left to remain warm in the incubator at 37°C and 5% CO_2. Infection state and virus distribution were monitored daily and documented photographically.

Protocol 2—Infection With Oncolytic Viruses While Passaging

Organoids were passaged according to the method described above. After centrifugation and removal of the supernatant the cell pellet was resuspended in 300 µL of breast cancer culture medium. Aliquots of 90 µL of this suspension were used for cultivation in 60% Matrigel according to the aforementioned method (passaging of organoids). Aliquots of 10 µL of suspension were used to count in an improved-Neubauer cell counting chamber. The remaining 200 µL of suspension were used for plating the cells in 10% Matrigel. Each well consisted of 225 µL of breast cancer culture medium with the cells/organoids, 25 µL of Matrigel, and 50 µL of breast cancer culture medium with the virus (in case of the control wells additional breast cancer culture medium was used). A total of 18 wells were required for each infection. The 200 µL organoid suspension was resuspended in breast cancer culture medium and Matrigel. From this suspension 500 µL were removed and 100 µL of the desired viral suspension added. Aliquots of 300 µL were plated out into one well at a time, resulting in the desired two wells for each viral concentration. This was repeated for all the desired viral concentrations. An untreated 48-well culture plate was used. The plate was then placed in an incubator at 37°C with 5% CO_2. The viral distribution was monitored daily through microscopy and photographically documented each day.

Protocol 3–Infection of Organoid Cultures With Oncolytic Viruses 7–10 Days After Passaging

Organoid cultures were passaged according to the method described above and plated out in 6 wells of a 48-well culture plate treated with 60% Matrigel. The organoids were placed in a CO_2 incubator for 7–10 days until the organoid cultures had reached a sufficient size and density for viral infection. Then aliquots of 100 µL of dispase II (1 mg/ml) were added to each well while mechanically scraping the Matrigel dome from the bottom of the well. The cell culture plate was returned to the CO_2 incubator at 37°C for 60 min. Then the contents of the wells were removed and transferred into a 15 ml tube. The wells were then washed with 1 ml of Dulbecco's PBS. This was also added to the 15 ml tube and centrifuged at 210 x g for 15 min. The supernatant was carefully removed with a pipette and discarded. The cell pellet was resuspended in 5,625 µL breast cancer culture medium. Aliquots of 625 µL of Matrigel were added. Subsequently, 520 µL of the suspension were pipetted into a 1.5 ml tube and 100 µL of the desired viral concentration were added. Then 300 µL of this suspension were transferred into a well of an untreated 48-well cell culture plate thereby resulting in two wells with the same viral concentration. The cell culture plate was then placed in an incubator at 37°C with 5% CO_2.

Viral Titration

We used a cell density of 25,000 cells per well and investigated the effects of MeV-GFP, MeV-SCD, GLV-0b347 and GLV-1h94 (see below). All viruses were used in a concentration termed multiplicity of infection (MOI) equal to 10 (meaning that the ratio of infectious viral particles to tumor cells had been adjusted to 10:1). In addition, MOI 1 was also used for MeV-GFP and MOIs of 0.1 and 1 were also used for GLV-0b347. The prodrug 5-fluorocytosine (5-FC) was added to infections with MeV-SCD and GLV-1h94 at a concentration of 1 mmol/L. Additionally, the following controls were measured for each organoid line: 1) 1 mmol/L 5-FC, 2) MeV-SCD/GLV-1h94 without the prodrug 5-FC and 3) 1 mmol/L 5-FU as well as 4) two wells containing breast cancer culture medium only.

Organoids were dissipated into single cells for counting with an improved-Neubauer cell counting chamber to enable an estimation of the cells seeded out to for organoid growth and viral infection. An aliquot of 10 µL of the organoid suspension used for viral infection was incubated with TrypLE Express for 20 min to allow for dissipation of the organoids into single cells. The solution was then used for cell counting with an improved-Neubauer cell counting chamber. Approximately 25,300 ± 10,300 cells (mean ± SD, N = 10) in the form of organoids were contained in each well.

Oncolytic Measles Viruses

Oncolytic measles viruses were genetically modified from the *Schwarz* vaccine strain. MeV-GFP is a live attenuated, recombinant oncolytic measles virus in which the genetic information for the GFP marker protein was integrated at genome position one (**Figure 2**). MeV-SCD is a live attenuated, recombinant oncolytic measles virus in which the genetic information for the prodrug converting enzyme super cytosine deaminase (SCD; i.e., a fusion protein of yeast cytosine deaminase and uracil phosphoribosyltransferase) was integrated at the same genome position (**Figure 2**). Expression of GFP allows the monitoring of both viral infection and spread.

Oncolytic Vaccinia Viruses

Vaccinia virus GLV-0b347 is derived from a *Western Reserve* vaccinia virus strain (**Figure 2**). The locus of the J2R gene, encoding for a thymidine kinase, has been replaced with a vaccinia synthetic early/late promotor and TurboFP635, a red fluorescent protein derived from the sea anemone *Entacmaea quadricolor* (**Figure 2**) (Wiedenmann et al., 2002). Disruption of J2R moreover results in reduced virulence (Zhang et al., 2009). GLV-1h94 contains a *Lister* vaccinia virus strain (LIVP) backbone (Zhang et al., 2009). In GLV-1h94 the A56R gene (encoding for a thymidine kinase) has been disrupted by the insertion of the vaccinia synthetic early/late promoter and the suicide gene FCU1, also leading to an attenuated virus (Zhang et al., 2007). GLV-1h94 expresses the Renilla luciferase–Aequorea green fluorescent protein (RUC-GFP) expression cassette in the gene locus of F14.5L, resulting in an inactivation of the F14.5L gene. The gene locus F14.5L encodes a protein important for cell adhesion and virulence (Izmailyan and Chang, 2008).

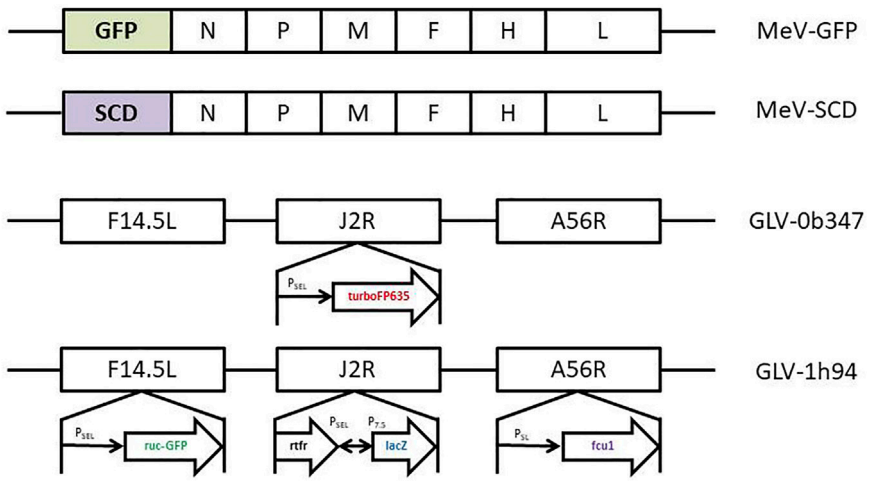

FIGURE 2 | Schematic depiction of the relevant sections of the oncolytic virus genomes for the viruses used in this study. MeV-GFP: The gene encoding for green fluorescent protein (GFP) has been inserted as a transgenic marker gene upstream of the N-gene of the Measles virus genome. MeV-SCD: The gene encoding for the SCD/FCU1 suicide gene has been inserted at the very same position. Key for the MeV genomes: N = nucleocapsid gene; P = phosphoprotein gene; M = matrix protein gene; F = fusion protein gene; H = hemagglutinin gene; L = large protein gene. GLV-0b347: The gene encoding red fluorescent protein (turboFP635) has been inserted in the J2R locus, thus deleting the thymidine kinase function of the respective vaccinia viruses. GLV-1h94: The SCD/FCU1 suicide gene has been inserted in the F14.5L locus. Key to the GLV genomes: F14.5L = open reading frame encoding 49 amino acids; J2R = non-essential gene encoding vaccinia thymidine kinase; A56R = non-essential gene encoding hemagglutinin; turboFP635 = far red mutant of the red fluorescent protein from sea anemone *Entacmaea quadricolor*; ruc-GFP = *Renilla reniformis* luciferase—*Aequorea victoria* green fluorescent fusion protein; rtfr = reverse gene of human transferrin receptor; lacZ = β-galactosidase; P_{SEL} = VACV (vaccinia virus) synthetic early/late promoter; P_{SL} = VACV synthetic late promoter; $P_{7.5}$ = VACV early/late promoter.

Fluorescence Microscopy

Starting 24 h after the infection, imaging was performed on all organoid cultures every 24 h to depict viral spread in the breast cancer cells. The microscope (Olympus IX50 inverted fluorescence phase-contrast microscope) used, was permanently connected to an F-view camera system (Soft Imaging System GmbH, Muenster Germany). Pictures taken with phase contrast (100 ms exposure time) and fluorescence (150 ms–5 s exposure time) were processed using AnalySIS version 3.1 software (Soft Imaging System GmbH, Muenster, Germany).

CellTiter-Blue® Viability Assay

We used the CellTiter-Blue® Assay (Promega, Walldorf, Germany) to measure the viability of organoids after infection. An aliquot of 60 µL was added per well. The plate was then placed back in the incubator for 90 min and measured using a Synergy HT microplate reader and Gen5.11 software (BioTek Instruments, Winooski, VT).

Statistical Analysis

To determine the percentage of surviving cells with the CellTiter-Blue® Assay we divided the read out of organoids treated with virus, 5-FC or 5-FU by the read out of untreated organoids (no virus, 5-FC or 5-FU). As Matrigel alone exhibits a small signal with the assay, this control value was deducted from all original values before calculating the percentage of surviving cells. All data are expressed as mean ± standard deviation (SD) of ten independent experiments performed in duplicate. Statistical analyses were performed using GraphPad Prism software (GraphPad Holdings, LLC, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed to determine whether there were any significant differences between the groups or between the different MOIs of 10, 1 and 0.1. Subsequent post-hoc Tukey's multiple comparisons tests were performed to determine statistical significance between any two groups. A significance level of $p < 0.05$ was used to reject the null hypothesis that there was no difference between the groups tested. We expressed the level of significance with the following annotations in the figures: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Stable Organoid Cultures Prepared From Breast Cancer Tissues

Organoids from breast cancer patients were mainly observed as being circular and dark colored (**Figure 3A**, left picture). In some cases, however, the organoids could also appear paler and appeared as a more cystic structure (**Figure 3A**, right picture). The cells from breast cancer tissue visible on day 1 [**Figure 3B**, top left picture; passage 0 (p0)] over time grew into clusters and formed organoids (**Figure 3B**, other pictures). Tumor grade appeared to influence the growth of organoids as follows: breast cancer tissue graded G1 (organoid line BC-ORG 1) and G2 (organoid line BC-ORG 2) appeared similar and showed growth in clusters, whereas breast cancer tissue graded G3

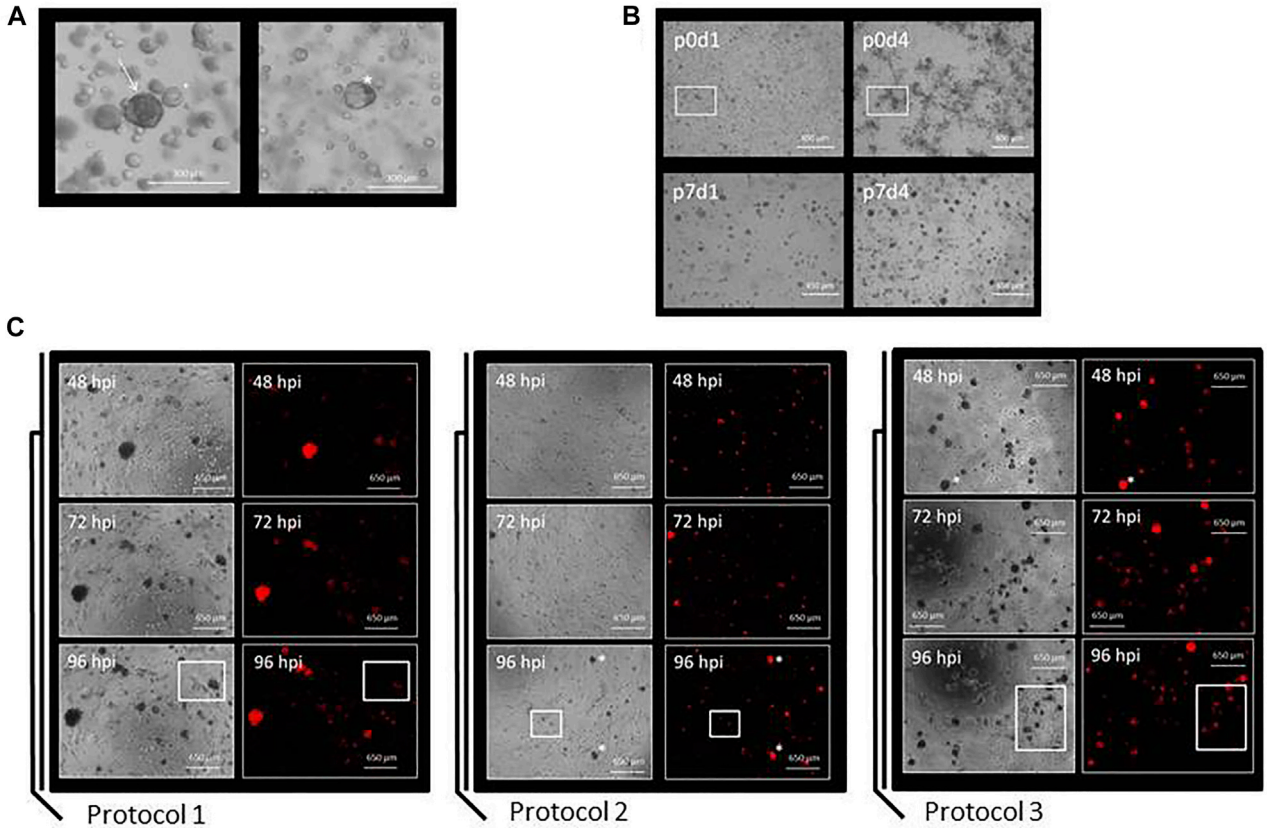

FIGURE 3 | Preparation of organoid cultures from breast cancer tissues and a comparison of the three different protocols used to infect the organoid cultures with oncolytic viruses. **(A)** Organoids from breast cancer patients were mainly observed as being circular and dark colored (arrow in left panel). In some cases the organoids could be paler and appeared as more cystic structures (asterisk in right panel). **(B)** Growth characteristics of breast cancer organoid line BC-ORG 3. Pictures were taken at different passages (p) and on different days (d). Single cells being visible on day 1 (upper left panel) grew into organoids of comparable size and density during the subsequent days (other panels). **(C)** Comparison of three different protocols used for infection of the breast cancer organoid cultures. Protocol 1 was based on standard methods for two-dimensional cell cultures and the images show cells from patient sample BC-ORG 3 harvested with TrypLE and plated out in 10% Matrigel before being infected 24 h later with oncolytic virus GLV-0b347 (MOI 1) and taking phase-contrast and fluorescence pictures at different hpi. Protocol 2 involved incubating the cells immediately with the oncolytic virus and not waiting 24 h. The images show patient sample BC-ORG 5 infected with GLV-0b347 (MOI 10) before taking phase-contrast and fluorescence pictures. Protocol 3 allowed the growth of organoids and even distribution of oncolytic viruses throughout the organoids. Organoids were harvested with dispase II rather than TrypLE after being cultivated in normal growth environment without addition of oncolytic viruses. The oncolytic viruses were then added to the organoid suspension and subsequently distributed into the wells before growth of the organoids. The images show cells from patient sample BC-ORG 5 infected with GLV-0b347 (MOI 10) before taking phase-contrast and fluorescence pictures.

(organoid line BC-ORG 3) displayed a more evenly distributed growth of organoids (**Figure 4**).

Establishing a Reliable Protocol for Infecting the Organoid Cultures Derived From Breast Cancer Tissues With Oncolytic Viruses

In Protocol 1 we first tested the infection of breast cancer tissue organoids based on established methods for testing oncolytic virotherapy in two-dimensional cell culture models. Here the organoids were plated out in 10% Matrigel after regular passaging and infected 24 h later with GLV-0b347 (**Figure 3C**) and MeV-GFP (data not shown). The distribution of the resulting fluorescence was used as an indicator for the distribution of viral spread throughout the organoids. Following infections with oncolytic vaccinia virus GLV-0b347, red fluorescent organoids could be seen first at 48 h post infection (hpi) (**Figure 3C**, Protocol 1, upper panels) and even more fluorescent organoids could be observed at 72 hpi and 96 hpi (**Figure 3C**, Protocol 1, middle and lower panels). However, even at 96 hpi some organoid clusters still were not found to be infected (**Figure 3C**, Protocol 1, lower panels). Beyond that infections did not appear to be distributed homogeneously throughout the wells.

Hence, we set out to improve the cultivation conditions to enable both higher infection rates as well as more evenly distributed infections. For this purpose, the timing of the infections was changed. Instead of infecting organoids at 24 h after passaging, oncolytic viruses now were directly added to

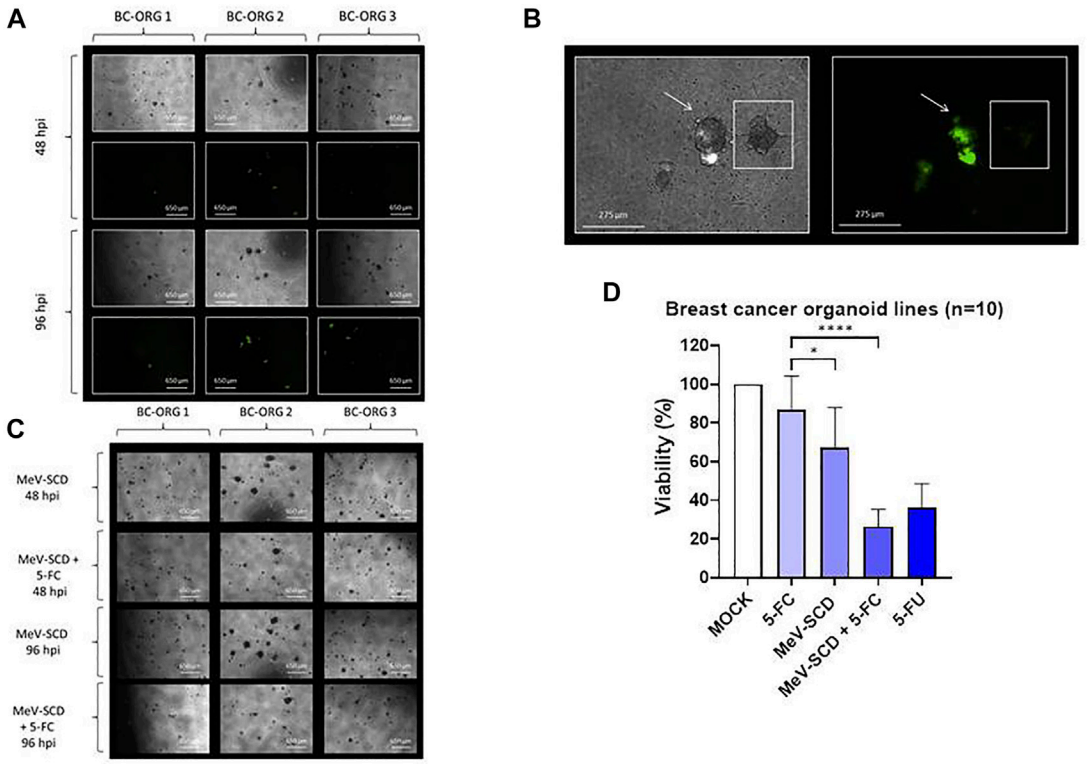

FIGURE 4 | The effects of different genetically engineered oncolytic measles viruses (MeV) on organoids derived from breast cancer tissues (n = 10). **(A)** Phase-contrast and fluorescent images of breast cancer organoid lines BC-ORG 1, BC-ORG 2 and BC-ORG 3, respectively, at 48 and 96 hpi with MeV-GFP (MOI 10) as representative images of all 10 infected breast cancer organoid lines. **(B)** Higher magnification phase-contrast and corresponding fluorescence images of breast cancer organoid line BC-ORG 2 infected with MeV-GFP (MOI 10) at 96 hpi. The organoid highlighted with an arrow (same image in the left and right panel) has been infected with oncolytic measles virus (MeV-GFP) as seen by the green fluorescence. The neighboring organoid highlighted with a small square box (same image in the left and right panel) was not infected and did not express GFP. **(C)** Phase-contrast images of the same breast cancer organoid lines taken at 48 and 96 hpi with MeV-SCD (MOI 10) with and without 5-FC. **(D)** The effects of oncolytic MeV-SCD (MOI 10) in presence (+5-FC) and absence of 5-FC, on mean viability (%) of organoids derived from all 10 breast cancer patients (*p < 0.05, ****p < 0.0001 with post-hoc Tukey's multiple comparisons tests). MOCK contained breast cancer organoids and culture medium.

the organoid culture suspension containing Matrigel and breast cancer culture medium. When applying this Protocol 2 more organoids were found to be infected by GLV-0b347 at 48 hpi (**Figure 3C**, Protocol 2, upper panels). The viral distribution appeared to be more even (**Figure 3C**, Protocol 2, panels to the right) and at 96 hpi the infection was found to have spread broadly across the wells (**Figure 3C**, Protocol 2, lower panels). However, fewer single cells grew out into organoids when compared with Protocol 1.

The next aim was to harvest the organoids without first dissipating them into single cells. For this purpose, organoids were harvested using 100 µL of 1 mg/ml dispase II rather than TrypLE after being cultivated in normal growth environment without addition of oncolytic viruses. Oncolytic viruses were then added to the organoid suspension and seeded into the wells (**Figure 3**, Protocol 3). This modification resulted in a greater number of large organoids (**Figure 3C**, Protocol 3) when compared to both previous protocols (**Figure 3C**, Protocols 1 and 2). This trend could be observed at all time points, but was clearest at 96 hpi (**Figure 3C**, Protocol 3, lower panels). This time point also showed the greatest total amount of GLV-0b347-mediated fluorescence. As similar data was obtained with the oncolytic measles virus MeV-GFP (data not shown), Protocol 3 was utilized for all subsequent infection experiments with the oncolytic viruses of both measles and vaccinia virus origin.

Effects of Oncolytic Measles Viruses

Employing Protocol 3, we next compared the oncolytic effects of MeV-GFP and MeV-SCD in ten organoid lines derived from 10 different breast cancer patients (**Table 1**). The mean values (±SD) of all 10 organoid lines are shown in **Figure 4D**. BC-ORG 1, BC-ORG 2 and BC-ORG 3 (**Figures 4A–C**) are depicted as typical representative images from all 10 breast cancer organoid lines shown in **Figure 4D**. All infections with MeV-GFP were successful as indicated by the green fluorescence at 48 and 96 hpi (**Figure 4A**; BC-ORG 1, BC-ORG 2 and BC-ORG 3).

Magnifications of phase-contrast and corresponding fluorescence images of breast cancer organoid line BC-ORG 2

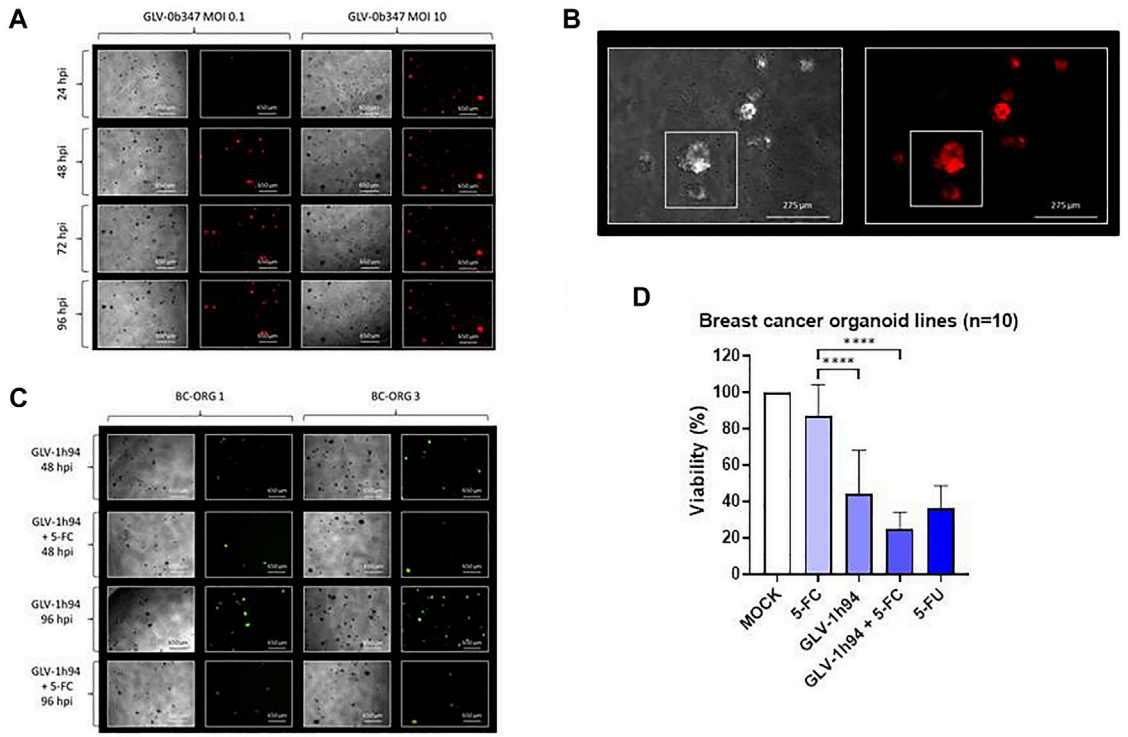

FIGURE 5 | The effects of different vaccinia viruses (GLV) on organoids derived from breast cancer tissues (n = 10)**. (A)** Phase-contrast and fluorescent images of breast cancer organoid line BC-ORG 4 taken at 24, 48, 72 and 96 hpi with two different titers of GLV-0b347 (MOI 0.1 and 10) typically representing all 10 infected breast cancer organoid lines. **(B)** Higher magnification phase-contrast and corresponding fluorescence images of breast cancer organoid line BC-ORG 4 infected with GLV-0b347 (MOI 1), taken at 96 hpi. The images show an infected organoid with intense red fluorescence and a partially infected organoid with partial red fluorescence (see small square boxes in the panels to the left and right). **(C)** Phase-contrast and fluorescence images of breast cancer organoid lines BC-ORG 1 and BC-ORG 3 infected with GLV-1h94 (MOI 10) with and without 5-FC at 48 and 96 hpi typically representing all 10 infected breast cancer organoid lines. **(D)** The effects of oncolytic GLV-1h94 (MOI 10) in presence (+5-FC) and absence of 5-FC, on mean viability (%) of organoids derived from all 10 breast cancer patients (*$p < 0.05$, ****$p < 0.0001$ with post-hoc Tukey's multiple comparisons tests). MOCK contained breast cancer organoids and culture medium.

taken at 96 hpi display an organoid being successfully infected with MeV-GFP (**Figure 4B**, highlighted by the arrows), while the neighboring organoid was found not to be infected (**Figure 4B**, highlighted with a square box).

Next, breast cancer organoid lines BC-ORG 1, BC-ORG 2 and BC-ORG 3 were infected with the suicide gene-enhanced vector MeV-SCD and then cultivated in presence or absence (control samples) of the prodrug 5-FC. When cultivation took place in presence of 5-FC, significantly fewer organoids could be detected (**Figure 4C**, compare images taken at 48 hpi [row 2 (+5-FC) versus row 1 (- 5-FC)] and at 96 hpi [row 4 (+5-FC) versus row 3 (- 5-FC)]. This effect also was quantified by employing the CellTiter-Blue® Viability Assay (**Figure 4D**). A mock infection containing breast cancer organoids and culture medium was defined as maximum viability (100%); 1) cultivation of MeV-SCD-infected organoids in presence of the prodrug 5-FC resulted in a highly significant drop in viability to 26% (**Figure 4D**, bar 4) when compared to the negative control (**Figure 4D**, bar 2; incubation only with the non-toxic prodrug 5-FC, no infections) ($p < 0.0001$); 2) cultivation of MeV-SCD infected organoids in the absence of the prodrug 5-FC displayed an intrinsic oncolytic effect (without additional tumor cell-bound conversion of 5-FC into 5-FU and derivatives), which resulted in a significant drop of viability ($p < 0.5$) to only 67% (**Figure 4D**, bar 3) compared to 5-FC alone (**Figure 4D**, bar 2). All CellTiter-Blue® Viability Assay values are expressed as the mean ± SD (n = 10).

Effects of Oncolytic Vaccinia Viruses

We also systematically investigated the oncolytic effects of vaccinia viruses GLV-0b347 and GLV-1h94 in ten organoid lines from different breast cancer tumors, again using Protocol 3 for infection (**Figure 5**). We observed titer-dependent effects of GLV-0b347-mediated oncolysis which are typified by the phase-contrast and fluorescence images of breast cancer organoid line BC-ORG 4 at 24, 48, 72 and 96 hpi shown as a representation of all 10 infected breast cancer organoid lines (**Figure 5A**, rows 1–4). Higher magnification phase-contrast and corresponding fluorescence images of breast cancer organoid line BC-ORG 4 infected with GLV-0b347 (MOI 1) at 96 hpi showed typically infected organoids with an intense red fluorescence (**Figure 5B**, small square box, upper organoid) and a partially infected organoid with less intense red fluorescence (**Figure 5B**, small square box, lower

organoid). **Figure 5C** shows typical phase-contrast and fluorescence images of two breast cancer organoid lines (BC-ORG 1, BC-ORG 3) at 48 and 96 hpi with the suicide gene-encoding oncolytic vaccinia virus GLV-1h94 cultivated in presence (+5-FC) and absence (- 5-FC) of the prodrug 5-FC. These images are typical of the effects seen on all 10 infected breast cancer organoid lines. The oncolytic effects of GLV-1h94 in the presence of 5-FC were found to be much stronger than in the absence of the 5-FC [**Figure 5C**, compare row 1 with row 2 (48 hpi time point) as well as row 3 with row 4 (96 hpi time point)].

Breast cancer organoid lines BC-ORG 1 and BC-ORG 3 were infected with suicide gene-enhanced GLV-1h94 and then cultivated in the presence or absence (control samples) of the prodrug 5-FC. Fewer viable organoids could be seen when cultivation took place with GLV-1h94 in combination with 5-FC in comparison to GLV-1h94 alone (**Figure 5C** compares images taken at 48 hpi [row 2 (+5-FC) versus row 1 (- 5-FC)] and at 96 hpi [row 4 (+5-FC) versus row 3 (- 5-FC)]. The CellTiter-Blue® Viability Assay enabled quantification of this effect (mean ± SD) for all 10 infected breast cancer organoid lines (**Figure 5D**). Maximum viability was defined by a mock infection containing breast cancer organoids and culture medium (100%); 1) cultivation of GLV-1h94 infected organoids in presence of the prodrug 5-FC resulted in a highly significant drop in viability to 25% (**Figure 5D**, bar 4) when compared to the negative control (**Figure 5D**, bar 2; incubation only with the non-toxic prodrug 5-FC, no infections with oncolytic viruses) ($p < 0.0001$); 2) cultivation of GLV-1h94-infected organoids in the absence of the prodrug 5-FC displayed an intrinsic oncolytic effect (without additional tumor cell-bound conversion of 5-FC into 5-FU and derivates), which resulted in a significant drop of viability ($p < 0.0001$) to only 43% (**Figure 5D**, bar 3) compared to 5-FC alone (**Figure 5D**, bar 2).

Taken together, our results show that we have developed a protocol to assess the effects of oncolytic viruses in stable organoid cell cultures derived from breast cancer tissues. This model provides a promising *in vitro* method to help further testing and engineering of new generations of virotherapeutic vectors for clinical applications. Beyond that we are opening the way for a future personalized pretesting and treatment of breast cancer patients with oncolytic viruses.

DISCUSSION

In this study we utilized an established experimental model of stable organoid cell cultures from breast cancer tissue first described and characterized by Sachs et al. (Sachs et al., 2018) to assess the effects of genetically engineered oncolytic viruses using two different types of oncolytic viruses, i.e., measles vaccine viruses and vaccinia viruses. Experimental investigation of personalized treatment of breast cancer patients requires a reliable patient-derived breast cancer model. This will facilitate the transfer of treatment options from bench to bedside.

Currently established models for breast cancer research include immortalized human tumor cell lines, rodent xenografts, and immunodeficient, xenograft mouse models (Bosma and Carroll, 1991; Kamb, 2005; Drost and Clevers, 2018). These models have major disadvantages and may only insufficiently represent the patterns of the original cancer patient tumor tissues (Bosma and Carroll, 1991; Kamb, 2005). In contrast, a three-dimensional organoid model based on patient-derived tumor samples should be able to recapitulate the structure of the original tumor and capture disease heterogeneity and the characteristics of the patient's individual tumor (Drost and Clevers, 2018; Sachs et al., 2018). Breast cancer organoid lines were successfully and reproducibly established from different patients in this study and several different tumor samples were used to capture the heterogeneity of breast cancer tissues.

The goal of the study was to establish a protocol for assessing oncolytic therapy in organoid cultures derived from breast cancer patients. All protocols used in this work showed success of oncolytic virotherapy as demonstrated by detection of fluorescence by microscopic images and reduction of organoid viabilities, measured with the CellTiter-Blue® Viability Assay. To improve viral spread, the time-point of infection was varied in relation to the passaging process to allow infection of organoids in a three-dimensional setup rather than aiming at single cells being infected in a three-dimensional setup. This resulted in a more homogenous distribution of the viral infections und enhanced the reduction of organoid viabilities through virus-mediated oncolysis. The CellTiter-Blue® Viability Assay was able to measure the reduction of organoid viabilities. However, it was important to note that Matrigel exhibits an inherent background signal in this assay and therefore needs to be corrected for when performing such measurements. We then set out to test whether single cells contained in the organoid setup interfere with the measurements *via* the CellTiter-Blue® Viability Assay.

At this step of our protocol development, cultivation of the breast cancer organoid lines did not yet include immune cells. For example, measles viruses normally induce an IFN response which triggers an immune response directed against the tumor cells (Krabbe and Altomonte, 2018). Tumor cells are known for mutations in IFN signaling thereby enabling an enhanced spread of oncolytic viruses throughout the tumor which also facilitates a subsequent anti-tumoral immune response (Kirn et al., 2001). The cultivation of breast cancer organoids without immune cells does not allow a measurement of the effects this immune response on the oncolytic virotherapy. Dijkstra *et al.* (Dijkstra et al., 2018) successfully incorporated autologous peripheral blood monocytes derived from patient blood samples into the corresponding colon and lung cancer organoid setup. In this setting, the T-cell infiltration of the patient's cancer organoids could be measured and displayed an efficient killing of cancer organoids. Accordingly, the addition of patient-derived peripheral blood monocytes to our breast cancer organoid setup could be a next step to improve our model yet further. It would allow a more accurate representation of the environment surrounding the tumor in the patient. The addition of these cells to oncolytic virotherapy also would allow a better assessment of the importance of the immune response on the efficiency of the oncolytic virotherapy.

The incorporation of immune cells into the organoid cultivation would also allow the evaluation of oncolytic viruses

that have been engineered for triggering an immune response against tumor cells specifically. For example, talimogene laherparepvec/T-VEC constitutes the first clinically licensed (for advanced malignant melanomas) oncolytic virus (IMLYGIC®) in the category of therapeutically armed oncolytic viruses. T-VEC is based on a recombinant herpes-simplex virus 1 (Hu et al., 2006) and also interferes with the IFN pathway resulting in enhanced tumor selectivity and effectivity (Liu et al., 2003). An additional genetic modification results in a higher production of class I major histocompatibility complex (MHC) molecules important for triggering an immune response against the host cells (Hill et al., 1994; Hill et al., 1995). The viral genome has been genetically modified to include the arming with a granulocyte-macrophage colony stimulating factor (GM-CSF) gene (Greig, 2016). Also T-VEC needs to be compared to other oncolytic viruses in our organoid model in future work.

Oncolytic viruses can be classified into three different types; 1) oncolytic viruses with natural anti-neoplastic properties, 2) oncolytic viruses designed for tumor-selective replication, and 3) armed oncolytic viruses (Hartkopf et al., 2011). Four oncolytic viruses derived from two virus families were tested in our organoid model. Two viruses exhibiting an intrinsic oncolytic activity (MeV-GFP, GLV-0b347) were used as well as the same two virus backbones additionally being armed with suicide genes to enhance naturally occurring oncolytic activity (MeV-SCD, GLV-1h94), thereby achieving an additional tumor-cell bound conversion of 5-FC into 5-FU and derivatives, i.e., a tumor cell-localized chemotherapy.

Measles viruses and Vaccinia viruses show innate oncolytic potential (Hartkopf et al., 2011). MeV-GFP and GLV-0b347 are recombinant vaccine viruses in which the marker genes 1) green fluorescent protein (MeV-GFP) or 2) red fluorescent protein (GLV-0b347) are encoded as transgenes. The insertion of the SCD/FCU1 suicide gene to the measles virus genome allowed evaluation of the additional influence of such a suicide gene on organoid viability. MeV-GFP and MeV-SCD showed a comparable reduction of organoid viability. Of note, the effect of MeV-SCD could be enhanced significantly when the prodrug 5-FC was added to the culture medium. Under this condition, organoid viabilities displayed a similar drop in organoid viability as when treated with the chemotherapeutic compound 5-FU. These results demonstrate that wild-type vaccine measles virus exerts an intrinsic oncolytic effect in the breast cancer organoid lines. This basic oncolytic effect of the measles virus is further enhanced when it encodes a suicide gene which in presence of the prodrug 5-FC conveys an additional tumor cell-localized chemotherapy.

GLV-0b347 and GLV-1h94 are both vaccinia viruses, yet include a different genetic backbone. GLV-0b347 is based on a *Western Reserve* strain backbone and GLV-1h94 on a *Lister* strain backbone. As previous research had demonstrated different distribution rates of the viruses in different types of tissues, vaccinia viruses with different backbones were used for the experiments (Zhang et al., 2007). However, the reduction in organoid viability between GLV-0b347 and GLV-1h94 was comparable. This suggests similar distribution of both vaccinia viruses in breast cancer tissues. Both, GLV-0b347 and GLV-1h94 also inhibited cell viability in breast cancer organoid lines. As expected, oncolytic effects of GLV-0b347 were found to be dependent on the virus titer used. The oncolytic effects of both MeV-SCD and GLV-1h94 were enhanced in the presence of the prodrug 5-FC which is converted to the active and cytotoxic metabolite 5-FU and 5-fluorouridine by the expressed suicide gene conversion enzyme SCD/FCU1.

The addition of 5-FC to the infection with GLV-1h94 led to a significant decrease in organoid viability in comparison to the infection with vaccinia viruses alone or 5-FC alone. Importantly, the combination of these agents resulted in greater reduction in organoid viability than 5-FU alone when using the same compound concentrations (1 mmol/L each). Therefore, the oncolytic effect of vaccinia viruses equipped with the suicide gene and the prodrug 5-FC cannot be based solely on the effects of the production of 5-FU. The oncolytic effect of the GLV-1h94 resulted in an additional reduction of organoid viability. The innate oncolytic effect of vaccinia viruses as well as the combination with a suicide gene seemed to work synergistically.

Taken together, our organoid model enabled oncolytic viral infection in breast cancer organoid lines. The next step would be to compare and contrast the effects of other oncolytic viruses such as T-VEC and test them on our breast cancer organoids to establish a panel most likely to be effective for oncolytic virotherapy of breast cancer. The viruses included in this panel should combine different approaches such as viruses with naturally occurring oncolytic potential, genetically modified virus for tumor selectivity and armed oncolytic viruses for enhanced cell killing or enhanced triggering of an immune response. Our study showed that infections with oncolytic viruses are possible in our organoid culture setup of primary breast cancers.

CONCLUSION

This study shows that it was possible to develop a protocol that could be used to assess the effects of two different oncolytic viruses on cell viability in established patient-derived organoid cell cultures from breast cancer tissue. The greatest oncolytic effects were observed for oncolytic viruses engineered to express a suicide gene (MeV-SCD; GLV-1h94) in the presence of the prodrug 5-FC. Thus the model provides a promising *in vitro* method for investigating the effects of different oncolytic viruses for treating breast cancer, thereby facilitating the correlation to *in vivo* results.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical commission of the Medical Faculty of the

Karl Eberhard University and University Clinic in Tuebingen (210/2019BO2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualisation: MC, AK, LV, AH, SB, UL Preliminary Establishment of the organoid model: AW, AK, MC Experimental Work: MC Data Curation: MC Writing: Original draft: MC Writing: Review and Editing: MC, AK, AH, UL Supervision: AK, AH, SYB, SB, UL All authors have read and approved the final manuscript.

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REVIEW
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Talimogene Laherparepvec: Moving From First-In-Class to Best-In-Class

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Talimogene laherparepvec (T-VEC) is a modified oncolytic herpes Simplex virus, type 1 (HSV-1) encoding granulocyte-macrophage colony stimulating factor (GM-CSF). T-VEC is adapted for selective replication in melanoma cells and GM-CSF was expressed to augment host anti-tumor immunity. T-VEC is indicated for the local treatment of melanoma recurrent after primary surgery and is the first-in-class oncolytic virus to achieve approval by the FDA in 2015. This review will describe the progress made in advancing T-VEC to the most appropriate melanoma patients, expansion to patients with non-melanoma cancers and clinical trial results of T-VEC combination studies. Further, strategies to identify predictive biomarkers of therapeutic response to T-VEC will be discussed. Finally, a brief outline of high-priority future directions for investigation of T-VEC and other promising oncolytic viruses will set the stage for a best-in-class oncolytic virus to bring the maximum benefit of this emerging class of anti-cancer agents to patients with cancer.

Keywords: biomarker, cancer, immunotherapy, oncolytic virus (OV), treatment

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INTRODUCTION

Oncolytic viruses (OV) are a new class of anti-cancer therapeutic agents that utilize native or genetically modified viruses to treat cancer. While early reports of tumor regression in patients infected with various viruses has been reports for over a century, advances in molecular genetics and virology have only recently allowed a more directed approach to therapeutic development in this area (Kucerova and Cervinkova, 2016). Based on these early observations, it was thought that OVs most likely mediated tumor regression by preferentially infected and killing tumor cells. Indeed, many cancer cells express high levels of viral entry receptors, and recent data demonstrating defects in the anti-viral machinery in cancer cells, provides a logical mechanism for selective tumor cell killing by oncolytic viruses (Kaufman et al., 2015). Some native viruses possess innate oncolytic activity, and functional tumor cell killing may be enhanced by serial passage through specific cancer cells selecting for viral clades with the highest lytic potential. Alternatively, many viruses can be genetically engineered through deletion or insertion of various viral genes designed to enhance tumor selective replication (Kaufman et al., 2015). While direct viral-mediated lysis of cancer cells was widely accepted as an important process for OV-induced anti-cancer activity, this now appears to not be the major mechanism of action for most OVs.

Viruses are among the most immunogenic agents recognized by the host immune system and the ability of OVs to induce immune responses likely explains the major mechanism involved in OV-mediated anti-cancer activity (Harrington et al., 2019). The induction of host immune responses against viral antigens is dependent on recognition of viral peptides within infected host cells, and this

process allows cancer cells to be specifically targeted for T cell-mediated effector functions (Milich, 1987). This process, which has been referred to as immunogenic cell death allows tumor-associated antigens to be released in the context of an active viral infection, which releases danger-associated factors, that results in immune recognition and eradication of OV-infected cells. In addition, release of soluble tumor antigens can also result in antigen spreading and this allows immune recognition, and in some cases eradication, of non-infected cells. This has been described as an "abscopal" or "anamnestic" response (Andtbacka et al., 2016a). Animal models have confirmed that injection of an index tumor can cause regression of uninjected tumors in an immune-dependent manner (Zamarin et al., 2014). Thus, OVs provide two independent mechanisms that can reinforce tumor-specific immune clearance.

Progress in molecular biology and cloning technology have also allowed expression of eukaryotic genes by viruses. Genomic stability and expression levels are dependent on the size of the gene or genes expressed, the size of the viral genome, the impact on viral integrity and likely additional epigenetic factors, large viruses have been shown to efficiently encode multiple human genes, which can be used to provide additional anti-tumor activity. In many cases, the genes selected for expression are cytokines to enhance local immune responses against the virally infected cancer cells, other strategies have included expression of suicide genes, apoptosis-inducing genes, and radiosensitizers among others (Kaufman et al., 2015). The contribution of transgene expression has not been fully elucidated but does offer an additional pathway for optimizing anti-tumor immunity and therapeutic responses.

While OVs have demonstrated proof-of-principle in a multitude of pre-clinical tumor models, clinical development has been slower. Globally, four OVs have been approved for cancer therapy. In the People's Republic of China, an oncolytic E1B-deleted adenovirus (H101; Oncorine®) is approved in combination with chemotherapy for treatment of head and neck cancers (Liang, 2018). An unmodified picornavirus (enterovirus, ECHO group, type 7; Rigvir®) is approved for the treatment of melanoma in several Eastern European countries (Alberts et al., 2018). In November 2021 a triple-mutated oncolytic HSV-1 (G47Δ), teserpaturev (Delytact), was approved in Japan for the treatment of malignant glioma (Nguyen and Saha, 2021). The only OV to achieve approval in the United States is Talimogene laherparepvec (T-VEC; Imlygic®), which was granted U.S. Food and Drug Administration (FDA) approval in 2015 for the treatment of melanoma (Andtbacka et al., 2015). T-VEC has subsequently been approved throughout Europe, and in Australia and Israel. Since approval, we have learned a lot about both the challenges and best clinical indications for T-VEC treatment. This review will describe the initial clinical development of T-VEC and then focus on our current understanding based on both real-world experience and new clinical trials with T-VEC. While T-VEC has provided another option for patients with melanoma, the integration of T-VEC into clinical practice occurred at a time of unprecedented therapeutic advances in melanoma, including the approval of BRAF/EK targeted therapy and single agents as well as combination immune checkpoint blockade (Luke et al., 2017). Nonetheless, the potential role for OVs, such as T-VEC, remains intriguing and a high priority for predictive biomarkers is needed to better select appropriate patients for effective therapy while avoiding potential toxicities. We will mention some recent insights into biomarkers of OV responses and complete the review by discussing anticipated future directions for T-VEC and other OVs in clinical development.

THE DEVELOPMENT OF TALIMOGENE LAHERPAREPVEC FOR MELANOMA

T-VEC is based on a modified oncolytic herpes Simplex virus, type 1 (HSV-1) that was originally isolated from a fever blister (Liu et al., 2003). The virus was selected for *in vitro* oncolytic activity against a range of tumor cell lines and further modified by deletion of the two viral infected cell protein (ICP) 34.5 genes, which encodes the neurovirulence factor and deletion improves tumor cell selective replication. In addition, the viral ICP47 gene is deleted and this encodes a viral inhibitor of peptide attachment to major histocompatibility complex (MHC) class I, which the virus uses to prevent immune detection during natural infections. The ICP47 deletion was thought to be important in allowing MHC class I loading of tumor-associated peptides, which would be necessary to promote anti-tumor immunity. Finally, T-VEC is modified by inserting two copies of the human granulocyte-macrophage colony stimulating factor (GM-CSF) genes to promote dendritic cell recruitment and activation following antigen uptake from lysing tumor cells. The virus demonstrated therapeutic activity in the murine A20 lymphoma model and was adapted for clinical translation (Liu et al., 2003).

The first clinical trial of T-VEC was reported in 2006 in a phase I study of 13 patients with a variety of cancers, including melanoma, breast, head and neck and gastrointestinal tumors (Hu et al., 2006). Virus was given by direct intra-tumoral injection into superficial, subcutaneous, or nodal accessible tumors. This study established the safety profile, which included low grade constitutional symptoms, such as fatigue, fevers, chills and nausea, and local injection site reactions. Biopsy of injected tumor sites revealed necrosis and other signs of inflammation with virus found only in viable tumor cells and evidence of local GM-CSF expression was confirmed. A series of dosing schedules was used and toxicity was generally lower in patients treated with a lower priming does of 1×10^6 plaque-forming units (pfu) to allow seroconversion in HSV-1-naïve patients, followed by a higher dose of 1×10^8 pfu. This was followed by a multi-institutional phase 2 single-arm study of T-VEC in patients with superficially accessible melanoma (Senzer et al., 2009). In this study an objective response rate of 26% was observed and the safety profile was similar to the profile seen in the phase I study.

Based on the emerging data from the early phase clinical trials, the OPTiM study, was developed as a prospective, multi-institutional randomized phase III clinical trial to determine the clinical benefit of T-VEC in patients with superficially

accessible melanoma. In this study, 436 patients with stage IIIB-IV melanoma were randomized in a 2:1 manner to treatment with T-VEC or recombinant GM-CSF. The control arm was selected to allow study participants to receive potentially active therapy and at the time there was interest in single agent GM-CSF for melanoma, although this was not supported by subsequent studies (Spitler et al., 2000). The study used a primary endpoint of durable response rate that was defined as an objective response per modified World Health Organization (WHO) criteria and persistent for at least 6 months. An objective response rate of 26.4% was seen compared to 5.7% for patients treated with GM-CSF; and durable response was 16.3% compared to 2.1%, which met the primary study endpoint. In addition, median overall survival was improved in T-VEC-treated patients compared to GM-CSF therapy [23.2 vs. 18.9 months (hazard ratio, 0.79; 95% CI, 0.62 to 1.00; p = .051)] (Andtbacka et al., 2015). At a median follow-up of 49 months, a final analysis was performed and demonstrated an objective response rate of 31.5 versus 6.4% for GM-CSF was reported and durable response rate of 19% for T-VEC vs. 1.9% for GM-CSF ($p < .0001$) was seen (Andtbacka et al., 2019). Further, the OS benefit for T-VEC compared to GM-CSF persisted (hazard ratio, 0.79; 95% CI 0.62, 1.00; p = .0494). The 5-year survival for patients was 33.4% and the impact on survival was most pronounced for patients with stage IIIB/C and IVM1a melanoma (hazard ratio, 0.57; 95% CI 0.41, 0.81; $p < .001$). In the OPTiM trial, 54% of patients exhibited some degree of disease progression based on caliper measurement or imaging prior to achieving an objective response, suggesting that pseudo-progression may be possible with T-VEC treatment. Based on these data T-VEC was approved by the U.S. FDA in 2015 for the local treatment of melanoma that recurs after initial surgery and T-VEC was approved in Europe in 2016 for the local treatment of melanoma patients with stage IIIB-IVM1a disease. Australia, Israel, and Switzerland have also approved T-VEC for the treatment of melanoma. The adverse events in the phase III clinical trial were similar to earlier phase studies establishing a favorable safety profile for T-VEC.

T-VEC was the first OV approved for cancer treatment and provided a new therapeutic strategy for patients with melanoma. Importantly, the approval in 2015–2016 corresponded to a time with major changes in the therapeutic landscape of melanoma. In 2011 the first BRAF inhibitor was approved for metastatic melanoma patients with BRAF V600E/K mutated tumors, which would be followed by combination BRAF and MEK inhibition therapy (Chapman et al., 2011; Flaherty et al., 2012). In addition, immune checkpoint inhibition achieved approval initially with ipilimumab, a monoclonal antibody that blocks the cytotoxic T lymphocyte antigen 4 (CTLA-4) negative T cell regulatory, in 2011 and then with pembrolizumab and nivolumab, both monoclonal antibodies that block the programmed cell death 1 (PD-1) checkpoint, in 2015. Thus, multiple new drugs in the targeted therapy and immunotherapy arena became accessible for melanoma patients. These drugs would also go on to be approved in the adjuvant setting (Eggermont and Dummer, 2017). Thus, the clinical implementation of T-VEC would take some time to integrate with other agents available for the treatment of advanced melanoma.

REAL WORLD EXPERIENCE WITH T-VEC

The launch of T-VEC was challenging for several reasons. First, OV storage and intratumoral delivery of the agent offered unique challenges for local pharmacies and healthcare providers, as outlined in **Table 1**. In addition, T-VEC was approved around the same time as the approval of immune checkpoint blockade and targeted therapy, which are given by intravenous and oral administration, respectively. Because several other promising agents were available, many patients were considered for T-VEC only after having progressive disease after other treatments. Over the last 5 years, however, there has been considerable real-world data published providing a better idea of how best to integrate T-VEC treatment into the clinical practice and how to optimize patient selection and management (Perez et al., 2018; Louie et al., 2019; Mohr et al., 2019; Perez et al., 2019; Louie et al., 2020; Sun et al., 2020; Kleemann et al., 2021; van Akkooi et al., 2021). In addition, techniques for injecting T-VEC have now been well established (**Figure 1**).

In a retrospective review of T-VEC in patients with stage IIIB-IV1a melanoma at a single German facility, 27 patients were treated with T-VEC between June 2016 and July 2017 (Mohr et al., 2019). All patients had undergone prior surgery and 63% of the patients received T-VEC as their first line of melanoma treatment. Of these patients, only one required subsequent systemic treatment. In a separate, multi-institutional U.S.-based retrospective review, 121 patients receiving T-VEC from October 2015 through October 2018 were identified with a median follow-up of 9 months and 80 patients were available for evaluation (Louie et al., 2019). Thirty-four (42.5%) of the patients evaluated received T-VEC as first-line treatment and an objective response was seen in 45 (57%) of patients after a median of six treatment cycles, including a complete response in 31 (39%) and partial response in 14 (18%) of patients, higher than that observed in the OPTiM phase III clinical trial (Andtbacka et al., 2015). In another independent review of T-VEC treatment at seven academic institutions, 76 patients were identified over a similar time period as these other trials but included a substantial number (43.4%) who had received prior checkpoint blockade prior to T-VEC treatment (Perez et al., 2019). Fifteen (19.7%) of patients achieved a pathologic complete response to treatment after a median duration of 3 months of treatment. Importantly, all these studies confirmed the initial safety profile of T-VEC and found that therapy was generally well tolerated with mostly low-grade constitutional symptoms and local injection site reactions. Collectively, these studies suggested that early use of T-VEC in the first-line setting may be preferrable to more advanced clinical settings.

A more recent real-world report was published on 127 patients in the national German prescription database (Louie et al., 2020). Of the patients identified, two-thirds were started in or after 2017 and most (88%) were treated by hospital sites. At the end of the study, 26 (36%) of the patients remained on T-VEC and the overall median duration of treatment was 18.7 weeks and was longer for those who started

TABLE 1 | Barriers and challenges to oncolytic virus clinical implementation.

Challenge to OV implementation	Comments
Requires storage at −80°C	• Many pharmacies do not have deep freezer capabilities
Live virus must be prepared in sterile biosafety cabinet	• Dedicated preparation space is often difficult in pharmacies preparing chemotherapy and other agents • Contamination of other drug products requires strict SOPs and dedicated time, space, and training for pharmacists
Drug dosing is different for initial injection vs. later timepoints	• Two different doses must be maintained and prepared appropriately
Drug volume is dependent on maximal tumor diameter	• Volume cannot be determined until the patient has tumor measured resulting in ordering delays and longer patient treatment wait times • May require new ordering forms/processes
Injection requires direct access to tumor site and manual administration	• Lesions may not be palpable or may regress to a size that is not detectable • Bedside ultrasound can help guide injections and may be used when lesion regress below levels of clinical detection • Technical training is required for optimal delivery • May be administered by non-physicians, such as nurse practitioners or physician assistants
Biosafety concerns	• OV are typically live, replicating viruses and clinics must adopt biosafety measures for spills and waste • Usually only requires standard universal precautions
Household and healthcare transmission	• Virus can be transmitted to close contacts • Acyclovir and other anti-virals which may be used in cases of inadvertent exposure • Transmission can be prevented by barrier bandages and educating patients to avoid direct contact between injection site and other individuals • Training for healthcare providers, affiliated clinic staff, patients and patient families may help prevent accidental spread
May require change to ambulatory practice	• Can improve process by dedicating specific room(s) and clinic day(s) for OV injection • Healthcare centers may require written SOPs and approval by biosafety and/or infection control committees • Practice deviations may be difficult if only a limited number of patients are treated with OV therapy at site

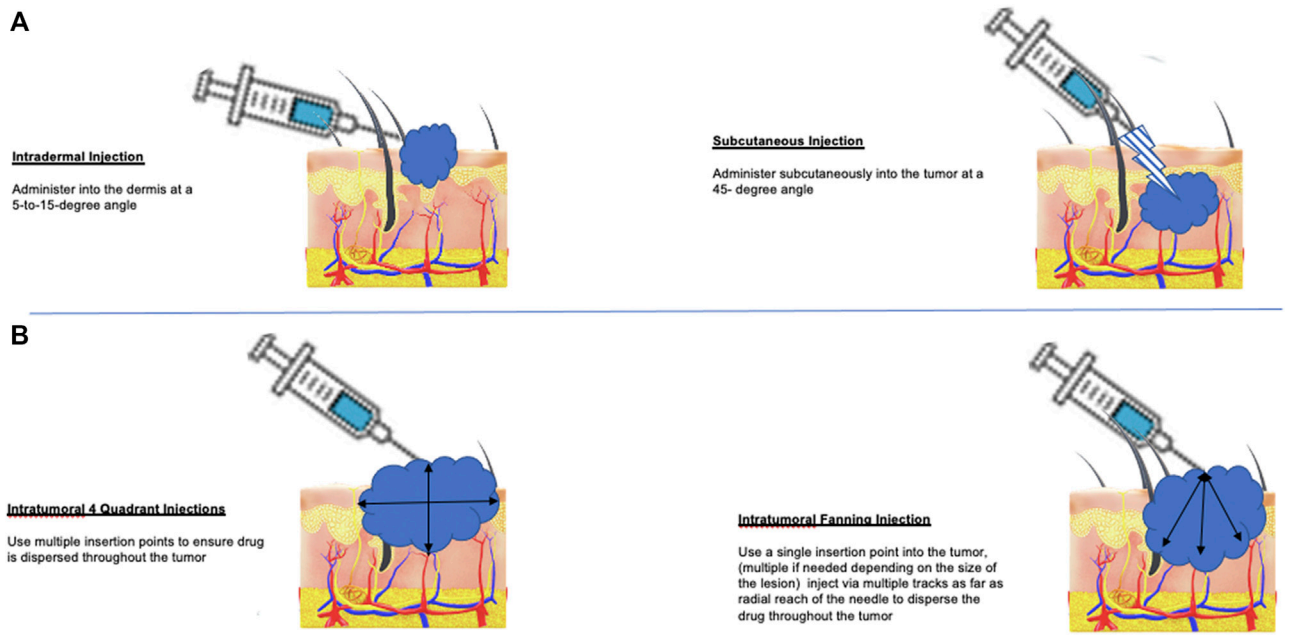

FIGURE 1 | Methods for T-VEC administration in patients with melanoma. **(A)** T-VEC can be administered by intradermal injection (left panel) at sites of cutaneous tumor or by subcutaneous injection (right panel) for tumors in the soft tissue or lymph nodes. If tumors are not clinically palpable or become undetectable after initiating treatment, portable ultrasound can be used to identify residual areas of tumor for injection. **(B)** The goal of injection is to distribute the virus as evenly as possible throughout areas of viable tumor cells. This can be done using a four-quadrant method (left panel), which allows re-insertion to reach all sites and may be preferred for large tumors; alternatively, a single injection site and then using a fan technique (right panel) for injection can also be used. In tumors with necrotic or liquid areas, peripheral injection at the edges where most viable tumor cells are located is also acceptable.

treatment in 2017 compared to those treated in 2016 (26.7 vs. 15.6 weeks, respectively. The authors concluded that with more clinical experience after 2017, patients were appropriately kept on treatment longer as more physicians recognized the possibility of pseudo-progression following T-VEC treatment. Another trial reported for patients in the 2017–2018 era evaluated the relationship between T-VEC and anti-PD-1 treatment (Sun et al., 2020). In 83 patients from multiple institutions, three patterns were observed. Twenty-two (26.5%) of the patients received T-VEC after anti-PD-1 therapy, 32 (38.6%) received T-VEC concurrently with anti-PD-1 therapy, and 29 (34.9%) of patients only received T-VEC. Across all groups the objective response rate was 25% and the authors concluded that T-VEC could be used in combination with checkpoint blockade and sequencing did not appear to influence therapeutic responses.

In another single institution retrospective study of T-VEC, 27 patients with a median age of 75 years were treated and results reported at a median follow-up of 8.6 months (Perez et al., 2018). In this study most patients had prior therapy, including four patients having isolated limb perfusion, five patients having prior systemic immunotherapy and four patients having both prior to T-VEC. Further, 22 (81.5%) of the patients had stage III disease and five patients (18.5%) had stage IV disease at the time of T-VEC treatment. Of the 27 patients, 23 met criteria for response assessment and there was disease control rate of 78.3% reported with ten patients (43.5%) having a complete response. The authors concluded that there is a high response to T-VEC and upfront selection of patients with limited disease burden, such as in-transit metastases, may be helpful in improving the likelihood of response.

In a small study of 12 melanoma patients with a median age of 83 years, T-VEC was used and resulted in an overall response rate of 58.3%, durable response rate of 41.7% and a complete remission rate of 25% (Kleemann et al., 2021). In this cohort there were no grade 3 or higher treatment-related adverse events noted. The authors concluded that T-VEC may be an important consideration for older patients with melanoma who may not be able to tolerate other systemic options. Overall, the real-world data suggests that objective responses and safety profile for T-VEC in melanoma patients are comparable to those observed in the OPTiM phase III clinical trials (van Akkooi et al., 2021). Clinical benefit may be especially high in older patients and in those receiving T-VEC as first-line treatment. Although these studies are subject to bias due to their retrospective nature and influenced by treatment changes in both adjuvant and metastatic melanoma therapy over time, they do support the concept of using T-VEC earlier in the disease course and that T-VEC may be a safe option for older patients who may not be eligible for other systemic treatments.

EXPANDING THE CLINICAL INDICATIONS FOR T-VEC IN MELANOMA

In a subset analysis of the OPTiM clinical trial, a higher response rate was noted in patients with head and neck melanomas (Andtbacka et al., 2016b). Of the 436 patients enrolled in the phase III randomized trial, 87 (19.9%) had melanomas located in a head or neck location. Of these 87 patients, 61 were treated with T-VEC and 26 with recombinant GM-CSF. The durable response rate was 36.1% for patients treated with T-VEC compared to 3.8% for GM-CSF and 16.3% for all patients treated with T-VEC). A complete response was seen in 29.5% of the head and neck melanoma patients treated with T-VEC. The probability of maintaining an objective response after 12 months was 73%. While the overall survival of the entire T-VEC-treated population was 23.2 months, the median overall survival had not been reached in the head and neck melanoma subset. While it is tempting to hypothesize that the head and neck melanomas may be more responsive due to the increased tumor mutation burden likely related to Sun exposure, this has not been formally confirmed. Nonetheless, the data suggests that there may be subsets of melanoma patients more likely to benefit from T-VEC treatment.

Another subset of melanoma patients that were not treated in the OPTiM trial are patients with organ allografts. This represents an important unmet medical need as malignancy is more common in transplant recipients with an increased incidence over time and cutaneous tumors, including melanoma are especially common. Because of the risk of allograft rejection, treatment with potent immunotherapy, such as immune checkpoint blockade, may not be possible. Thus, the potential benefit of OV therapy in this setting has gained recent attention. Indeed, there are two case reports demonstrating complete responses of locally advanced melanoma with T-VEC treatment in transplant recipients, one with a heart transplant and one with a heart and kidney transplant (Schvartsman et al., 2017; Ressler et al., 2019). In both cases, no new safety signals were reported. Further clinical studies are needed to better understand the full risk-benefit potential for T-VEC and other OVs in patients with transplant-related melanoma.

Although T-VEC was originally developed for patients with advanced melanoma. It is well suited for earlier use as, for example, in the neoadjuvant setting. The rationale for this is to provide an opportunity for T-VEC to induce host anti-tumor immunity by using established tumors as a source for *in situ* vaccination at an earlier time prior to extensive immunoediting as occurs in metastatic disease. A randomized phase 2 trial was conducted in 150 patients with resectable stage IIIB-IVM1a melanoma (Dummer et al., 2021). In this study 76 patients were randomized to six doses of T-VEC followed by surgery and 74 patients received surgery alone with a primary endpoint of 2-year relapse-free survival (RFS) in the intention-to-treat population. The 2-year RFS was 29.5% in the T-VEC arm and 16.5% in the surgery alone arm (overall hazard ratio 0.75, 80% CI, 0.58–0.96). In addition, 2-year overall survival was improved in patients treated with T-VEC followed by surgery compared to surgery alone (88.9 vs. 77.4%; overall hazard ratio 0.49, 80% CI, 0.30–0.79). The pathologic complete response rate in patients treated with T-VEC was 17.1%). This data is promising but requires larger sample size and longer follow-up to better define the true benefit of neoadjuvant T-VEC for melanoma. Given the high-risk for recurrence associated with some subsets of early stage I-II melanoma patients, successful demonstration of a neoadjuvant benefit could also help support clinical studies of T-VEC in high-risk stage II melanoma.

EXPANDING T-VEC TO OTHER CANCERS

While T-VEC was approved for the treatment of melanoma, the virus was able to demonstrate activity against tumor cells derived from other histologic tumors *in vitro* (Liu et al., 2003). This suggests that the agents may be useful in other types of human cancer. This is a concept that has been evaluated now in a small number of clinical trials with interesting yet inconclusive results. In general, accessible tumors for intratumoral injection have been a priority, and this has included head and neck squamous cell carcinoma, soft tissue sarcoma and breast cancer.

A small trial of 17 patients with stage III or IV squamous cell carcinoma of the head and neck was conducted with T-VEC in combination with *cis*-platinum chemotherapy and radiation therapy followed by surgery (Harrington et al., 2010). Fourteen (82.3%) of patients demonstrated objective responses by imaging or clinical exam with 93% showing pathologic complete response at the time of surgery. At a median follow-up of 29 months, disease-specific survival was seen in 82.4% of patients. Although the number was small, the results supported further studies in head and neck cancer. In a phase Ib/III multi-institutional clinical study, T-VEC was evaluated in combination with pembrolizumab in patients with recurrent or metastatic head and neck squamous cell carcinoma (Harrington et al., 2020). Thirty-six patients were entered into the phase Ib portion of the study and data was reported at a median follow-up of 5.8 months. Ten patients (27.8%) of the patients were not evaluable for response due to early mortality. A confirmed partial response occurred in 5 (13.9%) of patients. The authors concluded that while treatment was generally well tolerated combination therapy was not associated with a benefit compared to historical studies with pembrolizumab alone and the phase III portion was not pursued further. This trial only administered T-VEC into cutaneous, subcutaneous, and nodal tumor but did not allow injection into mucosal or visceral sites of disease.

Soft tissue sarcomas have also been targeted for clinical study of T-VEC with intriguing results to date. An open-label, single institution, phase 2 study of T-VEC and pembrolizumab was conducted in 20 patients with locally advanced or metastatic sarcoma patients who had disease progression after at least one other systemic therapy (Kelly et al., 2020). The study was designed with a primary endpoint of objective response rate at 24 weeks and all 20 patients were evaluable for response. The overall objective response rate was 35 and 20% of patients experience grade 3 treatment-related adverse events although no grade 4 events were seen. The authors concluded that the combination of T-VEC and pembrolizumab was worthy of further evaluation. In addition, another phase Ib/II study of T-VEC administered with standard pre-operative external beam radiation therapy was evaluated in patients with locally advanced soft tissue sarcomas of the trunk and extremities measuring more than 5 cm and for whom neoadjuvant radiation therapy was indicated (Monga et al., 2021). In this trial, one patient with a myxoid liposarcoma demonstrated a partial response and 7 (24%) patients had a 95% pathologic necrosis seen in resected tumor. The authors reported no dose-limiting toxicity and no patients had evidence of local recurrence after surgery. The 2-year overall survival was 88% and progression-free survival was 57%. The authors concluded the combination of T-VEC and pre-operative radiation was safe and further studies were warranted.

Breast cancer is another tumor that has been targeted for treatment with T-VEC since recurrent tumors are often accessible for direct injection. T-VEC was evaluated as a strategy for enhancing response to neoadjuvant chemotherapy in patients with triple-negative breast cancer (Soliman et al., 2021). In this phase I clinical trial, nine patients were treated with T-VEC at two dose levels in combination with paclitaxel followed by doxorubicin and cyclophosphamide for 8 weeks prior to surgery. The primary endpoint of the study was safety and no dose-limiting toxicities were reported. A complete pathologic response was seen in 55% of patients. In another phase 2 study, T-VEC was tested in breast cancer patients with inoperable locoregional recurrence (Kai et al., 2021). Nine patients were enrolled and six patients had locoregional disease only and three had additional metastatic lesions. While no significant adverse events were reported, no patients had an objective response. The authors suggested that further studies should consider combination approaches. A study of T-VEC in combination with atezolizumab, an anti-PD-L1 agent, in patients with operable HER2-negative breast cancer with residual disease after neoadjuvant chemotherapy is planned (Pascual et al., 2020). Other studies have been conducted to evaluate T-VEC in pancreatic cancer, hepatocellular carcinoma and non-melanoma skin cancers (NCT00402025; NCT02509507; NCT04163952).

EXPLORING T-VEC COMBINATION STRATEGIES

T-VEC infection triggers type 1 interferon production by infected cells, and this in turn can result in expression of immune inhibitory surface receptors, such as PD-1 ligand 1 (PD-L1) on tumor cells (Bommareddy et al., 2018). Even when interferon signaling in tumor cells is defective, local infection of normal cells can drive local interferon production and, it is now clear, PD-L1 expression can inhibit viral clearance and may also be associated with suppressed immune clearance of tumors. Thus, it is logical to combine T-VEC with immune checkpoint blockade to enhance anti-tumor immunity (Ribas et al., 2017). In a small phase I clinical trial T-VEC and pembrolizumab demonstrated a 62% objective response rate in melanoma patients (Ribas et al., 2017). Further, this study demonstrated that T-VEC was able to induce regression of lymphocyte-deficient tumors, which is a negative predictive feature of pembrolizumab responses. The high response rate observed was the impetus for a larger, prospective randomized phase III trial of T-VEC and pembrolizumab versus placebo and pembrolizumab (Gogas et al., 2021). Unfortunately, after enrolling 692 patients in this global clinical trial, no benefit was observed for the combination treatment. The combination group had an overall response rate of 48.6% compared to 41.3% for pembrolizumab alone, which was not statistically significant Furthermore, the median OS was also not different between treatment arms with a median of 49.2 months for pembrolizumab alone and it was not reached for the combination treatment arm (hazard ratio 0.96, 95% CI 0.76, 1.24, $p = .74$). The reasons for the lack of benefit are not entirely clear as the final data has not yet been published. It is possible

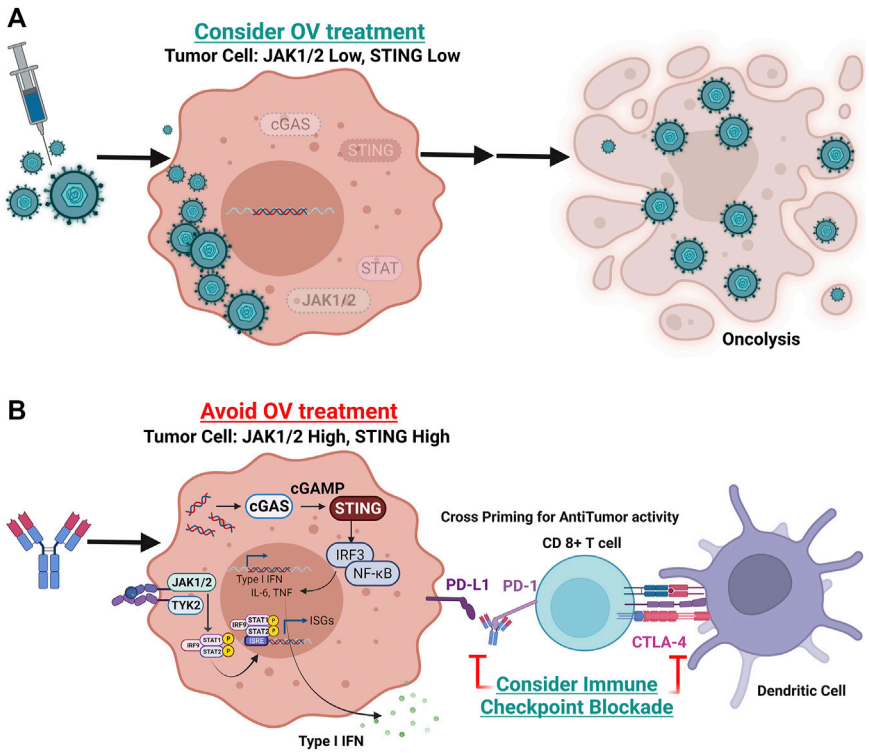

FIGURE 2 | Potential predictive biomarker strategy for oncolytic virus therapeutic response. Shown are tumor cells with variable gene expression and interferon pathway signaling status at the time of initial diagnosis or pre-treatment biopsy. **(A)** Tumor cell with loss of function or low levels of JAK1, JAK2, and/or STING expression. In these tumors, oncolytic viruses may replicate more efficiently and induce widespread immunogenic cell death (oncolysis). **(B)** Tumor cells with intact JAK1, JAK2 and STING signaling will be resistant to DNA viral replication but are more sensitive to immune checkpoint blockade. These tumor cells will express higher levels of PD-L1 making them permissive to PD-1 blockade and potentially other checkpoint blockade, such as CTLA-4 (Created with Biorender).

that the response rate of pembrolizumab alone in stage IIIB-IVM1a melanoma is higher than in stage IV disease and the study was not adequately powered to detect a narrower response difference between arms. Another difference between the phase I and III trial was that in the phase I study pembrolizumab was started after the second injection of T-VEC to allow seroconversion for HSV-naïve patients and avoid rapid vial clearance by enhanced anti-viral immune responses mediated by pembrolizumab. In the phase III trial, however, no T-VEC lead in was employed and both drugs were given on the first day. Further scrutiny of the data may be needed to better understand why this study was negative.

Interestingly, in another phase I study T-VEC was combined with ipilimumab, an anti-CTLA-4 monoclonal antibody in patients with advanced melanoma who were immune checkpoint inhibitor naïve (Puzanov et al., 2016). In this study a 50% response rate was seen with an acceptable safety profile comparable to adverse events seen with individual monotherapy. This was followed by a larger randomized phase II clinical trial in which 198 treatment-naïve melanoma patients were randomized to treatment with T-VEC and ipilimumab or ipilimumab alone (Chesney et al., 2018). The primary endpoint was objective response rate, which was more than doubled in the combination treatment arm (39 vs. 18%). In this trial, regression of un-injected visceral lesions was also seen in 52% of T-VEC and ipilimumab treated patients (vs. 23% with ipilimumab alone). The study met its primary endpoint but was not pursued for registration. A limitation of this study was that eligible patients were not allowed prior anti-PD-1 treatment, and thus, the therapeutic effectiveness of the combination in patients who have progressed after anti-PD-1 treatment is unknown.

TABLE 2 | Considerations for treating patients with T-VEC in the ambulatory setting.

- Establish institutional standard operating procedures
- Consider dedicating a single room and day for T-VEC treatment
- Provide education for healthcare providers handling T-VEC
- Before placing orders, measure the diameter of all tumors at each visit with calipers
- Select index lesions for injection (prioritize large > small size lesions; new > old lesions; avoid lesions near critical anatomic structures, e.g., carotid artery, mucosal surface)
- Use schema in **Table 3** to determine volume
- NOTE: the maximum volume at any visit is 4 ml
- Ensure first dose is 10^6 pfu/ml
- Ensure subsequent doses are 10^8 pfu/ml
- Lesions may be anesthetized with local ice pack prior to injection and/or local anesthetic
- May use four quadrant or fan technique (see **Figure 1**); may need to avoid necrotic areas and inject locations with viable tumor cells (i.e., periphery)
- Injector should use universal precautions
- Portable ultrasound may be useful if lesion regresses or is not clinically palpable

TABLE 3 | Tumor volume determination for T-VEC administration.

Lesion size (longest diameter)	T-VEC injection volume
>5 cm	UP to 4 ml
>2.5–5 cm	UP to 2 ml
>1.5–2.5 cm	UP to 1 ml
>0.5–1.5 cm	UP to 0.5 ml
≤0.5 cm	UP to 0.1 ml

Abbreviations: cm, centimeter; ml, milliliters; T-VEC, talimogene laherparepvec.

PREDICTIVE BIOMARKERS FOR ONCOLYTIC VIRUS RESPONSES

Predictive biomarkers of immunotherapy response have been important for better identifying patient populations likely to respond to treatment. For immune checkpoint blockade, several biomarkers are now recognized as clinically important, including a high tumor mutation burden, elevated local tumor PD-L1 expression, presence of tumor-infiltrating lymphocytes, and a high interferon gene expression pattern, all of which are associated with improved therapeutic responses (Cristescu et al., 2018). Biomarkers of OV response, however, have not been as well investigated but there are some new insights that have emerged from genomic studies of melanoma tumor cells.

In an intriguing study by Nguyen et al., next-generation sequencing and CRISPR-Cas9 screens identified mutations in the interferon-JAK-STAT signaling pathway in melanoma cells as associated with resistance to anti-PD-1 therapy (Nguyen et al., 2021). This study found a melanoma patient with disease progression after treatment with anti-PD-1 had mutations resulting in JAK1 and JAK2 loss of function. They showed that tumor cells without JAK1/JAK2 function, while resistant to anti-PD-1, were much more sensitive to OV infection. They also showed that genetic and pharmacologic inhibition of JAK function could enhance the oncolytic activity of OVs *in vitro*. These data suggest that JAK1 and JAK2 expression may be an important biomarker of OV activity but clinical validation is still required. In our lab we also found that loss of STING expression, a known biomarker for anti-PD-1 resistance, was associated with improved oncolytic activity of T-VEC *in vitro* (Bommareddy et al., 2019). Furthermore, low STING-expressing melanoma cells resistant to PD-1 blockade *in vivo*, were sensitive to T-VEC treatment supporting a role for STING expression as a biomarker of T-VEC response. Collectively, these data support a role for elements of the interferon signaling anti-viral machinery in tumor cells as possible predictive biomarkers of OV activity and merits further clinical investigation (**Figure 2**).

In addition to intracellular anti-viral machinery factors, other potential predictive biomarkers might include viral cell entry receptor expression on tumor and other stromal cells within the tumor microenvironment, high tumor mutation burden, high levels of tumor-infiltrating effector $CD8^+$ T cells, low levels of regulatory $CD4^+$ T cells, and the status of macrophage and myeloid-derived suppressor dendritic cells. In addition, there has been limited data on the association of anti-viral humoral and cellular immune responses with clinical outcome in OV clinical trials. Furthermore, metabolic, and nutritional factors, including the individual patient microbiome status, may impact viral infection and potentially OV-mediated anti-tumor therapeutic responses. Investigators should consider incorporating these biomarkers in future OV clinical trials to obtain exploratory data to identify those markers worth further prospective validation.

CONCLUSION AND FURTHER DIRECTIONS

T-VEC was the first-in-class OV approved for the treatment of melanoma. While treatment was initially approved for patients with melanoma that recurs after initial surgery, further real-world data has helped to better define which patients to treat and how best to implement T-VEC therapy in the ambulatory setting. The potential for objective responses is optimal when T-VEC is used in first-line therapy for locally and regionally advanced melanoma. As such, T-VEC should be considered early in the management of recurrent melanoma when surgical management may be technically feasible but is not considered curative, such as for management of in-transit melanoma metastases. In addition, an important aspect of T-VEC treatment is the potential for pseudo-progression, which occurs when tumors appear to increase in size or number by clinical exam or radiologic imaging but the increase is due to local inflammatory changes and not tumor progression. This has been seen with T-VEC alone and in combination approaches (Andtbacka et al., 2015; Chesney et al., 2019). Since the mean time to response in the phase III OPTiM trial was 4.1 months, it may be prudent to use immune related RECIST criteria or allow treatment past progression provided there no deterioration in clinical performance status. If there is uncertainty about the response, biopsy of the lesion can often resolve tumor progression or inflammation with regression. An outline of considerations in patient selection and treatment of patients in the clinic is shown in **Table 2**. The volume of T-VEC is based on the longest diameter of accessible tumors when patients present for treatment according to **Table 3**. Post-injection management pearls are provided in **Table 4**.

Subset analyses have suggested that certain populations may receive especial benefit from T-VEC, including melanomas of the head and neck, older patients who may also have other co-morbid conditions, and transplant recipients. Melanoma metastasis to the central nervous system (CNS) remains a significant clinical challenge

TABLE 4 | Considerations for patient management after T-VEC injection.

- Site should be wiped with alcohol prior to injection and after bandage is placed
- Sites of injection should be covered with dry gauze and virus impenetrable occlusive dressing (e.g., Tegaderm dressing)
- Biohazard waste receptacles for dry waste and needles should be in the treatment room
- Bandages should be maintained for 5–7 days
- Patient should be given extra bandages in case replacement is needed and provided with education on how to manage (e.g., hand washing, gloves, proper disposal of waste)
- Acyclovir can be used for accidental exposure
- Pregnant woman and immunosuppressed individuals should avoid direct contact with T-VEC-injected patients for 7 days

and recent evidence that oncolytic HSV-1 (teserpaturev) has activity in glioblastoma suggests that T-VEC could be considered for treating CNS melanoma. Direct access to the CNS for bimonthly injections remains a logistical challenge but further clinical studies may be warranted. Further studies are needed to confirm a role for T-VEC in the neoadjuvant setting and for other cancers. While studies of combination treatment with immune checkpoint inhibitors have been contradictory, other therapeutic combinations await clinical validation, including combinations of T-VEC with radiation therapy, targeted therapy, chemotherapy, and adoptive T cell therapy. Predictive biomarkers are also needed and early work suggests that elements of the intracellular anti-viral machinery may be important predictors of OV sensitivity and merit further evaluation. As techniques for single cell genomic analysis have matured, this will provide assays to interrogate tumor cells *in vitro* and *ex vivo*, which should accelerate better patient selection and more rational combination strategies.

Oncolytic viruses represent a new class of cancer therapeutics that have, thus far, resulted in limited approvals for cancer. Newer viruses with more rationally designed transgene payloads, coupled with a better understanding of the underlying biology, should lead to new approvals and best-in-class agents across a range of tumor types and clinical indications. Further studies to explore T-VEC injection of visceral lesions as well as determining the risks and benefits of intravenous delivery are needed. What is established is the tolerable safety profile of T-VEC and other OVs in clinical development with most exhibiting similar low grade and short duration constitutional and local injection site reactions. The safety profile may allow better patient acceptance and expansion of OVs into more immunologically sound combination clinical trials for patients with cancer.

AUTHOR CONTRIBUTIONS

HK was responsible for the initial concept, writing and approval of the manuscript. GI contributed to figure generation, editing and final approval of the paper. SS provided additional review of the manuscript, helped respond to reviewer critiques and generated graphics for the manuscript figures.

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Oncolytic Virotherapy in Peritoneal Metastasis Gastric Cancer: The Challenges and Achievements

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Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer death globally. Although the mortality rate in some parts of the world, such as East Asia, is still high, new treatments and lifestyle changes have effectively reduced deaths from this type of cancer. One of the main challenges of this type of cancer is its late diagnosis and poor prognosis. GC patients are usually diagnosed in the advanced stages of the disease, which is often associated with peritoneal metastasis (PM) and significantly reduces survival. This type of metastasis in patients with GC poses a serious challenge due to limitations in common therapies such as surgery and tumor resection, as well as failure to respond to systemic chemotherapy. To solve this problem, researchers have used virotherapy such as reovirus-based anticancer therapy in patients with GC along with PM who are resistant to current chemotherapies because this therapeutic approach is able to overcome immune suppression by activating dendritic cells (DCs) and eventually lead to the intrinsic activity of antitumor effector T cells. This review summarizes the immunopathogenesis of peritoneal metastasis of gastric cancer (PMGC) and the details for using virotherapy as an effective anticancer treatment approach, as well as its challenges and opportunities.

Keywords: oncolytic virotherapy, peritoneal gastric cancer, metastasis, anticancer, immunopathogenesis

1 INTRODUCTION

Gastric cancer (GC) is considered one of the most common human cancers, and it is the third leading cause of global cancer deaths (Jemal et al., 2011; Olnes and Martinson, 2021). Evidence showed that GC has higher cytologic, genetic, and architectural heterogeneity than other human gastrointestinal malignancies (Abdi et al., 2021). Due to the poor prognosis of GC, it has been shown that this type of cancer has a low 5-year overall survival (OS), which even after treatment with surgery and chemotherapy as well as other therapeutic approaches such as biological treatments, the OS rate in patients according to different continents has been reported between 20 and 60% (Sant et al., 2009; Wei et al., 2016; Olnes and Martinson, 2021). According to the available knowledge, due to the presence of immunosuppressive cells and mediators, as well as the overexpression of inhibitory

molecules on the tumor's surface cells in the tumor microenvironment (TME) of GC, cancerous cells have a strong tendency to invade and metastasize to other organs in the body (Yuki et al., 2020). Among patients with advanced GC, peritoneal implantation is one of the most common and worst metastasis forms. Studies have reported that the peritoneal metastasis (PM) rate of GC patients at the initial phase of the examination was about 14%, and also the median survival time was approximately 3–6 months (Thomassen et al., 2014). Until the early 1990s, PM of GC was considered a terminal disorder due to its unresectability as well as resistance to systemic chemotherapy (Yonemura et al., 2017). Nevertheless, in the late 1990s, conversional therapy was recommended by researchers as a novel therapeutic approach with the aim of *en bloc* resection of macroscopically obvious lesions employing gastrectomy, peritonectomy, and lymphadenectomy, along with the ample removal of peritoneal micrometastasis via perioperative chemotherapy (Yonemura et al., 2017; Wang et al., 2019). However, most clinical studies on peritoneal malignancies are challenged by the continual high rates of peritoneal recurrence and reduced patient survival (Thadi et al., 2018). In this regard, the growing use of novel therapeutic approaches, including immunotherapy-based methods and oncolytic virotherapy in the management of metastatic malignancies, has led to research into translation applications for primary and metastatic peritoneal diseases (Morano et al., 2016). The strength of virotherapy over other therapies is the direct killing of tumor cells without damaging normal and non-tumor cells and tissues, and this advantage clearly emphasized the need to study this treatment (Fukuhara et al., 2016).

Since the early 20th century, there has been speculation that viruses may be used to treat cancer, and some viruses, such as rabies virus, have been studied in the field since the mid-nineteenth century and have shown relatively satisfactory results in tumor regression (Pack, 1950; Southam and Moore, 1952; Sinkovics and Horvath, 1993; Sinkovics and Horvath, 2000). In the following years, the anticancer effects of several other viruses, such as flavivirus West Nile virus (strain Egypt 101), bovine enterovirus, Newcastle disease virus (NDV), oncolytic serotype adenovirus type 4, and the paramyxoviruses mumps, were used in human studies as well as animal models of cancer (Southam and Moore, 1952; Asada, 1974; Okuno et al., 1978). A major challenge in treating patients with peritoneal metastasis of gastric cancer (PMGC) is resistance to chemotherapy which can impair the effectiveness of systemic chemotherapy (Rau et al., 2019). To address this issue, researchers have used reovirus-based anticancer therapy in patients with the chemotherapy-resistant form of PMGC because it can activate dendritic cells (DCs), restore suppressed immune responses and ultimately lead to activation of antitumor $CD8^+$ T lymphocytes (Gujar et al., 2010). Experimental and human studies have so far yielded relatively acceptable outcomes from this type of treatment. In this regard, it has been reported that reovirus-based immunotherapy can delay the expansion of PM and increase animal survival via decreasing myeloid-derived suppressor cells (MDSC), regulatory T cells (Tregs), and increasing $CD3^+/CD8^+$ effector T cells and interferon-gamma (IFN-γ) production in studied mice (Gujar et al., 2013). Despite the advantages of virotherapy in the treatment of cancer, similar to other therapeutic approaches, this method is also encountered with relatively similar challenges, including the presence of immunosuppressive TME, lack of proper penetration into the tumor mass, and lack of specific therapeutic therapy biomarkers as well as off-target infections and anti-virus responses immune system.

Therefore, this review aimed to summarize the limitations of PMGC treatment and the reasons for the tendency to use other therapeutic tactics such as virotherapy. Furthermore, the details of the virotherapy are also discussed, along with the challenges facing this type of cancer therapy.

2 PERITONEAL METASTASIS OF GASTRIC CANCER

Evidence showed that PM is one of the most frequent types of metastasis in GC and up to 14% of newly diagnosed GC patients (Kang et al., 2021). Furthermore, the peritoneum is considered the most common site of recurrence upon radical surgery in GC patients (Sugarbaker et al., 2003; Thomassen et al., 2014). It has been reported that in patients with PMGC, due to low treatment efficacy and its challenges, the median survival time of these patients is short and about 3–6 months (Ishizone et al., 2006; Thomassen et al., 2014). However, the cellular and molecular mechanisms underlying PMGC are not yet fully understood. Metastasis of tumor cells as a multistage process is a complex phenomenon. Peritoneal metastasis of GC tumor cells consists of several steps based on available knowledge, including dissemination, adhesion, invasion, and proliferation. Primary malignant cells can migrate to other areas and tissues through the blood, lymph nodes, and local invasion (Bogenrieder and Herlyn, 2003) (**Figure 1**).

In PM, the primary tumor cells originate from the primary abdominal organs and propagate through the transcolumic mechanism. The specific type and direction of peritoneal fluid circulation can lead to the dispersion of tumor cells in a specific state that depends on multilevel cellular and molecular reactions between peritoneal components and the initial site of malignant cell growth. In this regard, it has been shown that the expression of TGF-β1, leukocyte-associated adhesive molecules such as CD44, selectins and integrins could up-regulate by peritoneal mesothelial cells and endothelial cells, resulting in epithelial-mesenchymal transition (EMT) of peritoneal mesothelial cells (Sun et al., 2017). Following these events, the proliferation of invasive species tumor cells could be increased (Mikuła-Pietrasik et al., 2018). Due to common gastrointestinal cancers, peritoneal carcinomatosis can occur through transversal growth (synchronous peritoneal carcinomatosis) and intraperitoneal spread (*metachronous* peritoneal carcinomatosis). Cancer cells exfoliate from the primary tumor into the peritoneal cavity in the more common transverse growth method, usually occurring before surgery. In the intraperitoneal spread due to surgical injury, malignant cells are inadvertently released and

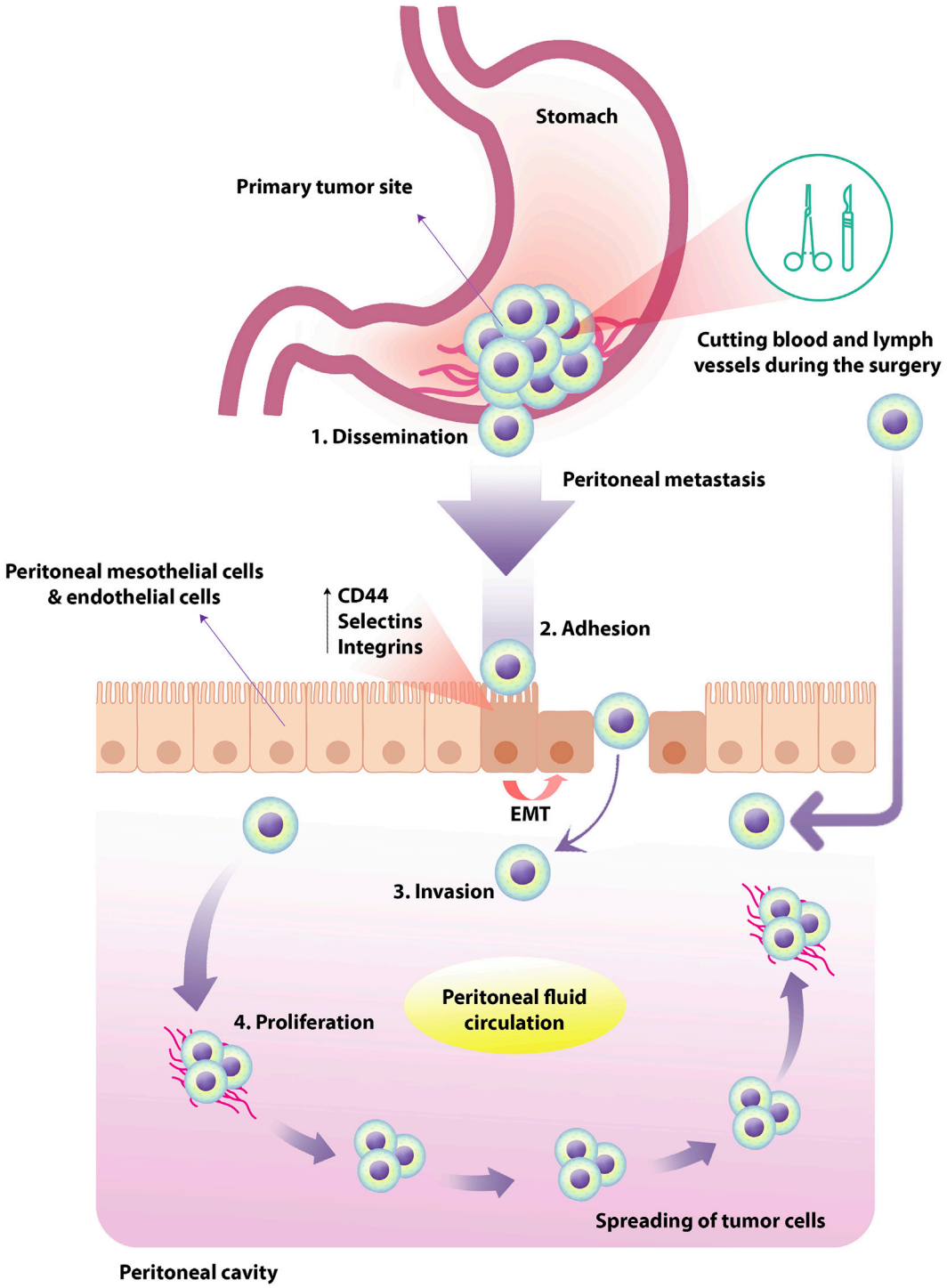

FIGURE 1 | Peritoneal metastasis of tumor cells in human GC. Primary tumor cells originate from the primary abdominal organs and spread through the transcolumic mechanism. The specific type and direction of peritoneal fluid circulation can lead to the tumor cells spreading in a particular order. In human GC, PM occurs in four steps; dissemination, adhesion, invasion, and proliferation. The expression of TGF-β1, leukocyte-associated adhesive molecules such as CD44, selectins and integrins could up-regulate by peritoneal mesothelial cells and endothelial cells, resulting in EMT of peritoneal mesothelial cells. Tumor cells exfoliate from the primary tumor into the peritoneal cavity in the more common transverse growth method, regularly occurring before surgery. In the intraperitoneal spread due to surgical injury, malignant cells are inadvertently released and spread through the peritoneum by manipulating the primary tumor, cutting blood and lymph vessels during the operation. GC, gastric cancer; PM, peritoneal metastasis; TGF-β1, transforming growth factor-beta1; EMT, epithelial-mesenchymal transition.

propagated through the peritoneum by manipulating the primary tumor, cutting blood and lymph vessels during the operation (Terzi et al., 2014). Previous studies in this field categorized the spread of peritoneal cancer into three types: Random Proximal Distribution (RPD), Complete Redistribution (CRD), and Wide Cancer Distribution (WCD). Understanding these patterns can greatly affect treatment management and clinical outcomes. It is useful because, for example, the best treatment for RPD is selective peritonectomy of macroscopically involved sections, while for WCD and CRD, complete peritonectomy and cytoreduction treatment are more desirable. Studies demonstrated that among these patterns, RPD occurs in early implantation of moderate and high-grade tumors such as GC in order to the existence of adherence molecules on the cancer cells near the tumor site (Kusamura et al., 2010).

3 PERITONEAL METASTASIS OF GASTRIC CANCER THERAPY

Based on available knowledge, systemic chemotherapy is considered the standard cancer therapy method for patients with PMGC (Ishigami et al., 2017). Regarding the outcomes of pivotal clinical trials, the combination of capecitabine or S-1 (Tegafur, Gimeracil, Oteracil) with oxaliplatin or cisplatin is suggested for first-line chemotherapy, and ramucirumab with paclitaxel is also recommended for second-line chemotherapy (Association JGC, 2017). Current improvement in systemic chemotherapy could enhance patients' prognosis; nonetheless, the median survival time has been extended to only around 1 year (Koizumi et al., 2008; Kang et al., 2009; Wilke et al., 2014; Yamada et al., 2015). Although it has been possible to improve the prognosis of patients with PMGC through chemotherapeutic agents and new molecular targeting, the effectiveness of treatment is still unsatisfactory (Wang et al., 2019). Researchers believe that combination therapy with surgery and chemotherapy can dramatically reduce the size and regression of metastatic tumor lesions and sometimes even the complete disappearance of the tumor (Bang et al., 2010). However, this type of treatment (gastrectomy and postoperative chemotherapy) could not lead to greater efficacy or survival than chemotherapy alone due to the lack of adherence to chemotherapy following surgery (Fujitani et al., 2016). In contrast, other studies aimed at R0 resection (a microscopically margin-negative resection) on cancers that are initially only partially resectable or non-resectable have shown that the use of a multidisciplinary model of conversion therapy through surgical intervention followed by chemotherapy (only in responders to chemotherapy) could be safe and lead to increased survival of patients with PMGC (Ishigami et al., 2017).

4 ONCOLYTIC VIROTHERAPY

Virotherapy has been studied for cancer treatment since the 19th century, but due to genetic engineering challenges and concerns about self-immune responses, it has not progressed much in the last 2 decades (Goradel et al., 2021). Genetic engineering aims to modify viral genomes to replicate in cancer cells selectively, and lysis is performed without affecting normal cells. Virotherapy is now considered a form of cancer immunotherapy because oncolytic virus therapy induces immune responses against viral, anti-epitopes in virus-infected tumor cells as well as the death of these tumor cells (Davis and Fang, 2005; De Munck et al., 2017). The United States food and drug administration (FDA) approved T-VEC, a modified form of herpesvirus type 1 (HSV-1), as the first oncolytic virus in 2015 to treat melanoma (Aurelian, 2016). Deleting specific genes in this type of virus can lead to selective proliferation in tumor cells and increase the presentation of tumor and viral antigens to immune effector cells (Pol et al., 2016). Regarding the use of genetic engineering in virotherapy, it has been shown that the gene of cytokines such as the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promotes the growth factor development and prolongation of cellular and humoral immune responses is inserted in the HSV-1 genome (Rehman et al., 2016). Moreover, in other countries, Oncorine and RIGVIR (enteric cytopathic human orphan type 7) have also been approved as oncolytic viruses for cancer therapy. Oncorine, a genetically modified type 5 human adenovirus (HAdV-C5) in which the E3 and E1B-55KD regions were deleted to stimulate selective virus replication in p53-impaired cells and enhance the safety of the treatment (Goradel et al., 2021). In 2005, China's state food and drug administration confirmed Oncorine (H101) for head and neck squamous cell carcinoma (Goradel et al., 2021). Furthermore, RIGVIR, a strain from the *Picornaviridae* family, is a no-genetically engineered virus employed to treat melanoma (Doniņa et al., 2015; Alberts et al., 2016). Recent studies show that among the wide range of oncolytic viruses that have been investigated so far, members of the *poxviruses* are the most hopeful candidates for different types of tumors. For example, the oncolytic myxoma virus (MYXV), as a member of the *Leporipoxvirus* genus, contrasting other oncolytic viruses, only infects rabbits and does not harm humans. However, MYXV can selectively infect tumor cells of humans, mice, and some other species, resulting in lysis of these infected tumor cells (Rahman and McFadden, 2020). As mentioned before, among the studied oncolytic viruses, only T-VEC has FDA-approved labeling for use in the treatment of melanoma and investigations on other viruses are underway. **Table 1** shows some of the most important completed clinical trials on the use of oncolytic viruses in human malignancies.

4.1 Oncolytic Viruses Mechanisms of Action

Studies have shown that oncolytic viruses can kill cancer through the two primary mechanisms of direct cell lysis and the induction of antitumor immune responses (**Figure 2**).

4.1.1 Tumor Cell Lysis

Virus replication in infected tumor cells leads to apoptosis in the cell lysis mechanism. Following virus replication in tumor cells and cell lysis, viral particles repeat the lytic cycle by infecting adjacent cancer cells, inducing and amplifying treatment at the target tumor site (Mullen and Tanabe,

TABLE 1 | Completed clinical trials of oncolytic viruses.

	Virus	Genetic manipulation	Tumor type	Phase	References
HSV viruses	G207	None	Brain tumor	II	NCT04482933
	ONCR-177	IL-12, CCL4, FLT3LG, αCTLA4 and αPD-1	Melanoma and other solid tumors	I	NCT04348916
	OH2 (HSV-2)	GM-CSF	Gastrointestinal tumors and other solid tumors	I and II	NCT03866525
	RP1	GALV-GP and GM-CSF	Cutaneous squamous cell carcinoma	Ib	NCT04349436
	RP1	GALV-GP and GM-CSF	Cutaneous squamous cell carcinoma	I	NCT04050436
	RP2	GALV-GP and GM-CSF	Advanced solid tumors	I	NCT03767348
	T-VEC	GM-CSF	Breast Cancer	I	NCT04185311
	T-VEC	GM-CSF	Angiosarcoma of skin	II	NCT03921073
	T-VEC	GM-CSF	Sarcoma	II	NCT03069378
	T-VEC	GM-CSF	Cutaneous melanoma	II	NCT03842943
Adenoviruses	CG0070	GM-CSF	Bladder cancer	II	NCT02365818
	Delta-24-RGD	None	Brain tumor	I and II	NCT01582516
	MG1-MAGEA3	MAGEA3	NSCLC	I and II	NCT02879760
	CG0070	GM-CSF	Bladder cancer	II	NCT02365818
Vaccinia viruses	Pexa-Vec	GM-CSF	Hepatocellular carcinoma	II	NCT01171651
	Pexa-Vec	GM-CSF	Hepatocellular carcinoma	II	NCT01636284
	Pexa-Vec	GM-CSF	Hepatocellular carcinoma	II	NCT01387555
	GL-ONC1	Luc-GFP β-Galactosidase β-glucuronidase	Head and neck cancer	I	NCT01584284
	GL-ONC1	Luc-GFP β-Galactosidase β-glucuronidase	Solid tumors	I	NCT00794131
	vvDD	Cytosine deaminase and somatostatin receptor	Solid tumors	I	NCT00574977

2002). The viral lytic cycle continues until infected host cells are depleted, or antiviral immune responses attenuate virus replication (Hamid et al., 2017). Immune responses can also lead to the death of tumor cells by breaking the tolerance of tumor cells (Workenhe et al., 2015; van Vloten et al., 2018). Non-infectious host cells can also be affected by oncolytic viruses in favor of treatment. In this context, it has been disclosed that the oncolytic vaccine virus can interrupt tumor angiogenesis, reduce blood flow to cancer cells, and ultimately cause hypoxia by affecting vascular cells, all of which are associated with inhibiting tumor growth and progression (Breitbach et al., 2007; Breitbach et al., 2013; Hashemi Goradel et al., 2018). Although lysis of tumor cells through the initiation of the lytic cycle is one of the inherent characteristics of oncolytic viruses, evidence suggests that further manipulations can increase their lytic capacity. For instance, the herpes simplex virus-1 thymidine kinase (HSV-1 TK) expresses adenovirus (Ad-OC-HSV-TK), in which the expression of HSV-1 TK is under the osteocalcin promoter, to target tumor cells in designed for bone malignancies (Kubo et al., 2003; Goradel et al., 2021). In this regard, HSV-1 TK can activate thymidine analogs such as ganciclovir as a competitive inhibitor of deoxyguanosine by conversion to monophosphates. Monophosphates can also disrupt and terminate DNA synthesis by inserting proliferating cells DNA, resulting in cell death (Alvarez and Curiel, 1997). Another suicidal gene under study is cytosine deaminase (CD), which can convert 5-fluorocytosine to 5-fluorouracil with high cytotoxic properties (Freytag et al., 2002). The insertion of the ADP gene into the adenovirus genome upsurges the lytic activity of the virus. ADP is also involved in encoding the adenovirus death protein (ADP), which is crucial for the infection of type C adenoviruses in the later phases of infection and the spread of viral particles (Doronin et al., 2000).

4.1.2 Enhancement of Anti-Tumor Immune Responses

The second mechanism of action oncolytic viruses is to increase antitumor immune responses. Studies have shown that following infection of tumor cells with oncolytic viruses, cell death and the release of tumor-related antigens such as viral pathogen-associated molecular patterns (PAMPs) and different cellular danger-associated molecular patterns (DAMPs) lead to the enhancement of tumor-specific immune responses and the killing of distant and non-infectious tumor cells (Pol et al., 2012). Tumor cell lysis can also induce the production and secretion of inflammatory mediators, including type I interferons (IFNs), interferon-gamma (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor-α (TNF-α) (Kaufman et al., 2015). The philosophy of using engineered oncolytic viruses is to enhance immune responses further. In this strategy, the insertion of an immune-stimulating molecule into the oncological genome of viruses could alter the immune-suppressive tumor microenvironment in favor of treatment. As previously mentioned, GM-CSF is the most obvious example of this type of genetic engineering. After incorporating the GM-CSF gene into the oncolytic genome, viruses can act as an immune responses stimulator, leading to the maturation and recruitment of antigen-presenting cells (APCs), particularly DCs, inducing antitumor effector T cells and NK cells which are specific for tumor antigens (Jhawar et al., 2017). In order to improve and increase the delivery of intracellular antigen to the proteasome and antigen presentation, the oncolytic adenovirus genome was modified for

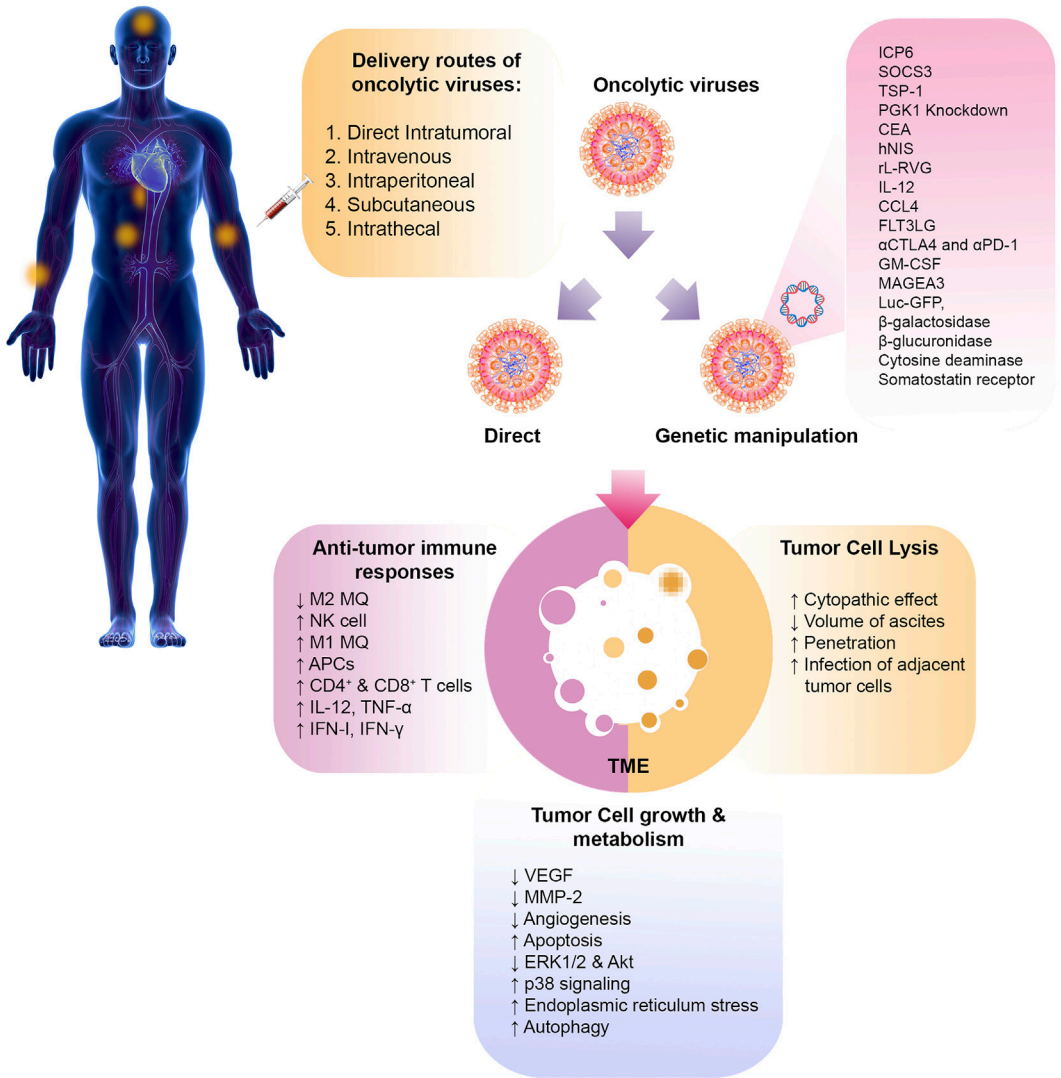

FIGURE 2 | Oncolytic virotherapy of cancer. The various routes of oncolytic virus delivery are shown. Oncolytic viruses can be manipulated through genetic engineering to express specific genes or administered directly without modification. Once they reach the tumor site, these viruses can directly cause lysis of tumor cells. They can also help remove the tumor by altering the immunosuppressive TME and inducingantitumor effector immune cells. Other mechanisms, such as angiogenesis, apoptosis, and autophagy, can also be altered by oncolytic viruses. PMGC, peritoneal metastasis of gastric cancer; SOCS3, suppressor of cytokine signaling 3; TSP-1, thrombospondin-1; PGK-1, phosphoglycerate kinase-1; CEA, carcinoembryonic antigen; hNIS, human sodium iodide symporter; rL-RVG, rabies virus glycoprotein; HSV, herpesvirus; 5-FU, 5-fluoro-uracil; PTX, paclitaxel; NDV, Newcastle disease virus; MQ, macrophage; VEGF, vascular growth factor; MMP-2, matrix metalloproteinase-2; FLT3LG, Fms related receptor tyrosine kinase 3 ligand; CTLA4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; MAGEA3, melanoma-associated antigen 3; Luc GFP, luciferase green fluorescent protein; IFN, interferon; TNF, tumor necrosis factor; APC, antigen presenting cell.

overexpression of heat shock proteins (Hsp70) protein, and the outcomes disclosed that the frequency of $CD4^+$ and $CD8^+$ T cells along with NK cells increased following the administration of this type of modified oncolytic adenovirus (Li et al., 2009). Correspondingly, due to the expression of Hsp receptors such as CD91 (α2-macroglobulin receptor or the low-density lipoprotein–related protein) and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), HSP70 in APCs, the delivery of tumor antigen to APCs is improved through this approach (Nishikawa et al., 2008).

4.2 Oncolytic Viruses Used in Cancer Therapy

Numerous oncolytic viruses have been used to treat malignancies. Among these viruses, adenoviruses, HSVs, vaccinia virus, Newcastle disease virus (NDV), coxsackievirus, measles virus (MeV), Seneca Valley virus, poliovirus, parvovirus, vesicular stomatitis virus, and the Maraba virus are the most investigated in cancer therapy (Davis and Fang, 2005).

4.3 Delivery Routes of Oncolytic Viruses

Studies on cancer treatment using oncolytic viruses have shown that non-optimal delivery is one of the main reasons for treatment failure. Several delivery routes for oncolytic virus therapy have been investigated, and their proper selection based on research objectives is essential to increase the effectiveness of treatment (**Figure 2**). This section briefly introduces common oncolytic viruses delivery methods in cancer therapy.

4.3.1 Direct Intratumoral Delivery

Direct intratumoral delivery is the most common route of administration of oncolytic virus in patients with cancer. In this method, the concentration of oncolytic virus in the desired site can be accurately managed and controlled, and on the other hand, the adverse effects caused by the improper transmission of the virus to other organs can be prohibited. According to the obtained outcomes, due to operational complications in direct intratumoral delivery, it is much more suitable for superficial tumors such as melanoma than deep tumors such as glioblastoma (Li et al., 2020).

4.3.2 Intravenous Delivery

Intravenous delivery of oncolytic viruses is a simple administration route for physicians in cancer therapy. Numerous researchers in clinical trials using oncolytic viruses prefer intravenous injections to intratumoral injections because they believe that intratumoral injections have several challenges and complexities, such as surgery for deep-seated tumors as well as delivery barriers in high metastatic malignancies (Waters et al., 2017; Komorowski et al., 2018; Samson et al., 2018; Tang et al., 2019). It has been shown in several human malignancies that intravenous injection of oncolytic viruses can induce tumor elimination through various mechanisms such as alteration of the immunosuppressive TME by reducing the expression of inhibitory molecules as well as affecting immune cells by increasing their antitumor function (Waters et al., 2017; Komorowski et al., 2018; Samson et al., 2018; Tang et al., 2019). Intravenous delivery of oncolytic viruses can also facilitate the passage of various barriers such as the extracellular matrix (ECM) and blood-brain barrier (BBB), which are the main challenge in the transmission of the oncolytic virus in solid tumors (Choi et al., 2012). However, the immune clearance of oncolytic viruses and insufficient concentration of viruses reaching the tumor site can disadvantage intravenous delivery.

4.3.3 Intraperitoneal Delivery

Because the peritoneal cavity is a large area, absorption of intraperitoneally injectable drugs and compounds is faster than drug administration via subcutaneous injection. While drug absorption by intraperitoneal injection is slower than intravenously injected drugs. Another advantage of intraperitoneal administration is the relative ease of injection, which does not require any specialized skills. It appears that if the organs inside the abdominal cavity are the target of treatment, intraperitoneal injection is an ideal and smart choice for the delivery of oncolytic viruses (Li et al., 2020; Chen et al., 2017; O'Leary et al., 2018).

4.3.4 Subcutaneous and Intrathecal Delivery

Subcutaneous injection is also a fairly common method of administering oncolytic viruses. This method is particularly used for small animals whose veins are hard to find (Kuryk et al., 2017). Additionally, the possibility of intrathecal injection is limited to the central nervous system (CNS)-related tumors. By way of explanation, due to the low efficiency of subcutaneous and intrathecal delivery approaches, these methods are less used and are principally limited to animal experiments (Ochiai et al., 2006).

5 ONCOLYTIC VIROTHERAPY IN TREATMENT OF PERITONEAL METASTASIS OF GASTRIC CANCER

As mentioned earlier, the prognosis of patients with PMGC is very poor, and related investigations are needed to find an effective treatment given the limitations and shortcomings of previous routine treatments such as surgery and chemotherapy. Few studies have been performed to evaluate the efficacy of oncolytic viruses in the treatment of PMGC.

5.1 *In Vitro* Studies

An investigation on GC cell lines including SGC-7901 and AGS infected with the NDV wild-type strain and the recombinant avirulent NDV LaSota strain expressing the rabies virus glycoprotein (rL-RVG) showed that the growth of studied cells in the rL-RVG-infected group was significantly inhibited compared with the wild-type NDV-infected group. RL-RVG and NDV also increase endoplasmic reticulum stress, autophagy, and apoptosis in SGC-7901 and AGS cells. Immunofluorescence analysis in this study disclosed that the mitochondrial membrane was collapsed. It has been revealed that beclin-1 participated in the Bcl-2/Bcl-xL complex activity and inhibition of the formation of the autophagosomes (Cho et al., 2009). In this context, the findings showed that the expression of beclin-1 increased in virus-infected cells, reducing the beclin-1 and Bcl-2/Bcl-xL interaction as well as inducing apoptosis and autophagy. These outcomes collectively suggested that NDV and rL-RVG could induce stomach adenocarcinoma cell death via apoptosis and autophagy along with dysfunction of the endoplasmic reticulum and mitochondria (Bu et al., 2015).

Based on previous studies, phosphoglycerate kinase 1 (PGK1) can participate in PMGC and impact the tumor stem cell's growth and differentiation in GC (Zieker et al., 2008; Zieker et al., 2013). A study by hairpin RNA knockdown of PGK1 through adenovirus-shPGK-1 and using the chemotherapeutic agents 5-fluoro-uracil (5-FU) and mitomycin showed that mitomycin and 5-FU alone could significantly reduce tumor cells viability. This study also showed that treatment with AdvshPGK-1 alone has an improved effect on reducing tumor cell viability. To determine the effect of combination therapy, 5-FU and mitomycin were used simultaneously with adenovirus-shPGK-1, and the outcomes disclosed that this treatment could be more

effective than using either 5-FU, mitomycin or AdvshPGK-1 alone. These findings indicate that inhibition of PGK-1 can increase the susceptibility of metastatic GC cells and tumor stem cells to overcome the chemotherapeutic therapy resistance (Schneider et al., 2015).

5.2 *In Vivo* Animal Model Studies

A study was performed using an experimental PMGC animal model to use serotype three oncolytic reoviruses to treat PM in human GC by evaluating the cytopathic effect of reovirus and activity of Ras in human GC cell lines *in vitro*. After reovirus infection, the cytopathic effect was reported in GC cell lines without affecting normal control cells. The Ras activation assay showed Ras's activity increased in all GC cell lines (MKN45p, NUGC4, MKN7) compared to control cells (KatoIII). Correspondingly, the animal model of PMGC using systemic delivery of reovirus showed that the mean number of tumor cells and weight of total peritoneal tumors along with the volume of ascites were significantly reduced in the treated group compared to the control group. The outcomes of this study indicate that intraperitoneal administration of reovirus might be useful as a novel treatment in PMGC (Kawaguchi et al., 2010).

It has been revealed that to inhibit the growth of human epidermal growth factor receptor 2 (HER2)-overexpressing GC cells, using trastuzumab (anti-HER2 receptor mononuclear antibody) could be effective. The question arises as to whether combination therapy employing oncolytic reovirus and trastuzumab could offer a novel and more effective treatment option for GC. A mouse GC xenograft transplantation model study explored the therapeutic impacts of oncolytic reovirus and trastuzumab to answer this question. Molecular analysis of pathways associated with cell damage was measured by PCR array, and the expression of proteins involved in cell proliferation and apoptosis was examined by western blotting. The results showed that reovirus could sensitize GC cells by overexpressing HER2 for apoptosis. The outcomes of *in vitro* and *in vivo* experiments provided evidence that the combination of oncolytic reovirus and trastuzumab is a more effective method against HER2-overexpressing GC cells than using reovirus or trastuzumab alone. Molecular analysis showed that oncolytic reovirus and trastuzumab could induce higher tumor necrosis factor-related apoptosis-inducing ligand or Apo 2 ligand (TRAIL/Apo2L) in cancer cells.

Moreover, in this study, antibodies against TRAIL strongly reduced combination therapy-associated cytotoxicity. These findings suggested that reovirus might upsurge trastuzumab-induced cytotoxicity in GC cells (Hamano et al., 2015). It appears that upon the combination therapy, released TRAIL from tumor cells might stimulate antitumor responses such as anti-angiogenic responses and antibody-dependent cellular cytotoxicity (ADCC) in an autocrine manner; because according to the findings of this study, tumor xenografts in the nude mice only eradicated in reovirus and trastuzumab treated group.

The employment of G47Δ, the third generation of oncolytic HSV-1, is considered a novel and attractive therapeutic approach for solid tumors. In this regard, a study examined the therapeutic potential of G47Δ for human GC, and the results showed that *in vitro* administration of G47Δ showed a satisfactory proliferative and cytopathic impact on several studied human GC cell lines. Moreover, intratumor injection of G47Δ was also able to significantly inhibit the growth of subcutaneous tumors by increasing the expression of immunostimulatory molecules (soluble CD80) and IL-12 and enhancing M1 macrophages polarization and infiltration *in vivo*. Furthermore, the frequency of cytotoxic NK cells increased following G47Δ administration (Sugawara et al., 2020). Studies on orthopedic tumor models and peritoneal diffusion models of GC disclosed that intratumoral or intraperitoneal administration of G47Δ could alter the immunosuppressive TME and its components, including Tregs, MDSCs, and TAMs resulting in more effective trafficking of effector immune cells in tumor site and further antitumor responses (Saha et al., 2017).

On the other hand, the mentioned effector immune cells can induce innate immune antiviral responses and reduce the effectiveness of virotherapy (Fulci et al., 2007; Alvarez-Breckenridge et al., 2012). It has been reported that HSV-induced M1 macrophages can participate in removing virus-infected cells by producing TNF-α (Meisen et al., 2015). However, another study reported that stimulated macrophages by oncolytic viruses that have infiltrated tumor tissue did not lead to virus clearance and had no significant effect on the effectiveness of virotherapy in cancers (Zemp et al., 2014). Since the immune system's behavior against different viruses is different and the mentioned study was performed on oncolytic myxoma virus in glioma, this finding cannot be generalized to all cancers and oncolytic viruses. Therefore, eliminating the clearance of the virus by the immune system can be of particular importance in the success or failure of cancer virotherapy and further studies are needed in this area. Another study used a telomerase-specific oncolytic adenovirus expressing TRAIL (Ad/TRAIL-E1) to express both the adenovirus early region 1A (*E1A*) and *TRAIL* genes under the control of a specific tumor promoter. The antitumor effect of Ad/TRAIL-E1 on GC cells was evaluated *in vitro* and *in vivo* in a xenograft model of peritoneal carcinomatosis. This investigation demonstrated that Ad/TRAIL-E1 induces TRAIL-mediated apoptosis in GC cell lines and has no effect on normal cell lines, which is beneficial for treatment. In addition, Ad/TRAIL-E1 was able to significantly inhibit PM and increase the survival of mice without long-term toxicity associated with treatment. Thus, tumor-specific TRAIL expressing adenovirus may offer a novel therapeutic approach to treating PMGC (Zhou et al., 2017).

Studies have shown that the low-pathogenic human enterovirus Echovirus 1 (EV1), an oncolytic virus, can selectively target and kill malignant ovarian and prostate cancer cells in xenograft models (Melnick and Ågren, 1952; Shafren et al., 2005; Berry et al., 2008). EV1 infection and the initiation of the lytic cycle in the target tumor cell require the surface expression of the α2β1, a type of integrin that disseminates GC cells into the peritoneum (Koike et al., 1997; Kawamura et al., 2001). Flow cytometry-based analyses have shown that α2β1 integrin is highly expressed on several GC cell lines, making these cells more susceptible to EV1 lytic

infection *in vitro* and leading to effective PMGC treatment. One of the animal models used for non-invasive monitoring of tumor burden in the peritoneum is the MKN-45-Luc SCID bioluminescence mice model, which can also be used to determine therapeutic dose-response (Haley et al., 2009). In this model, it has been reported that oncolytic EV1 could be effectively employed to control PMGC. Pre-existing immunity to EV1, such as antiviral neutralizing antibodies, could be a potential barrier in virotherapy. Although preliminary investigations have revealed that the prevalence of anti-EV1 neutralizing antibodies in the population is low (about 6%), this study is relatively old and more studies are needed on different populations to determine the precise prevalence of anti-EV1 neutralizing antibodies (Karttunen et al., 2003).

Thrombospondin-1 (TSP-1), an endogenous anti-angiogenic factor, is able to suppress tumor growth and progression through various mechanisms, such as inhibition of angiogenic pathways (Weinstat-Saslow et al., 1994; Sheibani and Frazier, 1995; Volpert et al., 1997). One approach to enhance the effects of oncolytic HSV is to produce an oncolytic HSV expressing TSP-1, which in addition to oncolysis of tumor cells, can induce anti-angiogenic mechanisms. In the treatment of human GC, a third-generation oncological HSV (T-TSP-1) expressing human TSP-1 was studied *in vitro* and *in vivo*, and the results demonstrated that TSP-1-mediated apoptosis was more inhibited in MKN1 than TMK-1 GC cell *in vitro*. Arming the viruses with TSP-1 had little effect on their proliferation in some GC cell lines but did not reduce their viral cytolysis and antitumor effects. Furthermore, *in vivo* administration of T-TSP-1 in addition to oncolysis could inhibit angiogenesis through suppression of TGF-β signaling (Tsuji et al., 2013). As discussed before, PGK-1 is likely involved in the metastatic spread of tumor cells in GC (Warburg et al., 1927). In addition, PGK-1 has a real effect on tumor stem cell characteristics. The presence of malignant stem cells is significant in therapeutic resistance and recurrence. It is hypothesized that targeting and inhibiting PGK-1 makes these cells more sensitive to chemotherapy, and thus therapeutic resistance can be overcome. A phase III clinical trial study reported promising results using intraperitoneal paclitaxel (PTX) for PMGC (Takashima et al., 2019). However, this treatment has not been effective enough to eradicate PMGC. Whether intraperitoneal oncolytic virus therapy with PTX could be effective in PMGC was investigated by a research team. OBP-401, an attenuated oncolytic adenovirus that can express green fluorescence protein (GFP) driven by the telomerase promoter, was employed in this study and the effect of its combination therapy with PTX on different human GC cell lines (GCIY and KATO III) and xenograft PM model was also evaluated. The results showed that OBP-401 in combination with PTX synergistically reduced the viability of human GC cells and increased the proliferative ability of the virus in cancer cells. This combination therapy also induced mitotic catastrophe, accelerated autophagy, and apoptosis. Administration of PTX in the human orthopedic PMGC model was also able to profoundly increase the penetration of OBP-401 into the disseminated nodules. In this study, a non-invasive *in vivo* imaging system (IVIS) was used, and the imaging results showed that combination treatment of OBP-401 with PTX significantly inhibited the growth of the metastatic peritoneal tumor reduced the volume of malignant ascites. Although based on these findings, intraperitoneal virus therapy with PTX is considered a promising treatment approach for PMGC; clinical trials are necessary to evaluate the effectiveness of this type of combination therapy in patients with PMGC (Ogawa et al., 2019).

Although adenoviral gene therapy has been described as a potentially promising therapeutic approach, dose-limiting toxicity and reported in clinical trials adverse effects, including flu-like symptoms, transaminitis and lymphopenia, are considered challenges of using adenovirus vectors (Lan et al., 1997; Heise et al., 1999; Reid et al., 2002). To solve this problem, a new system using adenoviral oncolytic suicide gene therapy targeting carcinoembryonic antigen (CEA) was constructed, and its beneficial effect and the possibility to decrease the total viral dose by preserving the antitumor effect were evaluated. Three types of adenoviruses were employed for this system: (I) Ad/CEA-Cre, (II) Ad/lox-CD::UPRT for a Cre/loxP system, and (III) Ad/CEA-E1 for persisting adenovirus replication. Then, the antitumor consequence of the oncolytic suicide gene therapy (I + II + III) was assessed *in vitro*. At the same viral dose, the present system (I + II + III) showed pointedly improved cytotoxic impacts for CEA-producing cell lines compared to suicide gene therapy (I + II) *in vitro*. Therefore, it is possible to decrease the total adenoviral dose along whit preserving the antitumor properties of the virus in oncolytic suicide gene therapy (Imamura et al., 2010).

It has been demonstrated that NDV-D90, as an oncolytic virus in Newcastle disease, could induce cell apoptosis in GC tumor cells in a dose-dependent manner in GC cell lines, including BGC-823, SGC-7901 but not in MKN-28 cells MKN-28 (Sui et al., 2017). Additionally, cell invasion was significantly reduced only in BGC-823 and SGC-7901 cells following this type of virus therapy. The decrease in cell growth and the increase in cell apoptosis in GC cells treated with NDV-D90 are probably due to the suppression of ERK1/2 and Akt signaling and the increase of p38 signaling. Moreover, orthotopic injection of NDV-D90 impaired tumor cells implantation and inhibited tumor growth with intra-tumor necrosis *in vivo*. In addition, it appears that NDV-D90 could suppress angiogenesis of gastric tissue by inhibition of vascular endothelial growth factor (VEGF)-A and matrix metalloproteinase-2 (MMP-2), all of which may prevent tumor progress and metastasis (Sui et al., 2017). Since this study explored the effects of NDV-D90 on human GC cells, the TME was in mice. Moreover, the immunodeficiency condition of nude mice may affect the data interpretation.

Based on previous studies, vaccinia-based virotherapy has had hopeful therapeutic impacts on various human cancers with proper safety (Chen et al., 2009). The therapeutic efficacy of a novel genetically-engineered vaccinia virus expressing the human sodium iodide symporter (*h*NIS) gene was investigated, and the outcomes showed that treatment of tumor cells by GLV-1 h153 could efficiently regress GC and permit deep-tissue imaging (Jun et al., 2014).

TABLE 2 | Oncolytic viruses used in the treatment of PMGC.

Oncolytic virus	Study details	Genetic manipulation	Route	Outcomes	Reference
NDV	*In vitro* • Human GC cell lines: SGC-7901 and AGS • rL-RVG	rL-RVG	-	Increasing endoplasmic reticulum stress, autophagy, and apoptosis	Bu et al. (2015)
Adenovirus +5-FU and mitomycin	*In vitro* • Human GC cell line: 23132/87 (ACC409) • Adv-shPGK1	Knockdown of PGK1	-	Reducing tumor cell viability, increasing the susceptibility of metastatic GC cells and tumor stem cells to overcome the chemotherapeutic therapy resistance	Schneider et al. (2015)
Reovirus	*In vitro*/Animal model • Human GC cell lines: MKN45p, NUGC4, MKN7 • Reovirus serotype 3 • Nude mice	None	IV/IP	Increase of cytopathic effect, increase of Ras activity, Reduce the mean number and weight of total peritoneal tumors along with the volume of ascites	Kawaguchi et al. (2010)
Reovirus + trastuzumab	*In vitro*/Animal model • Human GC cell lines: NCI-N87 & MKN-28 • Reovirus serotype 3 • Male BALB/c nude mice	None	SQ	Inhibition of HER2, sensitization of GC cells by overexpressing HER2 for apoptosis by reovirus, increase of TRAIL/Apo2L-mediated apoptosis, increasing anti-angiogenic responses and ADCC	Hamano et al. (2015)
HSV-1 (G47Δ)	*In vitro*/Animal model • Human GC cell lines: MKN45, MKN74, and 44As3 • G47Δ • Female athymic mice	None	IT/IP	Satisfactory proliferative and cytopathic effects, decreasing M2 macrophages and increasing M1 macrophages along with NK cells	Sugawara et al. (2020)
Adenovirus	*In vitro*/Animal model • Human GC cell lines: MKN45, HGC27, SGC-7901, MKN28, NHFB • Ad/TRAIL-E1 • BALB/c nude mice	E1A and TRAIL	IP	Antitumor effects, inhibit PM and lead to increase survival	Zhou et al. (2017)
Echovirus 1	*In vitro*/Animal model • Human GC cell lines: AGS, Hs746T, and NCI-N87 • MKN-45-Luc cells • (SCID)- BALB/c mice	None	IP	Antitumor effects, oncolysis of α2β1expressing tumor cells	Haley et al. (2009)
HSV	*In vitro*/Animal model • Vero (Africa green monkey kidney), AZ521, MKN1, MKN28, MKN45 and MKN74 (human GC cell lines) • T-TSP-1 female BALB/c nu/nu mice	TSP-1	SQ	Proliferative and cytopathic effects, Oncolysis of tumor cells, anti-angiogenic effects via inhibiting TGF-β signaling	Tsuji et al. (2013)
Adenovirus+ PTX	*In vitro*/Animal model • Human GC cell lines: GCIY and KATO III • OBP-401 Xenograft peritoneal metastasis model	None	IP	Reducing the viability of human GC cells and increasing the proliferative ability of the virus in tumor cells, induction of mitotic catastrophe, accelerated autophagy, and apoptosis, inhibiting the growth of the metastatic peritoneal tumor and reducing the volume of malignant ascites	Ogawa et al. (2019)
Adenovirus	*In vitro*/Animal model • Human GC cell lines: AGS, MKN1, MKN45 • Ad/CEA-Cre, Ad/lox-CD::UPRT, and Ad/CEA-E1 • BALB/c nu/nu mice	CEA	IP	Decreasing the total viral dose, preserving the antitumor effect	Imamura et al. (2010)
NDV	*In vitro*/Animal model • Human GC cell lines: BGC-823, SGC-7901 and MKN-28 • NDV-D90 • Male nude mice	None	IT	Inducing cell apoptosis in GC tumor cells, reducing tumor cell invasion, suppression of ERK1/2 and Akt signaling, anti-angiogenic effects by inhibition of VEGF-A and MMP-2	Sui et al. (2017)
Vaccinia	*In vitro*/Animal model • Human GC cell lines: AGS, OCUM-2MD3, MKN-45, MKN-74 and TMK-1 • GLV-1 h153 Female nude mice	*h*NIS	SQ	Efficiently regress GC and permit deep-tissue imaging	Jun et al. (2014)

(Continued on following page)

TABLE 2 | (*Continued*) Oncolytic viruses used in the treatment of PMGC.

Oncolytic virus	Study details	Genetic manipulation	Route	Outcomes	Reference
4th-generation oncolytic HSV	*In vitro/ex vivo* • Vero (African green monkey kidney normal cell line), MKN1, MKN28, MKN45, MKN74, NUGC3, NUGC4, KATOIII, and N87 (human GC cell lines) • T-hTERT • Human gastric adenocarcinoma specimens	ICP6	-	Antitumor effects, oncolysis of tumor cells	Kato et al. (2021)
3rd-generation HSV	*In vitro/ex vivo* • Human GC cell lines: MKN1, MKN28 and MKN74 cells • T-01	SOCS-3	-	Satisfactory proliferative and cytopathic effects	Matsumura et al. (2021)

IP, intraperitoneal; IT, intratumoral; SQ, subcutaneous; IV, intravenous.

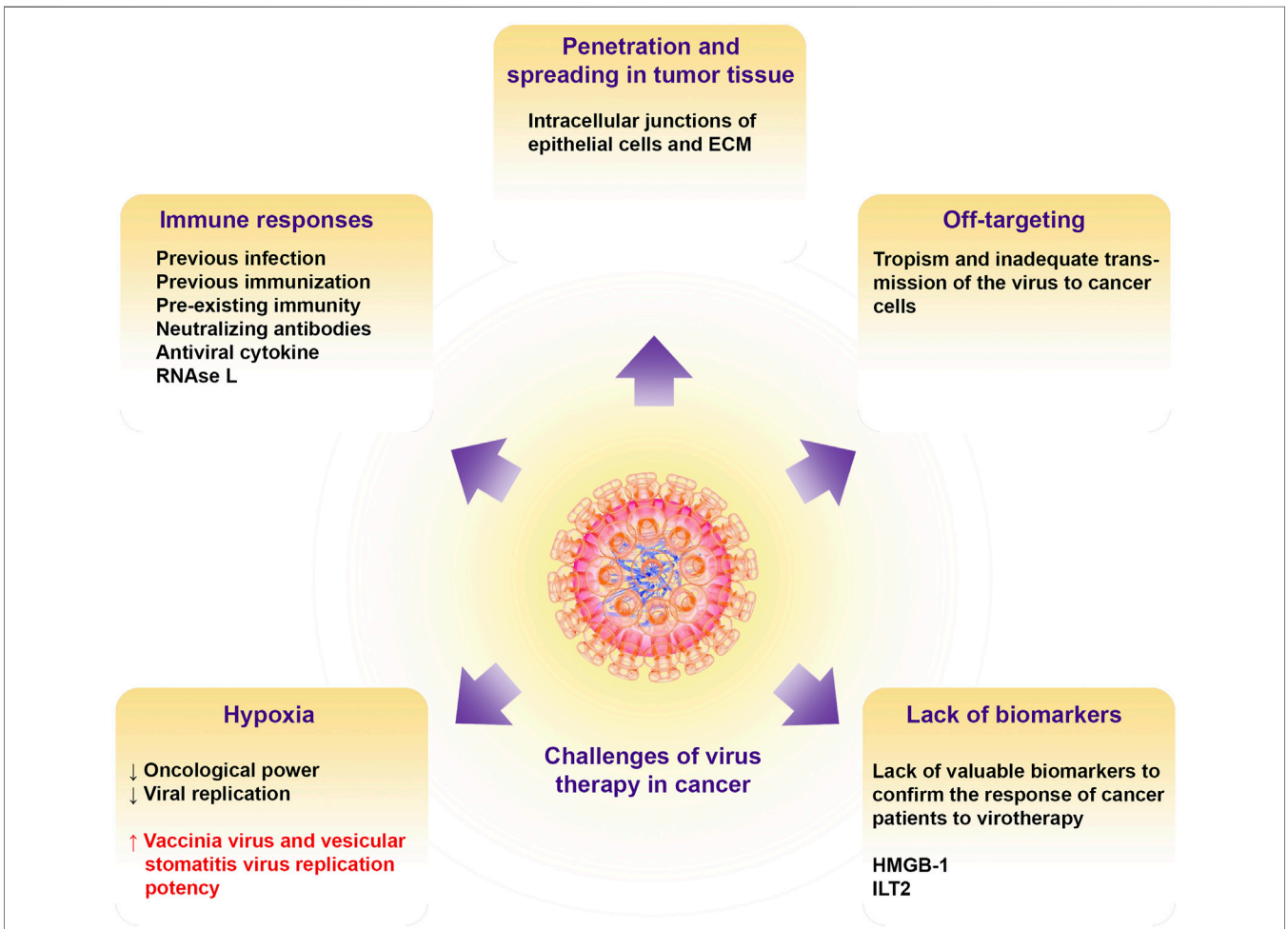

FIGURE 3 | Challenges of oncolytic virotherapy in cancer. Illustrate the barriers to oncolytic viral therapy including tissue penetration, off targeting, immune responses, hypoxic condition in the TME, and lack of putative biomarkers for patient virotherapy monitoring. ECM, extracellular matrix; HMGB-1, high mobility group box-1; ILT2, Ig-like transcript 2.

5.3 *In Vitro/Ex Vivo* Studies

As previously discussed, oncolytic virus therapy using HSV has emerged as a new therapeutic approach in treating human malignancies (Fukuhara et al., 2016). Evidence shows that telomerase is activated in many malignant tumors, including GC, and that human telomerase reverse transcriptase (hTERT) is one of the key components of the telomerase enzyme (Liu et al., 2012; Yano et al., 2017). Therefore, it can be clinched that the insertion of essential genes under the regulation of the hTERT promoter, such as the ICP6 in oncolytic HSV, may potentiate its antitumor effects. A study of fourth-generation oncolytic HSVs containing the ICP6 gene regulated by the hTERT promoter (T hTERT) showed that this type of virus could have enhanced cytotoxicity in MKN45, MKN28, and MKN1 cells *in vitro* compared to third-generation oncolytic HSV which the mentioned cytotoxicity of T hTERT especially was higher in MKN45 cells. In addition, *ex vivo* assessment of oncolytic HSV cytotoxicity in GC disclosed that a significant percentage of initial clinical tumors were lysed after infection with T null or T hTERT viruses. These findings suggest that the use of oncolytic HSVs containing the *ICP6* gene under the regulation of the hTERT promoter may be a beneficial and effective therapeutic approach for GC (Kato et al., 2021). Recently, another study examined the efficacy of a third-generation HSV oncolytic suppressor of cytokine signaling 3 (SOCS3). Intensification of viral replication and oncolysis of T-SOCS3 for different human GC cell lines was investigated *in vitro*, and the results showed that T-SOCS3 could increase its proliferation and its tumor cell lysis properties for the MKN1 cell line. T-SOCS3 also induces the destruction of tumor cells in human GC specimens (Matsumura et al., 2021).

Taken together, the studies and their results show that viral therapy using different types of oncolytic viruses and also amplifying them by arming these viruses with different genes with antitumor activity may be effective to treat PMGC via various mechanisms such as direct oncolysis, inhibition of angiogenesis and induction of apoptotic as well as autophagic pathways (**Table 2**).

6 WHAT ARE REMAINING CHALLENGES?

In this section, the challenges of virus therapy in the treatment of human cancers are discussed and also suggestions for removing these barriers and limitations to increase the effectiveness of treatment are presented (**Figure 3**).

6.1 Oncolytic Virus Penetration and Spreading in Tumor Tissue

Evidence has shown that intracellular junctions of epithelial cells and ECM in carcinomas prevent the penetration of therapeutic agents such as oncolytic viruses, especially adenoviruses, which leads to resistance to treatment and failure of cancer therapy (Lipinski et al., 1997; Green et al., 2002; Christiansen and Rajasekaran, 2006; Lavin et al., 2007). In addition, during metastasis, phenotype alteration through epithelial-to-mesenchymal transition (EMT) and then mesenchymal-to-epithelial transition (MET) makes epithelial junctions tighten, which this event is not in favor of effective treatment (Christiansen and Rajasekaran, 2006; Turley et al., 2008). Some types of adenoviruses, such as B14p, B14, and HAdV-B3, may overcome epithelial junctions by releasing Pantone-dodecahedron (Pt-Dd) in the early phases of infection and before oncolysis. Non-Pt-Dd adenoviruses such as HAdV-C5, which is most commonly used in the production of oncolytic viruses, begin to overproduce fiber protein in the mentioned phase of infection (Fender et al., 2005; Lu et al., 2013). For improved access to cancer cells and oncolysis, navigating the ECM barriers is necessary for oncolytic viruses (Wojton and Kaur, 2010). For this purpose, pretreatment of the tumor cells with collagenase or concomitant administration of hyaluronidase with oncolytic adenoviruses led to more spreading of the virus (Kuriyama et al., 2000; Ganesh et al., 2008). In order to increase therapeutic efficacy, the engineering of oncolytic viruses for the expression of MMP-1 and MMP-8 leads to the degradation of tumor-associated sulfated glycosaminoglycans, which increases virus penetration and dissemination (Mok et al., 2007). Induction of apoptosis by cytotoxic agents and activation of caspase-8 has been reported to increase intra-tumor infiltration and thus antitumor efficacy of oncolytic HSV. It has been interpreted that shrinkage or initiation of apoptotic pathways in tumor cells leads to the formation of channel-like structures and void spaces in the cells that enhance and facilitate the spread of oncolytic HSV (Nagano et al., 2008).

6.2 Off-Targeting

Although virus therapy has various benefits in controlling cancer, it has been shown to have little effect in the clinic after direct administration of HSV-1 (T-VEC) in people with melanoma due to tropism and inadequate transmission of the virus to cancer cells (Kloos et al., 2015a; Andtbacka et al., 2015). Therefore, surface alterations in oncoviruses can alleviate this problem to some extent (Jhawar et al., 2017). In tumor models, it has been revealed that insertion of a tripeptide Arg-Gly-Asp (RGD) motif in the HI loop of the adenovirus fiber knob domain can significantly enhance infection efficiency and cytotoxic effect via autophagy inhibition and apoptosis promotion (Xu et al., 2017). Another approach for targeting oncolytic adenovirus is to use different serotypes. In this regard, it has been revealed that HAdV-G52 is able to bind to polysialic acid on tumor cells, and due to the overexpression of polysialic acid on the surface of these cells, the use of HAdV-G52 can infect a variety of cancer cells. However, modifications seem to be potentially necessary to prevent neurotropism (Figarella-Branger et al., 1990; Lantuejoul et al., 1998; Tanaka et al., 2000; Suzuki et al., 2005). Other tactics for redirecting adenoviruses and targeting tumor cells by oncolytic viruses include the use of bispecific adapters capable of binding to viruses and tumor cells as well as antibody-based targeting of tumor cells by antibody single-chain variable fragments (scFvs) (Nakano et al., 2005; Belousova et al., 2008; Poulin et al., 2010; Baek et al., 2011; Kloos et al., 2015b; Bhatia et al., 2016).

6.3 Immune Responses

Evidence suggests that pre-existing immunity due to previous infection or immunization and shortening the virus half-life is one of the major challenges in cancer therapy with oncolytic viruses. To solve this problem, researchers mask the virus with different materials such as polymers, which can lead to virus protection, increase the virus half-life, and improve virotherapy's effectiveness (Carlisle et al., 2013). However, due to the non-genetic nature of these changes, progeny virions cannot have these characteristics and be protected. Neutralizing antibodies are another problem in virotherapy, which can be solved by using cellular carriers as delivery vehicles (Roy and Bell, 2013). Other immune system antiviral responses, such as interferons (IFNs), can inhibit the infection via delaying virus replication. To address this problem, the use of histone deacetylase (HDAC) inhibitors such as valproic acid to induce epigenetic modifications and suppress the expression of antiviral cytokine genes has been suggested (Otsuki et al., 2008; Cody et al., 2014). However, the use of these inhibitors can have adverse effects. For example, despite enhancing the proliferation of the oncolytic virus, valproic acid can inhibit viral DNA, reduce the recruitment of effector cells such as NK cells and macrophages into the tumor microenvironment (TME), and inhibit tumor cell apoptosis (Koks et al., 2015).

The pathways leading to RNase L production can also be activated in response to viral infection, eventually destroying cellular and viral single-stranded RNA (Liang et al., 2006). Studies showed that using RNase L inhibitors such as sunitinib, which also inhibits platelet-derived growth factor receptors (PDGF-R) and VEGF, can increase the effectiveness of oncolytic viruses in cancer treatment (Tang et al., 2020). The use of other anti-angiogenic agents such as bevacizumab (Anti-VEGF) as well as cytokine therapy with transforming growth factor-beta (TGF-β), and employment of immunosuppressive drugs such as cyclophosphamide can help increase the effectiveness of virotherapy (Fulci et al., 2006; Libertini et al., 2008; Tysome et al., 2013; Han et al., 2015).

6.4 Impacts of Hypoxia

Based on available knowledge, hypoxia is a feature of TME in solid tumors that occurs during tumor growth and development (Bosco et al., 2020). The effect of hypoxia can be different on oncolytic viruses. For example, hypoxic conditions in the TME can modulate the oncological power as well as replication in oncolytic viruses that are dependent on cell cycle progression (Shen and Hermiston, 2005; Shen et al., 2006). In this regard, researchers have designed an oncolytic adenovirus in which the expression of the E1A gene under the promoter's control contains the element of hypoxia response, and this genetic manipulation can lead to increased virus replication in hypoxic conditions (Hernandez-Alcoceba et al., 2002).

In contrast, under hypoxic conditions, other oncolytic viruses, including the vaccinia virus and vesicular stomatitis virus, can increase their replication potency (Connor et al., 2004; Hiley et al., 2010). Furthermore, the HSV-1 virus has been reported to exacerbate hypoxic conditions of virus replication. This ability of HSV viruses due to their tropism to low oxygen levels or oxygen-induced free radical DNA damage enhances the replication of these viruses (Aghi et al., 2009). Hypoxia-inducible factor-1 alpha (HIF-1α) has also been expressed in hypoxia that can stimulate HSV-1 proliferation-related genes (Aghi et al., 2009; Chaurasiya et al., 2018). However, infection with some oncolytic viruses, such as the Newcastle disease virus, degrades HIF-1α under hypoxic conditions and affects the expression of its target genes (Abd-Aziz et al., 2016).

6.5 Lack of Adequate Biomarkers for Patients Monitoring

The lack of valuable biomarkers to confirm the response of cancer patients to oncolytic viruses is another important challenge of virus therapy. Extensive tumor fluctuations also complicate the problem due to cancer patients' specific immune system conditions who have previously tried other anticancer therapies (Turnbull et al., 2015). Studies have revealed that high mobility group box-1 (HMGB-1) in virus therapy with oncolytic adenoviruses as well as human inhibitory receptors Ig-like transcript 2 (ILT2) in the treatment of cancer with vaccinia virus can be used as predictive, prognostic, and treatment monitoring biomarkers (Zloza et al., 2014; Liikanen et al., 2015). However, further studies are needed in this area.

7 CONCLUDING REMARKS

Considering the relatively satisfactory outcomes of studies in the field of treatment of solid cancers such as GC using oncolytic viruses, it seems that these viruses can be used more widely in combination therapies to increase the efficiency and effectiveness of cancer treatment. However, this therapeutic approach has several challenges, and more studies are needed. In PMGC, virotherapy can limit peritoneal metastasis and tumor metastasis to the peritoneum in various ways, such as direct oncolysis of tumor cells, as well as inhibition of mechanisms and molecules involved in angiogenesis. On the other hand, inserting genes with antitumor function in the genome of oncolytic viruses for expression in virus-infected tumor cells can enhance the therapeutic effect. Viruses seem to have a wide range of unknown functions, and due to their extraordinary capabilities, such as their ability to replicate in hypoxic conditions, which is one of the drawbacks of cancer therapy, in the near future, they can be used to treat cancers to the maximum benefited performance.

AUTHOR CONTRIBUTIONS

X.-Z.M. and P.-Y.H.: Conception, design and inviting co-authors to participate. S.S. and X.Y.: Writing original manuscript draft. Y.-N.Z., X.-J.W., K.L. and Y.-L.Z.: Review and editing of manuscript critically for important intellectual content and provided comments and feedback for the scientific contents of the manuscript. All authors read, revised and approved the final manuscript.

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REVIEW
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Alphaviruses in Cancer Therapy

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Alphaviruses have been engineered as expression vectors for different strategies of cancer therapy including immunotherapy and cancer vaccine development. Administration of recombinant virus particles, RNA replicons and plasmid DNA-based replicons provide great flexibility for alphavirus applications. Immunization and delivery studies have demonstrated therapeutic efficacy in the form of reduced tumor growth, tumor regression and eradication of established tumors in different animal models for cancers such as brain, breast, colon, cervical, lung, ovarian, pancreas, prostate cancers, and melanoma. Furthermore, vaccinated animals have showed protection against challenges with tumor cells. A limited number of clinical trials in the area of brain, breast, cervical, colon prostate cancers and melanoma vaccines has been conducted. Particularly, immunization of cervical cancer patients elicited immune responses and therapeutic activity in all patients included in a phase I clinical trial. Moreover, stable disease and partial responses were observed in breast cancer patients and prolonged survival was achieved in colon cancer patients.

Keywords: self-replicating RNA, recombinant particles, RNA replicon, DNA replicon, cancer vaccine, immunotherapy

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INTRODUCTION

Although significant progress has been made on many fronts of cancer treatment, the continuous increase in cancer cases because of pollution, unhealthy eating and lifestyle choices, and an aging population keep the suffering and mortality rates high (Magee et al., 2013). In addition to conventional chemo- and radiotherapy approaches, non-viral and viral based cancer therapies have been applied (Lundstrom and Boulikas, 2003). One approach has been to deliver cytotoxic or suicide genes (Zarogoulides et al., 2013) or anti-tumor genes (Liu et al., 2005) with the aim of killing tumor cells while normal tissue is unaffected. Several viruses have been referred as oncolytic demonstrating natural tumor targeting and specific replication in tumor cells leading to their death without affecting normal cells. For example, the M1 alphavirus possesses natural oncolytic activity (Zhang et al., 2021) and Sindbis virus (SIN) has showed natural tumor targeting (Tseng et al., 2004). Cancer immunotherapy has caught plenty of attention recently although its first use dates to William B. Coley's discovery of tumor regression in inoperable bone sarcoma after bacterial injection (Coley 1891; Kozlowska et al., 2013). The modern approach of cancer immunotherapy aims at boosting or restoring the ability of the immune system to detect and destroy cancer cells (Scott et al., 2012). Cancer immunotherapy comprises administration of various cytokines, and expression of tumor-associated antigens (TAAs) (Fortner et al., 2017). The advantage of using viral vectors relates to their features of excellent delivery and high level of recombinant protein expression. On the other hand, viral vectors can pose a safety risk, which has triggered the engineering of replication-deficient and suicide vectors. A variety of viral vectors based on adenoviruses, adeno-associated virus (AAV), alphaviruses, herpes simplex virus (HSV), lentiviruses (LV), measles virus (MV), Newcastle disease virus (NDV), and rhabdoviruses have demonstrated promising results in both preclinical animal

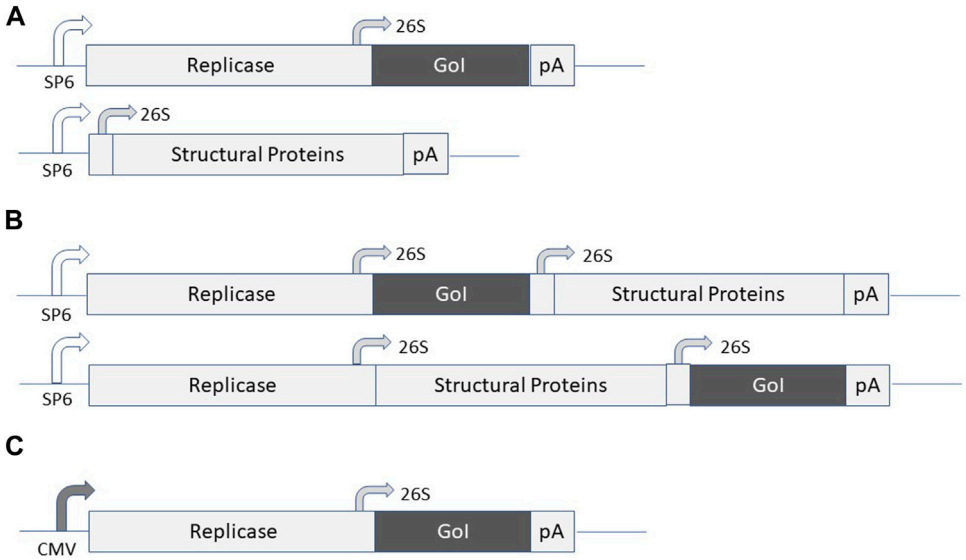

FIGURE 1 | Expression systems for alphaviruses. **(A)** Replication-deficient alphavirus particles. The alphavirus expression vector contains the non-structural protein (nsP) genes, the subgenomic 26S promoter, the gene of interest (GoI) and the poly A signal. The helper vector contains the subgenomic promoter, the structural protein (C-p62-6K-E1) genes and the poly A signal. SP6 RNA polymerase is used for *in vitro* transcription of RNA from expression vector and helper vector DNA and co-transfected/electroporated into BHK-21 cells for virus production. **(B)** Replication-proficient alphavirus particles. SP6 RNA polymerase is used for *in vitro* transcription of full-length alphavirus RNA genome including the GoI introduced either upstream or downstream of the structural protein genes followed by transfection/electroporation into BHK-21 cells for virus production. **(C)** DNA/RNA layered vector. The plasmid DNA replicon is transfected into mammalian cells for expression of the GoI.

models and clinical trials (Lundstrom, 2022). An essential part of viral vector-based cancer therapy has relied on the application of oncolytic viruses, which can provide selective killing of tumor cells while causing only minor or no damage to normal tissue (Kaufman et al., 2015). In this review, the focus will be on alphaviruses and their application in cancer therapy.

ALPHAVIRUS VECTORS

Alphavirus vectors have been engineered for the expression of recombinant proteins in mammalian cells lines, gene therapy applications and vaccine development (Lundstrom 2015). Briefly, alphaviruses are enveloped single-stranded RNA viruses (Strauss and Strauss, 1994). Due to the positive polarity of the genome, the ssRNA is directly translated in the cytoplasm of infected host cells. Expression of the alphavirus non-structural genes generates the replicase complex resulting in self-replication of RNA, production of structural proteins, encapsulation of RNA genomes, and assembly and release of new viral particles. The most commonly used expression vector systems are based on Semliki Forest virus (SFV) (Liljestrom and Garoff, 1991), SIN (Xiong et al., 1989) and Venezuelan equine encephalitis virus (VEE) (Davis et al., 1989). In principle, alphavirus vectors can be delivered as recombinant virus particles, RNA replicons or layered DNA/RNA plasmid vectors (**Figure 1**). In the case of viral particle delivery both replication-deficient and -proficient particles have been engineered. In the former case, the alphavirus genome is split on two or more plasmid vectors, where the expression vector carries the alphavirus non-structural genes (nsP1-4) and the gene of interest (GoI) and the structural genes are placed on one or several (split helper) helper plasmids. Recombinant virus particles are generated by *in vitro* transcription of RNA from linearized DNA plasmids followed by electroporation or transfection of mammalian cells such as BHK-21 cells (**Figure 1A**). Replication-proficient alphavirus particles are produced by introduction of the GoI in the full-length alphavirus genome downstream of either the nsP genes or the structural genes (**Figure 1B**). Alternatively, alphavirus vectors can be delivered as naked RNA, but due to the sensitivity of single-stranded RNA (ssRNA) to degradation, the delivery and stability of RNA can be significantly improved by encapsulation in lipid nanoparticles (Geall et al., 2012; Blakney et al., 2019). Moreover, DNA replicon vectors have been engineered by replacing the SP6 RNA polymerase promoter with a CMV promoter (DiCiommo and Bremner, 1998) (**Figure 1C**). In all cases, the vectors possess the self-replicating feature of single-stranded RNA viruses due to the presence of the nsP-based replicon complex, which can accumulate approximately 10^6 copies of viral RNA per cell in the cytoplasm of infected cells (Frolov et al., 1996). The RNA amplification in combination with the strong 26S subgenomic promoter present in alphavirus vectors generate high levels of GoI such as TAA or cytotoxic gene expression. Because of the degradation of the alphavirus ssRNA, only transient expression and no integration into host cell genome occur, ideal features for vaccine development. Concerning DNA replicons, the potential chromosomal integration risk is as low as determined for conventional DNA plasmids (Langer et al., 2013).

TABLE 1 | Examples of Alphavirus applications in cancer therapy.

Cancer	Vector	Strategy	Response	Ref
Brain				
GBM	SFV-Endostatin	VLPs	Tumor regression in mice	Yamanaka et al. (2001)
GBM	SFV-IL-18	VLPs + IL-12	Anti-tumor and protective immunity in mice	Yamanaka et al. (2003)
RG2	SFV-IL-12	VLPs	70–87% reduction in tumor volume in mice	Roche et al. (2010)
GBM	SIN-gp100/IL-18	DNA	Enhanced protection, prolonged survival	Yamanaka and Xanthopoulos, (2005)
GBM	SFV VA-GFP	Oncolytic SFV	Single injection > long-term survival	Heikkilä et al. (2010)
CT-2A	SFV4-miRT124	RPVPs	Tumor inhibition, prolonged survival	Martikainen et al. (2015)
GBM	LSFV-IL-12	LSFV	Phase I/II protocol for recurrent GBM	Ren et al. (2003)
Breast				
A2L2	SIN-HER2/neu	DNA + Ad-neu	Prolonged survival in mice	Wang et al. (2005b)
A2L2	SIN-HER2/neu	DNA	Tumor protection with 80% less DNA	Lachman et al. (2001)
A2L2	VEE-HER2 ECD/TM	VRPs	Complete prevention of tumor formation	Wang et al. (2005a)
HER2	VEE-HER2 ECD/TM	VRPs	Phase I: PR: 1 patient, SD; 2 patients	NCT03632941
HER2	VEE-HER2 ECD/TM	VRPs + Pembro	Phase II: study in progress	Crosby et al. (2019)
4T1	SFV-IL-12	VLPs + LVR01	Superior inhibition of metastases	Kramer et al. (2015)
TNBC	M1	oM1 + Dox	Enhanced anti-tumor activity with Dox	Zhang et al. (2021)
Cervical				
HPV16	VEE.HPV16 E7	VLPs	Protection against tumor challenges	Velders et al. (2001)
HPV16	SIN AR339	Oncolytic SIN	Significant tumor regression	Unno et al. (2005)
HPV16	SFVenh-HPV-E6-E7	VLPs	Complete eradication of tumors in mice	Daemen et al. (2003)
HPV16	SFV-sHELP-E7SH	VLPs	Tumor regression, protection of mice	Ip et al. (2015)
HPV16	SFV-HPV-E-E7	DNA	85% of immunized mice tumor free	Van der Wall et al. (2018)
HPV16	SFV HPV-E6-E7	VLPs (Vvax001)	Phase I: immune response in all patients	Komdeur et al. (2021)
Colon				
MC38	SFVenh-IL-12	VLPs	Complete tumor regression in 80% of mice	Rodriguez-Madoz et al. (2005)
MC38	SFV-IL-12	VLPs + anti-PD1	Synergism with immune checkpoint blockade	Quetglas et al. (2015)
MC38	VEE-IL-12/CEA	VLPs	Superior combination therapy in mice	Lyons et al. (2007)
CT26	SFV-VEGFR-2/IL-4	VLPs	Prolonged survival after combination	Ying et al. (1999)
CT26	SFV-LacZ	RNA	Immunogenicity, prolonged survival	Crosby et al. (2020)
CC	VEE-CEA	VLPs	Phase I: Prolonged overall survival	Osada et al. (2012)
Lung				
H358a	SFV-EGFP	VLPs	Complete regression of tumors	Murphy et al. (2000)
A549	SFV VA-EGFP	Oncolytic SFV	Superiority to adenovirus in mice	Määttä et al. (2008)
CL25	SIN-LacZ	VLPs	Complete remission, prolonged survival	Granot et al. (2014)
Melanoma				
B16	VEE-TRP-2	VLPs	Immune response, prolonged survival	Avogadri et al. (2010)
B16	VEE-TRP-2	VLPs + CTLA-4	Tumor regression in 50% of mice	Avogadri et al. (2014)
B16	VEE-TRP-2	VLPs + GITR	Tumor regression in 90% of mice	Avogadri et al. (2014)
B16	SFV-VEGFR-2/IL-12 SFV-surv/βhCG	Combination of 2 DNA vectors	Combination of SFV-VEGFR-2/IL-12 + SFV-surv/βhCG superior	Yin et al. (2015)
B16-OVA	SFV-IL-12	VLPs + anti-PD1	Synergism with immune checkpoint blockade	Quetglas et al. (2015)
MIII-IV	LSFV-IL-12	LSFV	Phase I: safe, 10-fold increase in IL-12	Ren et al. (2003)
Ovarian				
C33A	SIN AR339	Oncolytic SIN	Suppressed ascites formation in mice	Unno et al. (2005)
ES2	SIN-IL-12	VLPs + irinotecan	Long-term survival in mice	Granot & Meruelo (2012)
MOSEC	SFV-OVA	VLPs + VV	Enhanced anti-tumor activity	Zhang et al. (2010)
Pancreatic				
MePC	VEE-CEA	VLPs	Prolonged survival in patients	Morse et al. (2010)
LAPC	M1	oM1 + IRE	Prolonged survival in mice	Sun et al. (2021)
Prostate				
TRAMP	VEE-PSMA	VLPs	Th1-biased response, CTL activity	Durso et al. (2007)
CRPC	VEE-PSMA	VLPs	Phase I: safe, weak immunogenicity	Slovin et al. (2013)
TRAMP-C	VEE-STEAP	conDNA + VLPs	Prolonged survival, protection in mice	Garcia-Hernandez et al. (2007)
TRAMP	VEE-PSCA	VLPs	Long-term survival in 90% of mice	Garcia-Hernandez et al. (2008)

A2L2, breast cell line expressing HER2; Ad-neu, Adenovirus-neu; anti-PD1, anti-PD1 monoclonal antibody; CEA, carcinoembryonic antigen; CC, colon cancer; conDNA, conventional plasmid DNA; CRPC, castration resistant prostate cancer; CTLA-4, CTL antigen-4; Dox, doxorubicin; GBM, glioblastoma multiforme; GITR, glucocorticoid-induced tumor necrosis factor receptor; HPV, human papillomavirus; IRE, irreversible electroporation; LAPC, locally advanced pancreatic cancer; LSFV, liposome encapsulated SFV particles; LVR01, Salmonella typhimurium *aroC strain; M1, oncolytic M1 alphavirus; MePC, metastatic pancreatic cancer; MOSEC, murine ovarian surface epithelial carcinoma; OVA, ovalbumin; Pembro, Pembrolizumab; PR, partial response; PSCA, prostate stem cell antigen; PSMA, prostate specific membrane antigen; RG2, rat glioma 2; RPVPs, replication-proficient viral particles; SD, stable disease; SFV, Semliki Forest virus; SIN, Sindbis virus; TNBC, triple-negative breast cancer; TRP-2, tyrosine-related protein-2; STEAP, six-transmembrane epithelial antigen of the prostate; TRAMP, transgenic adenocarcinoma of the prostate; VEE, Venezuelan equine encephalitis virus; VEGFR-2, vascular endothelial growth factor-2; VLPs, virus-like particles; VV, vaccinia virus.*

ALPHAVIRUS VECTORS IN TISSUE-SPECIFIC CANCER THERAPY

Alphavirus vectors have been frequently used for cancer therapy in various animal models and to some extent in clinical trials. A common approach has been to overexpress TAAs to provide both therapeutic and prophylactic effects. In many cases, green fluorescent protein (GFP) and luciferase have been used as reporter genes to monitor location and delivery efficacy. Therapeutic activity has been achieved through alphavirus-based expression of cytotoxic and anti-tumor genes, but also due to the alphavirus induced apoptosis (Olkkonen et al., 1993). However, apoptotic events are not restricted to tumor cells as they also occur in normal cells infected by alphaviruses, which suggests that for systemic alphavirus administration it is recommended to use oncolytic or tumor-targeted viruses. Moreover, immunostimulatory and particularly cytokines have been targeted for cancer immunotherapy. Another approach comprises the application of oncolytic viruses for the therapy of existing tumors (Fukuhara et al., 2016). Examples of the above-mentioned strategies are described below and summarized in **Table 1**.

Among the different cancer types, brain tumors, especially glioblastomas have been targeted for alphavirus-based therapy. Recombinant SFV particles expressing endostatin (SFV-Endostatin) was compared to retrovirus-endostatin and SFV-LacZ particles based on their oncolytic activity in a B16 mouse brain tumor model (Yamanaka et al., 2001). The SFV-Endostatin particles provided superior tumor growth inhibition and reduced intratumoral vascularization compared to the other treatments. Moreover, endostatin serum levels were 3-fold higher after intravenous administration of SFV-Endostatin particles compared to intravenous endostatin. SFV particles expressing interleukin-18 (IL-18) were also applied for transduction of dendritic cells (DCs) combined with systemic administration of IL-12 (Yamanaka et al., 2003). The combination therapy was superior to IL-12 alone generating enhanced Th1 responses in tumor-specific $CD4^+$ and $CD8^+$ T cells and natural killer cells in mice with B16 brain tumors. Moreover, the anti-tumor and protective immunity were stronger. SFV particles expressing IL-12 have been evaluated in a syngeneic RG2 rat glioma model (Roche et al., 2010). Administration of a low dose of 5×10^7 SFV-IL-12 particles via an implanted cannula resulted in a 70% reduction in tumor volume and a significant prolongation of survival. The high dose of 5×10^8 SFV-IL-12 particles generated an 87% reduction in tumor volume but could also potentially induce vector-related lethal pathology. In another approach, SIN DNA replicons expressing the human gp100 and mouse IL-18 were intramuscularly administered to mice bearing B16-gp100 brain tumors (Yamanaka and Xanthopoulos, 2005). Co-delivery of SIN-gp100 and SIN-IL-18 DNA replicons enhanced the therapeutic and protective effect against brain tumors and significantly prolonged the survival of mice. The replication-proficient SFV(A774nsP) vector expressing enhanced green fluorescent protein (VA-EGFP) has demonstrated oncolytic properties in a subcutaneous orthotopic tumor model in BALB/c mice (Heikkilä et al., 2010). Stable expression of firefly luciferase (Luc) was completely inhibited in mice receiving a single intravenous injection of SFV VA-EGFP. Furthermore, 16 out of 17 immunized animals showed long-term survival. Therapeutic gene therapy applications of alphaviruses, particularly replication-proficient vectors, for brain tumors have presented some concerns because of their neurovirulence (Griffin 2016). In this context, the distribution of SFV particles and naked RNA replicons expressing Luc was compared in tumor-free and 4T1 mammary tumor-bearing mice after intravenous, intraperitoneal or intratumoral administration (Vasilevska et al., 2012). Intravenous administration of SFV-Luc RNA showed primary brain targeting in both mice without tumors and 4T1 tumor-bearing mice. In contrast, intratumoral injection led to high Luc expression in tumors. Intravenous and intraperitoneal administration of a high dose of SFV-Luc particles (6×10^9 particles/ml) demonstrated a broad distribution of expression, whereas a reduced viral dose (2×10^8 particles/ml) resulted in predominant tumor targeting. Furthermore, the neurotrophic affinity of SFV particles has been addressed by insertion of neuron-specific micro-RNA miRT124 sequences into the replication-proficient SFV4 vector (Martikainen et al., 2015). Significant tumor growth inhibition and prolonged survival was seen in C57BL/6 mice with CT-2A orthotopic gliomas after a single intraperitoneal injection of SFV4-miRT124 particles. No clinical studies on alphavirus-based treatment of brain tumors have been published, so far. However, a phase I/II protocol for the treatment of patients with recurrent glioblastoma with liposome encapsulated SFV particles expressing IL-12 (LSFV-IL-12) has been published (Ren et al., 2003). According to the protocol, the plan is to administer by continuous intratumoral infusion doses of 1×10^7 to 1×10^9 infectious particles.

Breast cancer is another indication, which has received attention as a therapeutic target. In one study, the HER2/neu gene was expressed from a SIN DNA replicon and an adenovirus vector (Wang et al., 2005b). SIN-HER2/neu DNA and Adeno-neu particles were administered into the mammary fat pad of mice or intravenously as a model for lung metastases. Immunization with SIN-HER2/neu DNA or Adeno-neu particles prior to tumor challenges with A2L2 cells expressing the rat HER2/neu gene showed a significant inhibition of tumor growth. In contrast, immunization with either vector 2 days after the tumor challenge was inefficient. However, a prime immunization with SIN-HER2/neu DNA followed by an Adeno-neu particle booster led to a significant prolongation of the survival of mice. Moreover, in comparison to a conventional DNA plasmid, intradermal administration of SIN-HER2/neu DNA replicons required 80% less DNA to elicit robust antibody responses and protection against tumor challenges in BALB/c mice (Lachman et al., 2001). VEE virus-like replicon particles (VRPs) expressing the extracellular domain (ECD) and transmembrane (TM) domains of HER2 were evaluated in a transgenic HER2 mouse model (Wang et al., 2005a). The VEE-HER2 VRPs prevented or inhibited the growth of HER2/neu-expressing mouse breast cancer cells either after injection into mammary tissue or administered intravenously. High levels of neu-specific $CD8^+$ T lymphocytes and serum IgG were obtained. Moreover,

complete prevention of tumor formation was seen in immunized mice. VEE-HER2 VRPs have been subjected to a phase I clinical trial in stage IV HER2 overexpressing breast cancer patients (NCT03632941). The immunization showed good tolerance and resulted in partial response (PR) in one patient and stable disease (SD) in two other patients. Additionally, a combination therapy phase II trial with VEE-HER2 VRPs and pembrolizumab combination in HER2-positive breast cancer patients has started (Crosby et al., 2019). In another approach, mice with 4T1 mammary tumor nodules were immunized with 2×10^8 SFV-IL-12 particles and 2×10^7 units of the *Salmonella typhimurium* aroC strain (LVR01) (Kramer et al., 2015). The treatment resulted in complete inhibition of lethal lung metastasis formation and provided long-term survival in 90% of vaccinated mice. The synergistic effect of SFV-IL-12 and LVR01 was superior to immunization with either agent alone. The oncolytic M1 alphavirus has been applied for experimental treatment of triple-negative breast cancer (TNBC), the most aggressive molecular subtype of breast cancer (Zhang et al., 2021). The oncolytic effect of M1 could be enhanced by 100-fold by co-administration of doxorubicin *in vitro* and it also reduced significantly tumor growth *in vivo*.

In the context of prophylactics and therapeutics against cervical cancer, the human papilloma virus (HPV) recombinant protein-based vaccine Gardasil was approved in 2006 by the FDA (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2006/ucm108666.htm). The viral vector-based vaccine platform has, however, been pursued because of the rapid high-level short-term transgene expression, favorable for vaccine development. For instance, VEE particles expressing the HPV16 E7 protein were administered to C57BL/6 mice, which induced robust $CD8^+$ T cell responses and provided protection against tumor challenges (Velders et al., 2001). Moreover, the replication-proficient SIN AR339 strain induced cytopathogenicity and apoptosis in HeLaS3 and C33A cancer cells, but not in normal keratinocytes *in vitro* (Unno et al., 2005). A single intraperitoneal or intravenous injection of SIN AR339 generated significant regression of established cervical tumors in nude mice. In the context of SFV, the HPV E6-E7 fusion was introduced into the SFVenh vector containing the translation enhancer signal from the SFV capsid gene to improve expression levels and immunogenicity (Daemen et al., 2003). Immunization of C57BL/6 mice showed a complete eradication of established tumors and a long-term high-level CTL activity lasting up to 340 days. In another approach to enhance immunogenicity, helper T-cell epitopes and an endoplasmic reticulum (ER) targeting signal were fused to the HPV E6 and E7 proteins (Ip et al., 2015). Immunization of C57BL/6JOlaHsd mice with as few SFV-sHELP-E7SH particles as 1×10^5 resulted in tumor regression and protection against challenges with tumors. SFV DNA replicons expressing HPV E6-E7 have also been applied for intradermal immunization of C57BL/6 mice followed by electroporation (van de Wall et al., 2018). In comparison to immunization with a conventional DNA vector, which did not prevent tumor growth, a 200-fold lower dose (0.05 μg) of the SFV HPV E6-E7 DNA replicon rendered 85% of mice tumor free. In the context of clinical trials, in a phase I study 12 individuals with a history of cervical intraepithelial neoplasia were immunized with doses of 5×10^5, 5×10^6, 5×10^7, or 2.5×10^8 of the SFVenh-HPV E6/E7 vaccine candidate (Vvax001) (Komdeur et al., 2021). The immunization was determined safe resulting in HPV-specific immune responses in all 12 patients.

Alphavirus vectors have also been used for colon cancer therapy. For example, IL-12 has been expressed from SFV vectors for evaluation in a poorly immunogenic MC38 colon adenocarcinoma mouse model (Rodriguez-Madoz et al., 2005). The two subunits of IL-12 were either expressed from a single subgenomic promoter from the SFV-IL-12 vector or from two independent 26S promoters from the SFVenh-IL-12 vector. In the latter case, IL-12 was expressed at 8-fold higher levels. A single intratumoral administration of 1×10^8 particles of either SFV-IL-12 or SFVenh-IL-12 resulted in complete tumor regression and long-term tumor-free survival in mice with implanted MC38 xenografts. The SFVenh-IL-12 induced more efficiently anti-tumor responses at lower doses compared to SFV-IL-12. The anti-tumor response was increased after repeated intratumoral injections and was superior to first generation adenovirus vectors in elimination of tumors. Furthermore, co-administration of SFV-IL-12 particles and the anti-PD1 monoclonal antibody showed a synergistic effect in the MC38 mouse tumor model (Quetglas et al., 2015). Similarly, the superiority of the combination therapy was demonstrated in a B16-OVA melanoma mouse model (Quetglas et al., 2015). In the case of VEE, C57BL/6 mice with MC38-CEA-2 tumors were immunized with VEE-IL-12 particles and VEE particles expressing the carcinoembryonic antigen (CEA) (Osada et al., 2012). VEE-IL-12 immunization induced stronger immune responses than administration of IL-12 protein. The anti-tumor activity and survival time were superior after immunization with both VEE-IL-12 and VEE-CEA compared to VEE-IL-12 or VEE-CEA alone. SFV particles carrying the vascular endothelial growth factor receptor-2 (VEGFR-2) were evaluated in a CT26 colon carcinoma mouse model (Lyons et al., 2007). A significant inhibition of tumor growth and metastatic spread was seen in mice either prophylactically or therapeutically immunized with SFV-VEGFR-2 particles. However, co-immunization with SFV-IL-12 particles completely abrogated antibody responses and anti-tumor activity seen for SFV-VEGFR-2 particles alone. In contrast, co-administration of SFV-VEGFR-2 and SFV-IL-4 particles enhanced VEGFR-2-specific antibody titers and extended survival of mice after tumor challenges compared to immunization with only SFV-VEGFR-2 particles. SFV RNA replicons have also been evaluated. In this context, a single intramuscular immunization of mice with 0.1 μg of SFV-LacZ RNA replicons elicited robust antigen-specific immune responses and protected mice from tumor challenges due to apoptotic activity (Ying et al., 1999). Moreover, the survival of mice with pre-existing tumors was extended after immunization with SFV-LacZ RNA replicons. In the case of clinical trials, a phase I study was conducted in patients with stages III and IV colorectal cancer immunized with VEE-CEA particles four times every 3 weeks (Crosby et al., 2020). The treatment induced antigen-specific immune responses and long-term survivors were identified among both stage III and IV patients indicating an extended overall survival.

In the case of lung cancer, SFV-EGFP particles demonstrated efficient killing of human H358a non-small cell lung cancer (NSCLC) cells and inhibition of H358a spheroid growth (Murphy et al., 2000). SFV-EGFP particles were injected into nu/nu mice with H358a xenografts resulting in complete tumor regression in three out of seven immunized mice. Replication-proficient SFV VA-EGFP particles were compared to the conditionally replicating adenovirus Ad-Delta24TK-GFP vector in nude mice with A549 lung adenocarcinoma implants (Määttä et al., 2008). The study demonstrated superior survival of mice locally immunized with SFV VA-EGFP. Neither vector system did elicit significant immune responses after systemic administration. Also, SIN particles have been subjected to studies in lung cancer models. Intravenous administration of SIN-LacZ particles to CT26.CL25 colon tumor-bearing mice showed complete tumor remission and provided long-term survival (Granot et al., 2014).

Melanoma is a cancer indication that has received plenty of attention, including prophylactic and therapeutic applications of alphaviruses (Zappasodi and Merghoub, 2015). In this context, VEE particles expressing the tyrosine-related protein-2 (TRP-2) showed humoral immune responses, anti-tumor activity and prolonged survival in a B16 mouse melanoma model (Avogadri et al., 2010). Combination therapy of VEE-TRP-2 with antagonist anti-CTL antigen-4 (CTLA-4) or agonist anti-glucocorticoid-induced tumor necrosis factor receptor (GITR) monoclonal antibodies (mAbs) led to tumor regression of 50 and 90% of mice, respectively (Avogadri et al., 2014). Additionally, combination therapy with two SFV DNA replicons expressing VEGFR-2 and IL-12 from one DNA replicon and survivin and β-hCG antigens from another DNA replicon showed tumor growth inhibition and prolonged survival in mice with B16 melanoma xenografts (Yin et al., 2015). The combination therapy showed superiority to immunization with either SFV DNA replicon alone. In the context of clinical evaluation, 18 patients with stage III or IV metastasizing melanoma and renal cell carcinoma have been subjected to intravenous administration of 1×10^8 or 1×10^9 liposome encapsulated LSFV-IL-12 particles per m^2 (Ren et al., 2003). The treatment caused no major toxicity. Patients receiving the higher dose had temporary and mild inflammatory reactions such as itching and slight fever with flu-like symptoms most likely due to enhanced IL-12 concentrations. In the peripheral blood 10-fold higher IL-12 concentrations were measured compared to the baseline, which lasted for 3–4 days.

In the case of ovarian cancer, SIN particles have demonstrated tumor targeting because of the overexpression of laminin receptors, which are utilized for host cell recognition by SIN (Tseng et al., 2004). The oncolytic SIN AR339 strain showed cytotoxicity and apoptosis in the HOC-1, HAC-2, and OMC-3 ovarian cancer cell lines (Unno et al., 2005). Moreover, in a metastatic ovarian cancer model ascites formation was suppressed after immunization of mice with SIN AR339. Alphaviruses have been subjected to combination therapy of ovarian cancers. For example, SIN-IL-12 particles administered together with the CPT-11 topoisomerase inhibitor irinotecan granted long-term-survival of SCID mice carrying highly aggressive human ES2 ovarian tumors (Granot and Meruelo, 2012). Furthermore, a prime-boost regimen with SFV expressing ovalbumin (OVA) and vaccinia virus (VV-OVA) was tested in C57BL/6 mice with implanted murine ovarian surface epithelial carcinoma (MOSEC) (Zhang et al., 2010). The immunization elicited OVA-specific $CD8^+$ T-cell responses and improved the anti-tumor activity.

Pancreatic cancer therapy has also been of interest due to the poor prognosis of patients and lack of efficient treatments. Based on preclinical studies, where a single dose or a prime-boost regimen with VEE-CEA particles showed high levels of T-cell antibody responses, VEE-CEA particles were repeatedly administered intramuscularly into patients with metastatic pancreatic cancer in a phase I trial (Morse et al., 2010). The treatment induced clinically relevant T cell and antibody responses. The outcome was cellular cytotoxicity against tumor cells and prolonged overall survival in cancer patients. Irreversible electroporation (IRE), also called Nanoknife, has been combined with the oncolytic M1 alphavirus for the treatment of pancreatic cancer (Sun et al., 2021). IRE triggered apoptosis in pancreatic cancer cells (PCCs) and when combined with M1 administration, the therapeutic efficacy was synergistically enhanced, illustrated by inhibition of tumor proliferation and prolonged survival of immunocompetent mice with implanted orthotopic pancreatic tumors.

Alphaviruses have also been evaluated in the context of prostate cancer. VEE particles expressing the prostate-specific membrane antigen (PSMA) induced robust PSMA-specific immune responses in BALB/c and C57BL/6 mice (Durso et al., 2007). A single injection of 2×10^5 VEE-PSMA particles elicited strong T- and B-cell responses, which were enhanced after repeated immunizations. Moreover, the immune responses were characterized by Th-1 cytokines, potent CTL activity and IgG2a/IgG2b antibodies. In another study, VEE particles expressing the mouse six-transmembrane epithelial antigen of the prostate (mSTEAP) were subjected to a booster immunization 15 days after the prime immunization with gold-coated conventional pcDNA-3-mSTEAP plasmids delivered by gene gun (Garcia-Hernandez et al., 2007). The immunization elicited specific $CD8^+$ T-cell responses against a newly defined mSTEAP epitope and significantly prolonged the overall survival of mice subjected to tumor challenges. Moreover, the immunization strategy showed a modest but significant delay in growth of previously established tumors. In another approach, VEE particles expressing the prostate stem cell antigen (PSCA) were administered to transgenic adenocarcinoma of the prostate (TRAMP) mice (Garcia-Hernandez et al., 2008). The immunization demonstrated long-term survival in 90% of mice lasting for at least 12 months. VEE-PSMA particles have also been subjected to a phase I clinical trial in patients with castration resistant metastatic prostate cancer (CRPC) (Slovin et al., 2013). Immunization with 0.9×10^7 or 3.6×10^7 IU of VEE-PSMA particles showed good safety and tolerance. However, the PSMA-specific immune responses were disappointingly weak.

CONCLUSION

In summary, alphavirus vectors have been evaluated for prophylactic and therapeutic use for a broad range of cancer indications in various animal models and in several clinical

studies as summarized in **Table 1**. Generally, SFV, SIN or VEE vectors have been used although application of the oncolytic M1 alphavirus has also been described. The properties of SFV, SIN, and VEE vectors are very similar although for vaccine development it has been claimed that VEE shows targeting of dendritic cells *in vivo* providing superior immune responses (MacDonald and Johnston, 2000). However, it was determined that a single amino acid substitution in the E2 glycoprotein rendered dendritic cells susceptible to SIN infection (Gardner et al., 2000). Although based on numerous vaccine studies it has not been possible to demonstrate superiority of any alphavirus system regarding immune responses or therapeutic efficacy. In most cases robust immune responses have been obtained, including both humoral and cellular responses. The Th1-biased immunogenicity confirmed the potential of alphavirus-based cancer vaccine. The possibility to include alphavirus-based delivery of cytotoxic genes, anti-tumor genes, immunostimulatory genes, the apoptosis induced by alphaviruses, and RNA interference in the form of short interfering RNAs and micro-RNAs expands the possibilities of therapeutic interventions. Moreover, alphavirus vectors can be applied as recombinant viral particles, including replication-deficient, replication-proficient, and oncolytic viruses as well as RNA replicons and DNA replicons. It has been demonstrated that the stability of RNA and its resistance against degradation can be improved by RNA encapsulation in lipid nanoparticles. Several studies have also confirmed that due to the presence of alphavirus replicons, both RNA replicons and DNA replicons can induce the same immune response at 100–1000-fold lower doses compared to synthetic mRNA and conventional DNA plasmids, respectively. Although alphaviruses have demonstrated good safety and efficacy in various animal models, the transfer to humans have often generated disappointingly weak immune responses in clinical trials. A number of issues such as targeting, delivery, dose optimization and potential combination therapy still needs to be addressed.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Perspectives for Combining Viral Oncolysis With Additional Immunotherapies for the Treatment of Melanoma

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Melanoma is the deadliest type of skin cancer with steadily increasing incidence worldwide during the last few decades. In addition to its tumor associated antigens (TAAs), melanoma has a high mutation rate compared to other tumors, which promotes the appearance of tumor specific antigens (TSAs) as well as increased lymphocytic infiltration, inviting the use of therapeutic tools that evoke new or restore pre-existing immune responses. Innovative therapeutic proposals, such as immune checkpoint inhibitors (ICIs), have emerged as effective options for melanoma. However, a significant portion of these patients relapse and become refractory to treatment. Likewise, strategies using viral vectors, replicative or not, have garnered confidence and approval by different regulatory agencies around the world. It is possible that further success of immune therapies against melanoma will come from synergistic combinations of different approaches. In this review we outline molecular features inherent to melanoma and how this supports the use of viral oncolysis and immunotherapies when used as monotherapies or in combination.

Keywords: immunotherapy, gene therapy, adenovirus, melanoma, checkpoint inhibitors, CAR-T cells, p53, interferon

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INTRODUCTION

Immunotherapy has revolutionized cancer treatment in unprecedented ways (Mellman et al., 2011). It consists of mobilizing the immune system's own defenses to recognize and eliminate neoplastic cells, thus reinstating cancer immunosurveillance (Zitvogel et al., 2013). The promising early results generated high expectations and gave hope to many patients to reach long-term remission (Cable et al., 2021). However, only a minority of patients with advanced cancer undergoing this therapeutic modality increase survival in a lasting way (Hegde and Chen, 2020). In this scenario, the use of rational combinations of immunotherapies has been increasing and may link different approaches and technologies in order to improve patient benefit of the treatment (Finck et al., 2020). Oncolytic viruses (OV) are therapeutic tools with the property of not only selectively inducing oncolysis, but also attracting cells of the immune system, activating them and thus mobilizing innate and adaptive antitumor responses (Vähä-Koskela et al., 2007; Russell et al., 2012). This property is suitable for combination with therapies aimed at cell-mediated cytotoxic effect, whether adoptive or naturally intrinsic to the immune system, acting synergistically at different stages of the cancer-immunity cycle

(Chen and Mellman, 2013). Here we provide a historical and biological perspective regarding the advent and clinical implementation of immunotherapies, including oncolytic viruses, for the treatment of melanoma and we explore the possible combinations of oncolytic viruses with additional immunotherapy strategies.

MOLECULAR ALTERATIONS IN MELANOMA THAT MAY SERVE AS THERAPEUTIC TARGETS

Cancer is a multifactorial disease characterized by heterogeneous subpopulations of cells with different phenotypes and genetic properties leading to uncontrolled proliferation, migration, invasion as well as metastasis and drug resistance. Skin cancer can be classified as basal cell carcinoma, squamous carcinoma and melanoma. Non-melanoma skin cancers account for nearly 98% of skin cancer in the United States (Force et al., 2018). Although melanoma is the least frequent type of skin cancer, it is the deadliest with steadily increasing incidence worldwide during the last few decades, especially in Caucasian populations (Siegel et al., 2020). The latest data released by Global Cancer Observatory (GLOBOCAN) estimated more than 280,000 new cases of skin melanoma (1.6% of all cancers) with nearly 60,000 deaths in 2020 (Global Cancer Observatory–https://gco.iarc.fr/). Melanoma arises from malignant transformation of melanocytes, melanin-producing cells found in the epidermal skin layer, due to mutagenic damage that activates many oncogenes and inactivates tumor suppressor genes (Kobayashi et al., 1998; Cancer Genome Atlas, 2015; Hayward et al., 2017). Some studies demonstrated that incidence of skin cancer (non-melanoma and melanoma) is inversely related to skin pigmentation, with higher risk and incidence in individuals with fair skin along with the presence of nevi and freckles. Furthermore, a family history of melanoma, immunosuppression related to organ transplantation, and HIV or HPV infection also increase the predisposition to this neoplasm (Veierod et al., 2010; Force et al., 2018).

One of the main risk factors for the development of melanoma is intermittent excessive exposure to solar ultraviolet (UV) radiation, being particularly harmful when it occurs in childhood. In addition, there is growing evidence that exposure to artificial UV radiation through the use of artificial tanning chambers increases the propensity to develop melanomas (Veierod et al., 2010; Sun et al., 2020). UV radiation induces DNA damage directly (DNA photoproducts) or through ROS production that indirectly causes oxidative DNA damage, leading to DNA mutations and alterations in the transcriptional profile, resulting in dysregulation of several tumor suppressor genes and oncogenes (Kvam and Tyrrell, 1997; Anna et al., 2007). Moreover, UV radiation can also downregulate cutaneous immunity by apoptosis of epidermal immune cells (Langerhans cells) and inhibition of antigen presentation together with release of immunosuppressive cytokines, favoring tumor development and progression (Fisher and Kripke, 1982; Shreedhar et al., 1998). The UV-induced DNA damage response is modulated by the tumor suppressor gene *TP53* that can be found downregulated, contributing to UV-mediated mutagenesis in non-melanoma and melanoma skin cancer (Smith et al., 2000; Decraene et al., 2001). In addition, UV exposure can alter *TP53*, resulting in cooperation with *BRAF* mutations to induce melanoma (Viros et al., 2014).

Sequencing studies revealed the genetic landscape of cutaneous melanoma and classified them into four subgroups: mutant *BRAF*, mutant *NRAS*, mutant *NF1* and triple-wild type (Cancer Genome Atlas, 2015; Hayward et al., 2017; Zhou et al., 2019). In another study, the whole genome sequence analysis of melanoma samples also found mutations in other genes, such as *TERT*, *TP53*, *CDKN2A* and *CDKN2B*. Some of these mutations as *BRAF*, *NRAS* and *TERT* are also found in benign lesions whereas *CDKN2A*, *TP73* and *PTEN* are observed only in invasive melanoma (Amaral et al., 2017; Consortium, 2020). Other mutations less frequently found, especially in melanomas missing heritability, are *BPA1*, *POT1*, *ACD* and *TERF2IP* (Potjer et al., 2019).

BRAF mutations are highly prevalent in melanoma and found in 40–60% of cultured primary melanoma cells but are not sufficient for melanoma progression and development since they are found in benign nevi (Pollock et al., 2003; Tschandl et al., 2013). The most frequent oncogenic mutation for *BRAF* in melanomas is the substitution of amino acid valine for glutamic acid at position 600 (V600 E), representing 70–90% of *BRAF* mutations. Other *BRAF* mutations, although less frequent, can be found in melanoma, including V600K, V600R, V600D for example (Rubinstein et al., 2010; Long et al., 2011; Lovly et al., 2012). Mutations in *BRAF* are not related to UV radiation exposition as 30–60% of patients without chronic sun-induced damage have been identified with somatic BRAF mutation (Curtin et al., 2005; Brash, 2015). These mutations have important clinical significance since mutated BRAF protein is active as a monomer instead of dimer and the monomer conformation is the target for the binding of BRAF inhibitors, such as vemurafenib, dabrafenib and encorafenib, used in melanoma therapy (Czarnecka et al., 2020). Moreover, the presence of *BRAF* mutations (*BRAF* (+)), despite not impacting recurrence-free survival from diagnosis of primary melanoma (stage I/II) to metastases development (stage IV) compared to *BRAF* WT patients, they do have a negative impact on median overall survival (OS) of patients who are newly diagnosed, untreated and with metastatic disease, since in *BRAF* (+) patients the OS is 5.7 months and for *BRAF* WT it is 8.5 months (Long et al., 2011). BRAFV600 E mutation resulted in altered BRAF protein conformation, increasing its kinase activity, leading to constitutive MAPK pathway activation, resulting in uncontrolled proliferation, cell survival and immune evasion which contribute to melanoma growth (Yang et al., 2019). The MAPK pathway is also activated by *NRAS* mutations that are frequently found in several tumor types and in 15–20% of melanoma patients but not concomitant with *BRAF* mutations (Wan et al., 2004; Chiappetta et al., 2015). Moreover, 15% of melanomas have *NF1* mutations with loss of function that also result in MAPK hyperactivation (Wan et al., 2004; Krauthammer et al., 2015). Deregulation of RAS/MAPK/ERK pathway is found

in nearly all melanomas (Hayward et al., 2017). The signaling pathway RAS/MAPK/ERK impacts more than 50 transcription factors involved in the regulation of genes that control cell growth, division, proliferation and differentiation (Molina and Adjei, 2006). The pathway is activated by cytokines, growth factors and hormones which interact with a membrane tyrosine kinase receptor, inducing its phosphorylation and leading to signal transduction by subsequent phosphorylation of a series of proteins from RAS, RAF (ARF, BRAF, CRAF), MEK (MEK1 and MEK2) and MAPK/ERK family. The activated ERK goes to the nucleus where it activates transcription factors such as cMyc and CREB by phosphorylation (Molina and Adjei, 2006). The activated MAPK pathway also has an immunosuppressive effect due to downregulation of tumor antigens and decreased recognition by immune cells together with upregulation and infiltration of immunosuppressive cells after cytokine secretion (Ott and Bhardwaj, 2013; Yang et al., 2019).

Another important pathway commonly upregulated in melanomas is that of PI3K/AKT/mTOR which regulates cell proliferation, cellular response during stress and quiescence, contributing to tumor growth, metastasis and angiogenesis induction in melanomas (Porta et al., 2014). The most common mutations contributing to this activation are found as upregulation of the oncogene *NRAS* (15–20%) and loss of function or expression of the tumor suppressor *PTEN* (20–30%), yet these are largely mutually exclusive events (Hocker and Tsao, 2007; Aguissa-Toure and Li, 2012). On the other hand, *PTEN* loss can occur concomitantly with *BRAF* mutations, resulting in activation of RAS/RAF/MAPK and PI3K/AKT pathways (Tsao et al., 2004; Goel et al., 2006). Activated AKT phosphorylates several proteins, including antiapoptotic proteins (XIAP, BAD, BIM), MDM2, p21 and many others, allowing survival and progression of melanoma cells together with apoptosis inhibition (Madhunapantula et al., 2011). Interestingly, *PTEN* loss is also related to immunosuppressive properties such as lower sensitivity to T cell mediated cell death and reduced infiltrations of T cell infiltration in the tumor site, contributing to melanoma immune resistance (Peng et al., 2016).

Melanomas bearing BRAFV600 E mutations commonly also have altered MITF expression and activity (Levy et al., 2006). The *MITF* gene encodes a central regulator of melanocyte differentiation, development and function, besides several biological processes such as DNA repair, senescence, cell metabolism, survival, differentiation, proliferation and metastases formation (Goding and Arnheiter, 2019). MITF can be employed as a diagnostic marker for tumors from melanocytic origin, however, with different levels of expression correlating with distinct behavior of malignant cells (Levy et al., 2006). High MITF expression is associated with highly proliferative and poorly invasive phenotype while low MITF expression correlates with a slowly proliferative and highly invasive profile. *In vivo* studies demonstrated that although different in MITF expression, both phenotypes can establish tumors when inoculated into nude mice but with the invasive phenotype requiring a longer period to develop palpable tumors (Hoek et al., 2008; Vachtenheim and Ondrusova, 2015). However, both invasive and proliferative phenotypes can be present simultaneously since melanoma progression is not associated just with differential gene expression (Harbst et al., 2012; Cancer Genome Atlas, 2015), but also with dynamic transcription signature plasticity which contributes to tumor metastization due to the adaptive response to the tumor microenvironment (Roesch et al., 2016) (**Figure 1A**) Also reported for melanoma is the P29 S mutation in the *RAC1* gene that is found in approximately 3% of melanomas but in almost 20% of patients resistant to BRAF inhibitors (Watson et al., 2014). RAC1 is involved in cellular adhesion, motility and differentiation, and the consequences of mutation in this gene are melanocytic to mesenchymal phenotype, increased tumor size and presence of metastasis (Lionarons et al., 2019).

The expression of immune-related genes also correlates with prognosis and response to melanoma immunotherapy as demonstrated after analysis of 45 patients submitted to anti-CTLA-4 therapy that had tumors with increased expression of genes related to immune signature (Ji et al., 2012). The interferon pathway is one of the key players in the response to immunotherapy and the type I and II interferons are mainly responsible for antitumor response due to increased immune recognition and apoptosis induction in tumor cells (Benci et al., 2016; Gao et al., 2016; Sharma et al., 2017). Many tumors have impaired interferon signaling resultant from alterations in regulatory genes such as loss of *IFNGR1*, *IRF1*, *JAK2* and amplification of *SOCS1* and *PIAS4* (Gao et al., 2016). Analysis of the TCGA dataset and studies *in vitro* and *in vivo* showed that nearly 30% of melanoma samples present mutations in the interferon signaling pathway, which is associated with shorter overall survival (Gao et al., 2016). Moreover, increased expression of interferon-related genes (e.g., *CXCL4*, *CXCL5*, *CXCL10*, *ID O 1*, *IRF1*, *STAT1* and others) was associated with benefit from anti-PD-1 and anti-CTLA-4 immunotherapy in melanoma patients (Ji et al., 2012; Gide et al., 2019). On the other hand, the suppression of the interferon pathway is associated with poor response to immunotherapy protocols due to immune evasion (Jerby-Arnon et al., 2018).

Melanoma patients' deaths are mostly associated with distant metastasis development, showing a 5-years survival rate of around just 20% (Bomar et al., 2019). However, some melanoma patients have metastatic disease without evident primary lesion (**Figure 1B**) and in this case, the disease development is associated with immunoediting mechanisms together with loss of immunohistochemical melanocytic markers like S100 protein, HMB-45, Melan-A, SOX10 and MITF (Gyorki et al., 2013; Bankar et al., 2015). The exhaustion of the immune system and immune evasion are among the key factors that enable melanoma growth and metastasis formation (Passarelli et al., 2017; Motofei, 2019). Moreover, *BRAF* mutations are present in about 50–60% of metastatic melanoma cases (Zaman et al., 2019). Indeed, studies have revealed that cutaneous melanoma has a high mutation rate compared to other common tumors, with a mean tumor mutation burden (TMB) of over 20 mutations per megabase, one of the highest TMB among solid tumors (Cancer Genome Atlas, 2015; Zhang et al., 2016). Malignant melanoma is highly genetically heterogeneous, with prevalence of

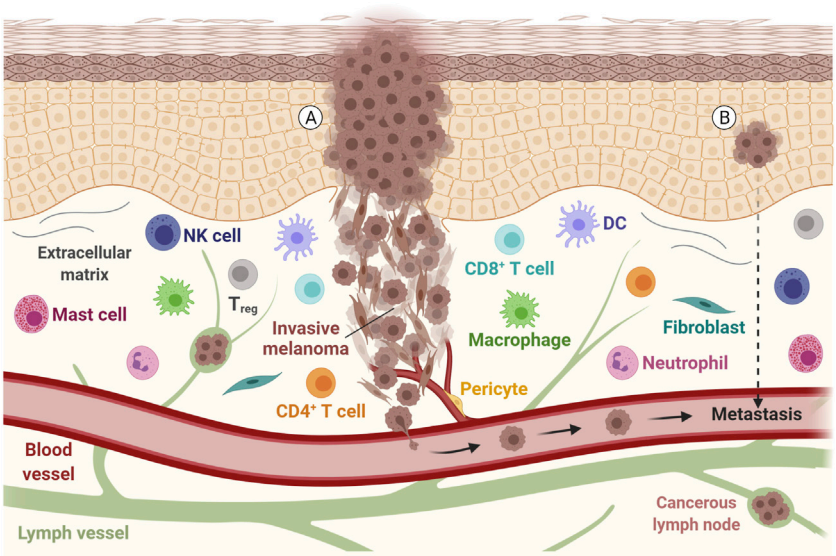

FIGURE 1 | Representation of a melanoma tumor during invasion and metastasis**.** Tumor cells with epithelial and mesenchymal-like morphology are shown, along with other components of the tumor microenvironment, such as the extracellular matrix, immune cells and fibroblasts. The communication between these components, with lymph vessels, blood vessels and tumor cells may allow the tumor to spread. **(A)** Metastatic disease is found in patients with clinically identified proliferating melanoma, **(B)** but also in patients with an undetected source of tumor cells or evident primary lesion. DC, Dendritic cell; NK, Natural killer; T_{reg}, Regulatory T cell. Adapted from "Melanoma Staging", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

somatic mutations in primary tumors and metastatic lesions that also acquire numerous mutations during their formation (Hoek et al., 2006; Swick et al., 2012).

MELANOMA ANTIGENS AND IMMUNOGENICITY

The immune system has the inherent property to distinguish self from non-self-antigens (Yarchoan et al., 2017). Despite the fact that tumor cells arise from healthy tissues, hence self, the ability of the immune system to recognize them is based on an important concept: neoantigens (also referred to as neoepitopes), which arise from tumor-specific mutations (Alexandrov et al., 2013). Since melanomas have a high mutational burden, which is reflected in the higher levels of neoantigens, they are more likely to promote immune response and be recognized by the immune system (Maleki Vareki, 2018). Described immunologically as "hot", these tumors offer a huge repertoire of potential targets for T cells that, in principle, reflect a greater inflammatory infiltrate (Maleki Vareki, 2018). This point has been extensively explored in several approaches in cancer treatment, such as cancer vaccines against neoantigens and adoptive T cell transfer, which can be combined with immunotherapy targeting T cell inhibitory receptors, including cytotoxic T-lymphocyte associated antigen (CTLA)-4 and programmed cell death (PD)-1 (Peng et al., 2019). The clinical benefits of immune checkpoint inhibitors are often observed in high mutational load tumors, which may be related to the presence of tumor associated antigen-specific T cells (Banchereau and Palucka, 2018).

The antitumor immune response is mainly mediated by the adaptive immune system, especially the tumor-infiltrating lymphocytes (TILs) (Lugowska et al., 2018) that can recognize through the T cell receptors (TCRs) antigenic peptides presented *via* major histocompatibility complex molecules (Durgeau et al., 2018). In the case of human melanomas, the high degree of TILs and, more specifically, cytotoxic T cell infiltration, together with elevated expression of checkpoint receptors make melanoma patients more likely to respond successfully to immunotherapy (Galon and Bruni, 2019).

Antigen targets of immunotherapy can be divided into tumor associated antigens (TAAs), which include the cancer testis antigens (CTAs), and tumor specific antigens (TSAs) (Aurisicchio et al., 2018). TAAs include proteins encoded in the normal genome, usually expressed at low levels, and might be over-expressed in malignant cells. CTAs are normally expressed in testis, fetal ovaries, and trophoblasts, but can also be expressed in cancer cells. Because TAAs and CTAs are found in normal cells, their antigenicity depends on abnormal expression levels and, frequently, their presence in the tumor microenvironment can lead to immunological tolerance. The third class comprises antigens that are not encoded in the normal host genome and are originated by somatic mutations in the coding sequence, creating a unique peptide sequence (Gubin et al., 2015), or by insertion of oncogenic viral genes, such as E6 and E7 encoded by human papillomavirus type 16 that drive oral and cervical tumors (Walboomers et al., 1999).

Many TAAs have been used for years to assist clinical practice. For example, the human epidermal growth factor receptor (HER2) is routinely used for breast cancer prediction and prognosis (Patani et al., 2013). Melanoma TAAs include the

type 1 melanoma antigen recognized by T cells (MART-1, also known as Melan-A) and the melanoma-associated antigen (MAGE). In a phase 1/2 clinical trial, a three-dose vaccine strategy using autologous DCs transduced with an adenoviral vector encoding the MART-1 antigen for metastatic melanoma patients showed that at least half of the treated patients had significant MART-1–specific T cell responses (Butterfield et al., 2008). Similarly, in a phase II study DCs were pulsed with a cocktail of melanoma-associated antigens, including MART-1 or MAGE-A1, MAGE-A2, MAGE-A3, gp100 and tyrosinase, and were subcutaneously injected in metastatic melanoma patients, for which 75% had an antigen-specific CTL response. Notably, patients in the vaccinated group with two or more peptide-specific responses had a significantly longer mean survival time (21.9 months) compared to treated patients who had less than two peptide-specific responses (8.1 months) (Oshita et al., 2012). Recently identified potential melanoma biomarkers, in addition to the more than 45 already studied (Belter et al., 2017), include metabolic components, for instance aminomalonic acid and phosphatidylinositol (PI) (Kim et al., 2017), as well as immune-related genes and TCRs (Charles et al., 2020; Huang et al., 2020). Although the clinical trials using TAA have shown initial immune response, most of them have failed to demonstrate durable beneficial effects. The main reasons are the lower TCR affinity to TAA and peripheral tolerance of TAA-reactive T cells (Melero et al., 2014).

Beyond TAAs, TSAs are attractive targets for immunotherapy. These neoantigens are expressed only in cancer cells and can be recognized by TCR with high affinity. Neoantigen-specific T cells are not subject to central and peripheral tolerance and, consequently, their activation leads to a lower induction of autoimmunity (Yarchoan et al., 2017). As a result, the antitumor immune responses to TSAs are more robust as compared to TAAs. An important advance in the understanding of TSAs and immune response was published in 2005 by Wölfel and colleagues. The authors found that the T cells of a patient were reactive against five mutated epitopes and the immunoreactivity against melanoma neoantigens predominated over the response to TAAs (Lennerz et al., 2005). In addition, Rosenberg's group showed that the adoptive transfer of *ex vivo*–expanded TILs reactive against two neoantigens into a melanoma patient promoted complete tumor regression. All these studies support the role of neoantigens in the natural antitumoral T cell response (Zhou et al., 2005).

Besides its high TMB (Lawrence et al., 2013) and despite most human melanomas having a mutational load above 10 somatic mutations per megabase of coding DNA, which are generally sufficient to lead to the formation of neoantigens, T cell reactivity is not always observed (Linnemann et al., 2015). Recent studies revealed that both TMB and PD-L1 are not effective biomarkers for identifying patients who will have clinical benefit from checkpoint inhibitor therapy. An important point that may be considered is the clonality of neoantigens. Some evidence suggested that a minimum quantity of cells is required to generate T cell-mediated immune rejection (Gejman and Chang, 2018) and subclonal neoantigens are not presented by every cancer cell, so they are less effective in immune control of disease (Mcgranahan and Swanton, 2019). Furthermore, the majority of neoantigens are considered passenger events and, usually, their loss during tumor progression may be tolerated. However, when the mutations occur in genes required for tumor cell survival (such as cancer driver genes and genes required for cancer cell viability) and these genes are retained despite the events of copy number loss or transcriptional repression through methylation, the neoantigens are considered as essential neoantigens. Due to positive selection, these high-quality neoantigens cannot be repressed or deleted during tumor progression. Thus, both the quantity and the quality of neoantigens, more emphatically the quality, may explain why some patients are good responders to immunotherapy and others are not (Mcgranahan and Swanton, 2019).

However, the study of neoantigens has encountered barriers due to the lack of effective tools for their identification. In 2012, using a combination of next generation sequencing and algorithms for predicting the binding of peptides to MHC class I and class II molecules, Castle and coworkers identified TSAs in B16-F10 mouse melanoma cells (Castle et al., 2012). In parallel, exome sequencing and high-throughput MHC tetramer screening showed higher expansion of pre-existing T cells specific for tumor neoantigens in a human melanoma patient after treatment with checkpoint blockade immunotherapy (Van Rooij et al., 2013). To improve the adoptive transfer of T cells, Lu et al. genetically engineered autologous T cells to have neoantigen-specific TCRs. Isolated TILs were cultured and screened for the identification of neoantigen-reactive T cells to be further co-cultured with peptide-pulsed APCs. The single-cell RNA-sequencing allowed the identification of different neoantigen-specific TCRs, for instance a mutated KRAS-specific TCR, which could be successfully transduced into autologous T cells and recognize the specific neoantigens presented by the donor APCs (Lu and Robbins, 2016). The use of genomics and bioinformatics approaches in both mouse and human studies supported the rapid identification of mutant proteins expressed exclusively in cancer cells that act as neoantigens compared to conventional antigen-cloning approaches (Gubin et al., 2015) and highlight the potential of personalized cancer vaccines targeting neoantigens.

In a phase I study, 10 patients with stage IIIB/C or IVM1a/b melanoma were vaccinated with 13–20 personalized neoantigens peptides synthesized from sequencing of the tumors. After 20–32 months from vaccination, four patients with stage III disease were recurrence-free. Two patients with lung metastases had a complete response with the anti-PD-1 antibody, indicating the expansion of neoantigens specific T cells (Ott et al., 2017). Similar results were found by Sahin and colleagues who used personalized RNA-based 'poly-epitope' vaccine in 13 patients with stage III or IV melanoma. Each patient developed an immune response against at least three mutations. One patient with relapse and progressive disease at the time of vaccination presented a complete response after administration of anti-PD-1 antibody and eight continued disease-free 12–23 months later (Sahin et al., 2017). Both studies revealed that immune response was generated by $CD4^+$ T cells and the

vaccination provided the expansion of the neoantigen-specific T cells (Li L. et al., 2017). These studies confirm the potential of the immunogenic melanoma neoantigens and open novel possibilities for approaches using neoantigens as vaccinogenic agents associated with diverse delivery vehicles such as synthetic and biological nanoparticles and adenoviral vectors.

Although neoantigens are a promising strategy, the non-synonymous mutations that will originate the mutated protein depend on several factors that need to be present; the sequence with the mutation must be translated into protein, the mutated protein must be processed, and the peptides must be presented by MHC molecules. At the end of the process, the affinity between the mutated peptide and the patient's MHC molecules will determine recognition by the TCR (Schumacher and Schreiber, 2015). All of these processes are susceptible to complications that can alter the TCR-MHC binding, contributing to tumor escape from the immune system and also confounding *in silico* approaches for the prediction of the most effective neoantigens.

CURRENT IMMUNOTHERAPEUTIC STRATEGIES AND CHALLENGES

More than 10 different drug types have been approved by the FDA for the treatment of melanoma, including dacarbazine chemotherapy, BRAF and MEK-targeted therapy, recombinant interferon alpha-2b and IL-2, immune checkpoint inhibitors (ICIs), and oncolytic viral therapy (T-VEC). These strategies, together with radiation therapy and surgery comprise the clinical arsenal against primary and metastatic melanoma (Garbe et al., 2020; Jenkins and Fisher, 2020). During the past 20 years, ICIs (commonly referred to as immunotherapy) have taken on a leading role and occupied center stage in the melanoma treatment scene. Extraordinary results were achieved with anti-CTLA-4, anti-PD-1 and anti-PD-L1 in a time when targeted therapy only offered reasonable short-term, but poor long-term, overall survival (Ribas et al., 2012; Spagnolo et al., 2016; Li X. et al., 2017; Karlsson and Saleh, 2017). Their success turned ICIs into the standard of care for advanced melanoma, after surgery, demonstrating that when the immune system is activated properly, it may lead to durable long-term responses.

In terms of improved efficacy and reduced toxicity, studies have found that anti-PD-1 (Nivolumab or Pembrolizumab) is superior to anti-CTLA-4 (Ipilimumab) (Li X. et al., 2017; Karlsson and Saleh, 2017). No statistically significant differences have been found between Nivolumab and Pembrolizumab, although Nivolumab presents a slight improvement in terms of median overall survival (Moser et al., 2020). Attempts to reduce toxicity through the combination of Nivolumab and Ipilimumab in reduced doses resulted in greater efficacy, but less tolerability than monotherapy (Karlsson and Saleh, 2017; Turajlic et al., 2018; Moser et al., 2020). Nevertheless, most patients still fall in the non-responder-to-ICIs category and many experience immune-related adverse events (irAE), suggesting that despite their potential, resistance and toxicity continue to be their major hurdles.

The lack of ICI response in melanoma patients may be due to different immunological reasons, such as the absence or exclusion of T cells within the tumor microenvironment (TME) or insufficient antigen presentation and priming. Combinations of strategies that turn ICI resistant tumors into responders are being pursued, but, in addition to immunotherapies that directly aim to activate the immune system, all therapeutic strategies would potentially offer some degree of immune activation as well, either by inducing immunogenic cell death of tumor cells and providing antigenic supplies to antigen-presenting cells (APCs), or by triggering inflammatory pathways that will set the tone of the microenvironment towards a possible antitumor response (Chen and Mellman, 2013). Thus, the most obvious way to tackle resistance and perhaps reduce toxicity is through the combination of currently approved therapies.

Recently, studies in melanoma have focused on combining targeted therapies such as BRAF and MEK inhibitors with ICIs, aiming to increase efficacy by tackling resistance; the biology behind this strategy is to promote immune changes within the TME, particularly the release of cancer cell antigens and antigen-presentation by APCs to T cells in a context of checkpoint inhibition, which in turn lead to the activation of effector, tumor-specific T cell clones (Chen and Mellman, 2013). The idea of the strategy is to combine the rapid and deep response of targeted therapy with the durable response of ICIs (Kim et al., 2014; Dummer et al., 2020). *In vivo* melanoma models have shown tumor growth delay, reduced tumor size and prolonged overall survival with the combination of anti-PD-1/anti-PD-L1 and BRAF and MEK inhibitors (Dummer et al., 2020). Currently, there are two studies in clinical phase III, IMspire150 to assess the combination of Atezolimumab (anti-PD-L1) and vemurafenib (BRAF inhibitor), and COMBI-I part 3, assessing the combination of Spartalizaumab (anti-PD-1) plus dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor); both studies are ongoing. Still, in terms of timing of administration, it is debatable if targeted therapy should be administered before, at the same time or after ICIs; although, the changes induced in the microenvironment by BRAF inhibitors including reducing immunosuppressive cytokines, increasing availability of tumor antigens and infiltration of tumors by immune cells may sensitize tumors to ICIs (Dummer et al., 2020).

Due to the important role that checkpoint molecules play in the homeostasis of the immune system, the intravenous administration and systemic action of ICIs are known to induce undesired irAE. A variety of inflammatory and autoimmune events, ranging from grade 1 to 4 toxicities, have been observed as a result of ICI usage; the most prevalent are the dermatologic toxicities (from grade 1 to 2 rash, pruritus, vitiligo, dermatitis to grade 3 to 4 Stevens-Johnsons syndrome and epidermal necrolysis), present in 50% of melanoma patients treated with anti-CTLA-4 and up to 40% for anti-PD-1 and anti-PD-L1. Other less frequent, but not less relevant, irAEs include gastrointestinal toxicities, from diarrhea to severe colitis, hepatitis with or without elevation of transaminases or bilirubin and fulminant hepatitis; endocrinopathies that include a range of thyroid and pituitary toxicities, adrenal insufficiency and type I diabetes; neurologic toxicities such as autoimmune

encephalitis, myasthenia gravis and Guillain-Barré syndrome; renal toxicities include hematuria and acute interstitial nephritis and lupus-like nephritis; ocular toxicity such as uveitis, ulcerative keratitis and retinopathy; cardiac toxicities such as myocarditis, pericarditis, fibrosis, arrhythmias and heart failure and finally, hematological toxicities including hemolytic anemia, thrombocytopenia and eosinophilia (El Osta et al., 2017; Calvo, 2019; Kennedy and Salama, 2019). Despite the increased efficacy, the occurrence of irAEs is indeed more frequent with combinations of different ICIs (Li X. et al., 2017; El Osta et al., 2017; Karlsson and Saleh, 2017; Turajlic et al., 2018). As expected, irAE clinical manifestations are mostly managed with steroids or immunosuppressants, that when used carefully, ameliorate irAE without compromising ICIs efficacy (Karlsson and Saleh, 2017; Calvo, 2019). Even though the possibility of the occurrence of a grade 3 to 4 or chronic irAE is rare, future studies must consider the importance of segregating, through biomarkers, which patients will actually benefit from ICI therapy. Unfortunately, to date, the ideal biomarker for the indication of immunotherapy has not yet been identified (Jessurun et al., 2017). Recent studies in murine melanoma models have suggested a pivotal role of the gut microbiome for ICI efficacy, showing that the presence of some bacterial populations may be associated with increased response to ICIs, while others may be associated with the lack of response (Sivan et al., 2015; Vétizou et al., 2015). Theoretically, through the modulation of microbial populations, non-responders could be turned into responders and perhaps even become less susceptible to ICI toxic effects. In addition, microbial populations may comprise potential biomarkers of ICIs response (Vetizou and Trinchieri, 2018).

Still, there is a clear need for more effective and less toxic strategies. Other checkpoint molecules such as LAG-3, TIM-3, TIGIT, VISTA and B7-H3 and other TME molecules such as IDO have been demonstrated to be promising targets for melanoma and other advanced solid tumors in preclinical studies, granting their passage into clinical trials, although all of them are currently ongoing (Kwiatkowska et al., 2019; Qin et al., 2019). The most studied target, with more than 60 open clinical trials, is the LAG-3 molecule; after binding MHC-II molecules or fibrinogen-like protein 1 (FGL1), LAG-3 restrains the activation, proliferation and cytokine production capacity of Th1 cells while contributing to the suppression activity of Tregs (Qin et al., 2019; Murciano-Goroff and Warner, 2020). In an ongoing phase 1/2 study, anti-LAG-3 antibody (Relatlimab) as monotherapy or in combination with anti-PD-1 is being tested for melanoma patients who were resistant to classical ICIs; early results suggest that the combination is safe and can even increase the antitumor activity of anti-PD-1 alone in ICI-resistant melanoma patients (Kwiatkowska et al., 2019).

Stage IV metastatic melanomas have also been subjected to a personalized strategy developed by Steven Rosenberg at the NIH, resulting in outstanding outcomes and durable complete responses in a few melanoma patients (Prickett et al., 2016). From the lessons learned with IL-2 immunotherapy, Dr. Rosenberg recognized the potential of expanding functional T cells while circumventing the toxicity induced by IL-2 systemic administration, through the *ex vivo* activation with IL-2 of tumor infiltrating lymphocytes which, upon reinfusion, can then target tumor neoantigens (Rosenberg, 2011; Lu et al., 2014; Rosenberg, 2014; Prickett et al., 2016). Despite the great potential of this kind of strategy, known as adoptive cell therapy (ACT), the sophisticated methods required could hamper their inclusion in the clinical routine.

Other strategies that trigger important pathways of the innate immune response have been recognized in preclinical models for their importance in setting the tone of tumors for antitumor responses. Different agonists that trigger innate receptors and sensors such as TLR, STING, RIG-1 and NLR are currently being developed and some, such as STING agonists, have been considered in clinical trials as ICI adjuvants for advanced melanoma and other solid tumors (Hu and Li, 2020); the activation of these receptors is intended to mimic the immune response against viruses, that ultimately trigger cytokines and chemokines that will break the suppressive TME and allow the infiltration of immune cells inside tumors (Clavijo-Salomon et al., 2017). Consistent with the idea of harnessing innate receptor agonists and antiviral responses to fight tumors, oncolytic viruses have demonstrated tremendous potential for the treatment of melanoma, since in addition to awakening antiviral immunity, they can also directly kill tumor cells.

USING ENGINEERED VIRUSES FOR CANCER IMMUNOTHERAPY

The continued study of viruses has brought countless advances not only to the understanding of the molecular basis of diseases, such as cancer itself, but also perspectives for its use as a genetic and therapeutic tool (see **Figure 2** and **Supplementary Table S1** for key publications in the development of gene therapy), including in Brazil, the first Latin American country with a defined regulatory process for the registration of advanced therapy products. Intriguingly, on one hand viruses that trigger cancer have been discovered (Rous, 1910), yet on the other it has been suggested that some viral infections could improve clinical outcomes for some patients with different types of cancer (Hoster et al., 1949; Newman and Southam, 1954). Though unthinkable today, in 1949 Herman A. Hoster and coworkers used, deliberately, wild type hepatitis B virus in clinical trials of patients with Hodgkin's disease. In this study, 21 patients were intentionally exposed to the hepatitis virus and 4 of them had improvement in the clinical course, at least with regard to Hodgkin's. (Hoster et al., 1949). This study was one of the first to intentionally use viral activity to alter the progression of cancer. As presented below, current approaches use a deeper understanding of viral properties, the molecular basis of cancer as well as recombinant DNA techniques in order to use viruses as anti-cancer agents, an approach known as virotherapy or oncolytic viruses (**Figure 3**). The term "oncolytic viruses" (OV) is typically used to describe genetically modified viruses that selectively infect cancer cells inducing their death, theoretically, without affecting non-malignant tissues (Vähä-Koskela et al., 2007; Russell et al., 2012). Some viruses offer oncolytic

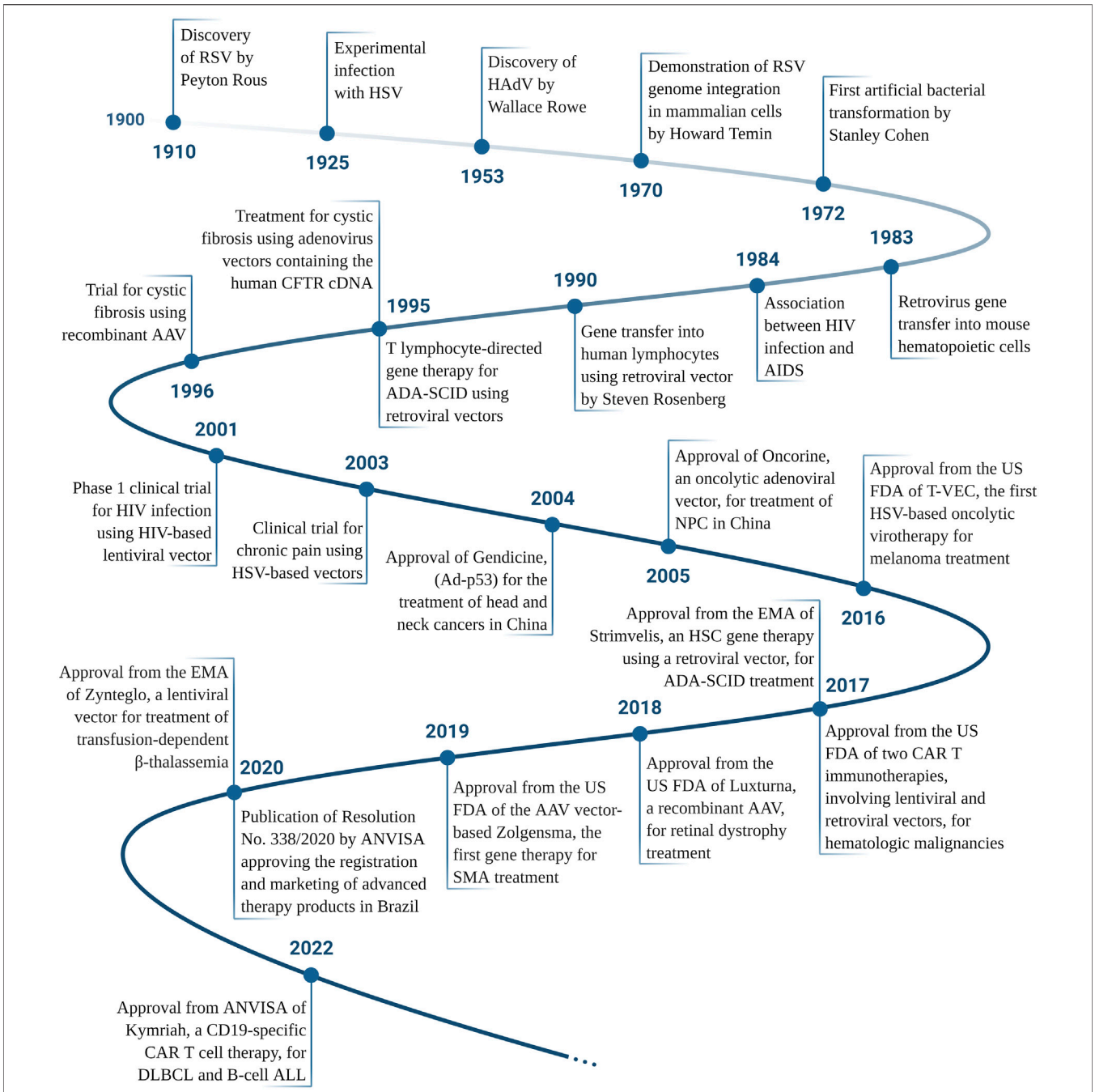

FIGURE 2 | Timeline of the main achievements in the gene therapy and virotherapy fields, according to the date of publication. RSV, Rous Sarcoma Virus; HSV, Herpes Simplex Virus; HAdV, Human adenovirus; HIV, Human Immunodeficiency Virus; AIDS, Acquired Immunodeficiency Syndrome; ADA-SCID, Adenosine Deaminase Severe Combined Immunodeficiency; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; AAV, Adeno-Associated Virus; NPC, nasopharyngeal carcinoma; US FDA, The United States Food and Drug Administration; EMA, European Medicines Agency; CAR, Chimeric Antigen Receptor; SMA, Spinal Muscular Atrophy; ANVISA, Agência Nacional de Vigilância Sanitária (National Agency for Sanitary Vigilance, the federal body in Brazil that regulates new drugs, among other health related items); DLBCL, Diffuse Large B-Cell Lymphoma; ALL, Acute Lymphoblastic Leukemia. Please see **Supplementary Table S1** for references. Created with BioRender.com.

properties naturally and do not require modification, for example, Vesicular stomatitis virus, Myxoma, Reovirus, and Newcastle disease virus (Roberts et al., 2006; Kelly and Russell, 2007; Jhawar et al., 2017). Although oncolytic viruses may enter normal cells, progression of the viral life cycle should be inhibited due to molecular components that block viral replication. In tumor cells, many of these mechanisms are dysfunctional or have been suppressed during tumor progression and thus provide a

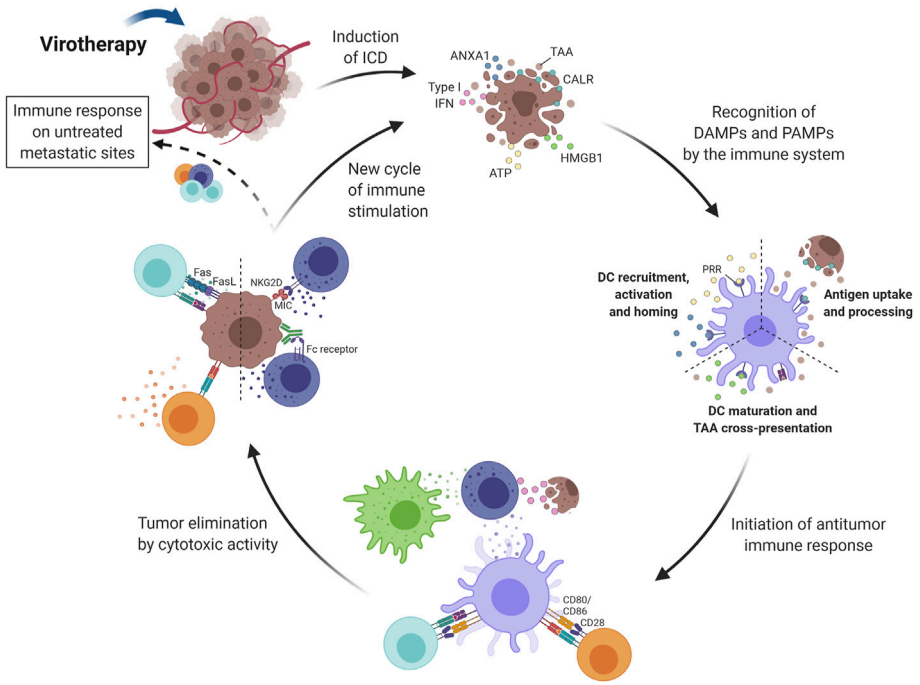

FIGURE 3 | Induction of immune activity in response to virotherapy. Virotherapy can induce ICD, which is characterized by the release of DAMPs (i.e., CALR, HMGB1, ANXA1 and type I IFN) during cell death. These danger signals, along with TAAs and PAMPs also released by OV-treated tumor cells, promote the initiation of an immune response after recognition by antigen-presenting cells (mainly DCs). ATP and ANXA1 are responsible for DC recruitment, activation and homing; CALR increases antigen uptake and processing; and HMGB1 promotes DC maturation and antigen cross-presentation. Next, TAAs are presented to T lymphocytes that can differentiate into both helper and cytotoxic cells since presentation occurs by class I and II MHC proteins. The cytotoxic activity of NK cells is also important for tumor elimination and type I IFNs play an important role in their stimulation together with signals provided by activated DCs and macrophages. Activated effector cells are then capable of recognizing and eliminating tumor cells by different mechanisms, for example, Fas-FasL interaction with $CD8^+$ T lymphocytes and MHC recognition by the NKG2D receptor on NK cells. Another key advantage of activating the immune system relies on the possibility of reaching untreated metastatic sites through circulating immune cells. The cycle restarts as dying tumor cells release more antigens and intracellular molecules that keep on activating the immune system. DC, Dendritic cell; NK, Natural killer; T_{reg}, Regulatory T cell; ICD, Immunogenic Cell Death; TAA, Tumor-Associated Antigen; CALR, Calreticulin; HMGB1, High Mobility Group Box-1; ATP, Adenosine Triphosphate; IFN, Interferon; ANXA1, Annexin A1; DAMPs, Damage-Associated Molecular Pattern; PAMPs, Pathogen-Associated Molecular Pattern; PRR, Pattern Recognition Receptor; NKG2D, Natural Killer Group 2D; OV, Oncolytic Virus; MHC, Major Histocompatibility Complex. Created with BioRender.com.

selective advantage for replication and dissemination of viral progeny (Kaufman et al., 2015). For example, resistance to apoptotic cell death, a critical hallmark of cancer, implies that tumor cells are lacking in a fundamental anti-viral defense, a point that may be exploited in order to promote viral replication (Russell et al., 2012). The interferon pathway was originally identified due to its anti-viral properties and, as mentioned above, plays an essential role in inducing innate and adaptive immune responses. While normal cells can defend themselves from viruses using the interferon pathway, tumor cells frequently present deficiencies in interferon response. Thus, this characteristic of tumor cells can be deliberately exploited for the development of OV (Murira and Lamarre, 2016; Gessani and Belardelli, 2021). Tumor cell killing in response to virotherapy occurs due to virus replication and induction of anti-viral responses. As we will detail below, the anti-viral response, which includes activation of innate and adaptive immunity, may be just as important, if not more so, than viral replication.

Our understanding of anti-viral and immunostimulatory properties of both normal cells and neoplasms advanced in the late 1980 s with the discovery of Toll like receptors (TLRs), and later families of Nod (nucleotide-binding and oligomerization domain) -like receptors (NLRs) (Takeda and Akira, 2004; Hansson and Edfeldt, 2005; Inohara et al., 2005). TLRs are present in antigen-presenting cells, such as DCs, macrophages and B cells, as well as T cells, NK cells, and non-immune cells (epithelial and endothelial cells, and fibroblasts), and recognize pathogen-associated molecular patterns (PAMPs), present in both viruses and bacteria, which attract and activate other cells that mediate adaptive immune responses (Fitzgerald and Kagan, 2020). These receptors provided evidence for understanding Coley's strategy (Coley's Toxin) (Coley, 1991) and the successful application of BCG in cases of bladder cancer (Lamm et al., 1980), both containing bacterial PAMPs, as well initial works that explored adjuvant effects of viral preparations or natural infections (Dock, 1904; Hoster et al., 1949; Newman and Southam, 1954). In the case of OV, the vector itself provides PAMPs in the form of viral proteins, DNA and RNA that are detected by the cell and initiate the anti-viral cascade through TLRs and NLRs.

In addition, viral infections naturally trigger danger-associated molecular patterns (DAMPs), stress-signaling proteins and

TABLE 1 | Clinical trials for treatment of melanoma with oncolytic viruses.

Oncolytic Vector	System	Transgene Load//Vector Main Modifications	Selectivity	Study Phase	Combination	Ref./Number clinical trial
T-VEC (Imlygic, talimogene laherparepvec)	Herpes simplex (HSV-1)	GM-CSF//Deletion: ICP34.5 (blocks PKR-eIF2 pathway) and ICP47 (reduces immune activation) genes	Replication in cells with low protein kinase R (PKR) levels	I, II, III, Approved[a]	—	Conry et al. (2018)
				Ib	Pembrolizumab	Ribas et al. (2017)
				Ib/III	Pembrolizumab	NCT02263508
				Ib/II	Ipilimumab	Chesney et al. (2018)
Pexa-Vec (JX-594, pexastimogene devacirepvec)	Vaccinia	GM-CSF and β-galactosidase//Deletion: thymidine kinase gene (promotes DNA synthesis)	Replication in cells high cellular thymidine kinase activity and active EGFR signaling	Ib/II	Anti-PD-L1 mAb (ZKAB001)	NCT04849260
Telomelysin (OBP-301)	Adenovirus	E1A and E1B regions under control of the human telomerase reverse transcriptase (hTERT) promoter	Replication in cells with telomerase activity	I	—	Nemunaitis et al. (2010)
TILT-123	Adenovirus	IL2 and TNF-α//D24 deletion in the E1A gene (inactivates pRB); E2F promoter	Replication in cells with high expression of E2F and with a dysregulated retinoblastoma pathway	I	TILs	NCT04217473
ICOVIR-5	Adenovirus	D24 deletion in the E1A gene (inactivates pRB); human E2F-1 promoter	Replication in cells with high expression of E2F and with a dysregulated retinoblastoma pathway	I	—	García et al. (2018)
LOAd703 (delolimogene mupadenorepvec)	Adenovirus	4-1BBL and TMZ-CD40L// D24 deletion in the E1A gene (inactivates pRB)	Replication in cells with a dysregulated retinoblastoma pathway	I/II	Atezolizumab	NCT04123470
ONCOS-102 (Ad5/ 3Δ24 GMCSF, CGTG-102)	Adenovirus	GM-CSF//D24 deletion in the E1A gene (inactivates pRB)	Replication in cells with a dysregulated retinoblastoma pathway	I	Pembrolizumab, cyclophosphamide	NCT03003676
GEN0101 (HVJ-E; TSD-0014)[b]	Hemagglutinating virus of Japan (HVJ)	RNA fragmentation by UV irradiation (inactivation); envelope presents fusion activity	Apoptosis and type-1 IFN response mediated by retinoic acid-inducible gene-I (RIG-I) activation in tumor cells upon viral RNA recognition	I/IIa	—	Kiyohara et al. (2020)
				Ib/II	Pembrolizumab	NCT03818893

[a]*by the U.S. FDA, Food and Drug Administration.*

[b]*Non-replicating vector.*

EGFR, epidermal growth factor receptor; GM-CSF, Granulocyte Macrophage Colony-Stimulating Factor; IFN, interferon; IL2, Interleukin 2; TNF-α, tumor necrosis factor alpha.

inflammatory cytokines (Matzinger, 2002; Tang et al., 2012). As a consequence, strategies employing viruses can induce immunogenic cell death (ICD) of cancer cells, generating chemo-attractants for cells of the immune system (Naik and Russell, 2009). Considering the characteristics of the tumor microenvironment, the use of these oncolytic strategies has the possibility of reversing the immunosuppressive profile and promoting the presentation of the repertoire of tumor antigens in an immunostimulatory context (Bartlett et al., 2013). Besides that, oncolysis triggered by viral particles, replicative or not, has the potential to aggregate immune responses against viral proteins and subvert this for antitumor immunity. When viral systems with replicative capacity are used, tumor selectivity gives these cells new viral epitopes, in addition to the TAAs and/or TSAs. This increases the exposure of these cells to both innate and adaptive immune responses, which may break the vicious cycle of tumor immunoediting. This is the main difference between oncolysis triggered by viral vectors and the approaches outlined above, and for this reason oncolytic virotherapy provides additional advantages over existing therapies that trigger ICD.

As shown in **Table 1**, several clinical trials have been performed using OV for the treatment of melanoma and novel approaches are being developed. Melanoma was the first neoplasm for which an oncolytic virus therapy was registered by the U.S. Food and Drug Administration (FDA). Approved in 2015, T-VEC, also known as Imlygic (OncoVex, talimogene laherparepvec) is prescribed for patients with advanced melanoma (Stage IIIB, IIIC or IV) that cannot be completely removed with surgery (Fukuhara et al., 2016). The history of this virotherapy exemplifies the path of a new biotechnological tool, from its conception in basic science to clinical trials aimed at proving its safety and efficiency for use in humans. Based on a modified herpes simplex virus (HSV-1), T-VEC was engineered deleting ICP34.5 and ICP47 viral genes. ICP34.5 blocks a cellular stress response to viral infection promoted by IFN-γ and ICP47 impairs the immune system's CD8 T-cell response against infected cells, thus these viral components render normal cells

susceptible to HSV replication. In T-VEC, the deletion of ICP34.5 and ICP47 prevents replication in normal cells, but tumor cells support viral replication due to defects in specific cellular pathways. The deletion of ICP47 also leads to upregulation of the viral protein US11, which further propels virus replication (Goldsmith et al., 1998; Liu et al., 2003). In addition, a constitutive expression cassette was inserted to provide granulocyte-macrophage colony-stimulating factor (GM-CSF) that, in conjunction with other cytokines, contributes to the attraction, differentiation and activation of APCs, such as DCs and macrophages, in the treated areas. Moving forward to phase I clinical trials, T-VEC was well tolerated and caused only mild adverse events such as local erythema and fever (Hu et al., 2006). Next, phase II clinical trials were realized in 50 patients with melanoma, stages III and IV, revealing a 26% response rate, including 8 with complete remission and another 5 with positive partial responses (RECIST) (Senzer et al., 2009). Finally, approval by the FDA and EMA was granted after an open-label phase III study that demonstrated the higher durable response rate (DRR) with a positive impact on overall survival compared to appropriate controls (Andtbacka et al., 2015). Kaufman and collaborators demonstrated that treatment with T-VEC induced a weakening of T cells responsive to MART-1 (melanoma-associated antigen) and, concomitantly, there was a decrease in regulatory T lymphocytes (Kaufman et al., 2010). Intriguingly, T-VEC is administered intratumorally, virus spread is only local, but immune response can mediate tumor regression in non-treated foci. This leads us to question the importance of viral replication itself *vs*. the induction of antitumor immunity for the success of the modality. Another point to be debated is if viral epitopes would indeed be needed for the effectiveness of these immune responses to contain and eliminate the primary tumor, as well metastases.

Oncolytic viruses have also been developed based on other viral systems. Pexa-Vec (JX-594) is derived from vaccinia virus inactivated by the deletion of the thymidine kinase gene, and modified for the expression of GM-CSF and β-galactosidase transgenes, is in the clinical testing phase for colorectal cancer and hepatocellular carcinoma (Breitbach et al., 2015; Park et al., 2015). A phase I/II clinical trial suggests that intratumoral injection of Pexa-vec is safe and with promising results being effective in treating both injected and distant disease in patients with surgically incurable metastatic melanoma (NCT00429312) (Mastrangelo et al., 1999). Some clinical trials testing Pexa-vec for melanoma are in progress (NCT04849260, NCT02977156). These findings reinforce interest in the use of OV as an immunotherapeutic for melanoma.

Adenovirus is another viral system widely used in immunotherapy for cancer. Oncorine (Onyx-015, H101), for example, is an oncolytic adenovirus-based used for the treatment of head and neck squamous cell carcinoma (Liang, 2018). It was designed to be replicated only in cells that have lost p53 activity, which would cover a large percentage of human neoplasms (Wei et al., 2018). Oncorine was approved in 2005 by State Food and Drug Administration, China (SFDA) (Wei et al., 2018). Although clinical trials of Oncorine for human melanoma have not yet been performed, Hu and colleagues found evidence that the use of ZD55-IL-24 (similar to Oncorine) in an animal model of melanoma prevents tumor growth and induced systemic antitumor immunity (Hu et al., 2020).

Telomelysin (OBP-301) is an oncolytic adenovirus utilizing the human telomerase reverse transcriptase (hTERT) promoter to control the expression of E1A and E1B, key genes that regulate adenoviral replication. In normal cells, the hTERT promoter should not be active, thus the lack of E1A/E1B prevents viral replication. Since hTERT is generally over active in tumor cells, E1A/E1B will be expressed, thus providing selectivity of virus replication (Trager et al., 2020). A phase I clinical trial showed good tolerability, with patients presenting only mild symptoms (grades 1 and 2), such as pain, induration, fever, and chill, and none of them had severe symptoms (grades 3 and 4). Despite having a small cohort, the results were promising, with seven of the twelve patients fulfilling RECIST criteria for stable disease at 56 days after the treatment (Nemunaitis et al., 2010; Trager et al., 2020). In 2016, a phase II clinical trial was initiated testing Telomelysin in patients with unresectable stage III and IV melanoma, though results are not yet available (NCT03190824). In addition, several phase I clinical trials involving replicative adenoviral vectors for different types of cancer have already been carried out, such as TILT-123, ICOVIR-5, LOAd703, ONCOS-102, as shown in **Table 1**.

As mentioned above, the induction of ICD is essential for the success of oncolytic approaches, bringing into question the importance of virus replication to achieve this goal. Many approaches are being developed for the induction of oncolysis even when the virus, such as adenovirus, does not replicate (Tessarollo et al., 2021). Our research has focused on the use of non-replicating adenoviral vectors for the transfer of genes intended to induce both cell death and immune activation including reversal of the immunosuppressive TME. That is to say, our approach induces oncolysis without the need for a replicating vector. In the first instance, the objective of the gene transfer is to induce immunogenic cell death in cancer cells, and subsequently, a second wave of death due to cytotoxicity mediated by cells of the immune system, mainly T and NK cells. Evidence from our studies indicates that the combined use of interferon-β (IFNβ) and p19Arf (alternate reading frame, p19Arf in mice and p14ARF in humans) induces melanoma cell death by necroptosis and is associated with an anti-viral response and the release of immunogenic factors (such as HMGB1, ATP and calreticulin) (Merkel et al., 2013; Ribeiro et al., 2017; Cerqueira et al., 2020). In our pre-clinical models, this therapy was well tolerated in animals where no side effects, such as liver transaminase induction, were observed and showed promising results in inhibiting tumor growth in s.c. tumors after *in situ* gene therapy, as well as prolonging the survival of treated animals (Cerqueira et al., 2020; David et al., 2020). We have also confirmed the induction of an antitumor immune response in vaccine and immunotherapy settings, with critical involvement of NK cells, $CD4^+$ and $CD8^+$ T cells, when our vector is used in immunocompetent C57BL/6 mice and B16-F10 mouse melanoma cells (Medrano et al., 2016). When using the human melanoma cell line SK-MEL 147 we demonstrated that transduction with adenoviral vectors encoding p14ARF and IFNβ resulted in activation of monocyte-derived DCs (Cerqueira et al.,

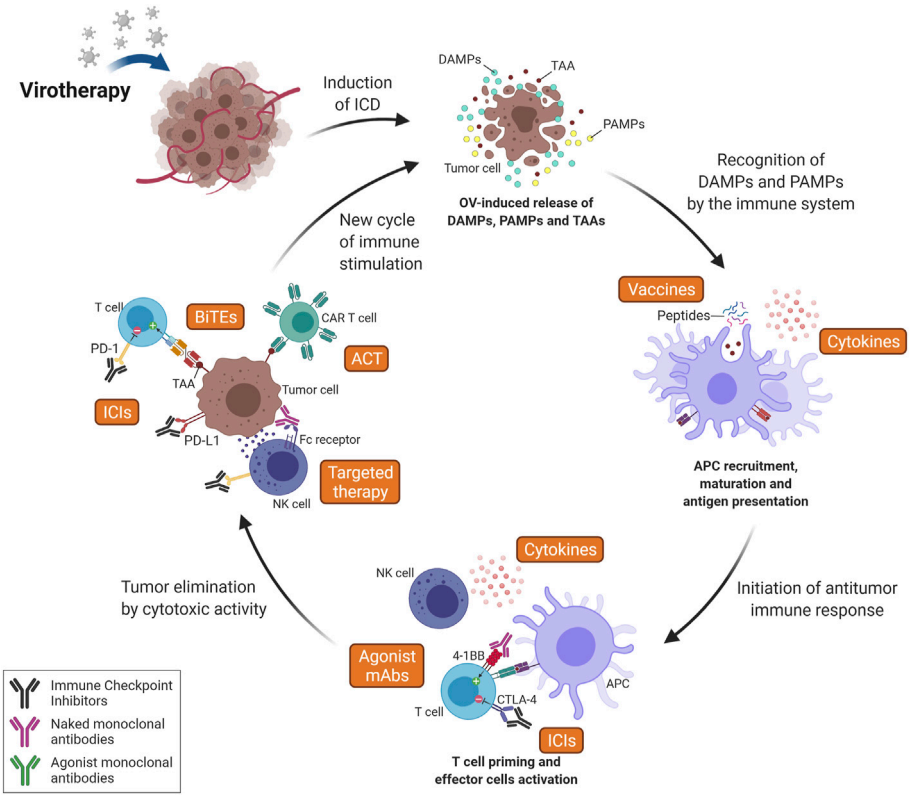

FIGURE 4 | Combining Oncolytic Virus (OV) therapy with other immunotherapy strategies. Since OV acts at the first step of the cancer-immunity cycle, releasing DAMPs, PAMPs and TAAs, its association with additional interventions aiming to establish an antitumor response is favored. The induction of ICD leads to recruitment and activation of antigen-presenting cells (APCs), which can increase the efficiency of vaccine-based approaches (especially peptide vaccines) and generate a stronger T cell response. Immune Checkpoint Inhibitors (ICIs) can increase activation of T cells during priming (here represented by anti-CTLA-4 mAb) and also increase T-cell effector activity in the tumor (i.e., anti-PD-(L)1 mAbs) following OV-induced immune cell infiltration. Agonist monoclonal antibodies (mAbs) are also an interesting intervention to increase T cell activation against TAAs (released after oncolytic therapy) by recognizing and activating costimulatory T cell receptors (i.e., 4-1 BB). Targeted therapy can also potentiate the immune responses to target tumor cells by increasing the effector activity of the innate immune system, including NK cell-mediated ADCC, macrophage-mediated ADCP and complement-mediated CDC. It is important to note that the cytotoxic activity of innate immune cells increases antigen release and, consequently, T cell recruitment. Virotherapy can also increase efficiency of Adoptive Cell Therapy (ACT), here represented by CAR T cells, by promoting a pro-inflammatory microenvironment, enabling T cells enhanced functions and leading to a higher recognition and elimination of tumor cells even in solid tumors. Therapy using bi-specific antibodies, such as BiTEs, can also be enhanced by oncolytic therapy as OVs precondition tumors in terms of T cell recruitment and activation. Finally, cytokines can act at many key steps of the process, such as antigen presentation and T cell priming, activation and recruitment. Furthermore, these factors have an important role at maintaining a favorable microenvironment for the survival of activated immune cells and the sustainment of the immune response. ICD, Immunogenic Cell Death; TAA, Tumor-Associated Antigen; DAMPs, Damage-Associated Molecular Pattern; PAMPs, Pathogen-Associated Molecular Pattern; NK, Natural killer; CTLA-4, Cytotoxic T-Lymphocyte-Associated protein 4; PD-1, Programmed cell Death protein 1; PD-L1, Programmed cell Death Ligand 1; CAR, Chimeric Antigen Receptor; BiTEs, Bi-specific T-cell Engagers; ADCC, Antibody-Dependent Cell-mediated Cytotoxicity; ADCP, Antibody-Dependent Cellular Phagocytosis; CDC, Complement-Dependent Cytotoxicity. Created with BioRender.com.

2020). In turn, these promoted the activation and priming of T cells, as well as the pro-inflammatory cytokine profile (Cerqueira et al., 2020). Together, these studies show that our gene transfer approach is a promising immunotherapy for melanoma (Hunger et al., 2017; Medrano et al., 2017; Cerqueira et al., 2020). The use of non-replicating vectors may be an advantage for our approach since the delivery of IFNβ can be counterproductive for replicating OVs (Cerqueira et al., 2020; Geoffroy and Bourgeois-Daigneault, 2020; Tessarollo et al., 2021). The results to date are encouraging and research will continue, with critical development using clinically relevant models, such as testing with patient-derived tumor samples, including PDO and immunological *ex vivo* models (Strauss et al., 2018).

COMBINING VIROTHERAPY WITH DIFFERENT IMMUNOTHERAPEUTIC INTERVENTIONS

Since the immune system consists of multiple components that act in an ordered and coordinated manner, immunotherapies that target a single step may not promote the entire cascade of events. Instead, combined approaches, especially those that facilitate different steps in the immune response, may provide an improved clinical outcome. As described above, the role of OV is to induce ICD, but this does not guarantee the effectiveness of the steps that follow, including antigen presentation, T cell priming and cytolytic activity. With the success of

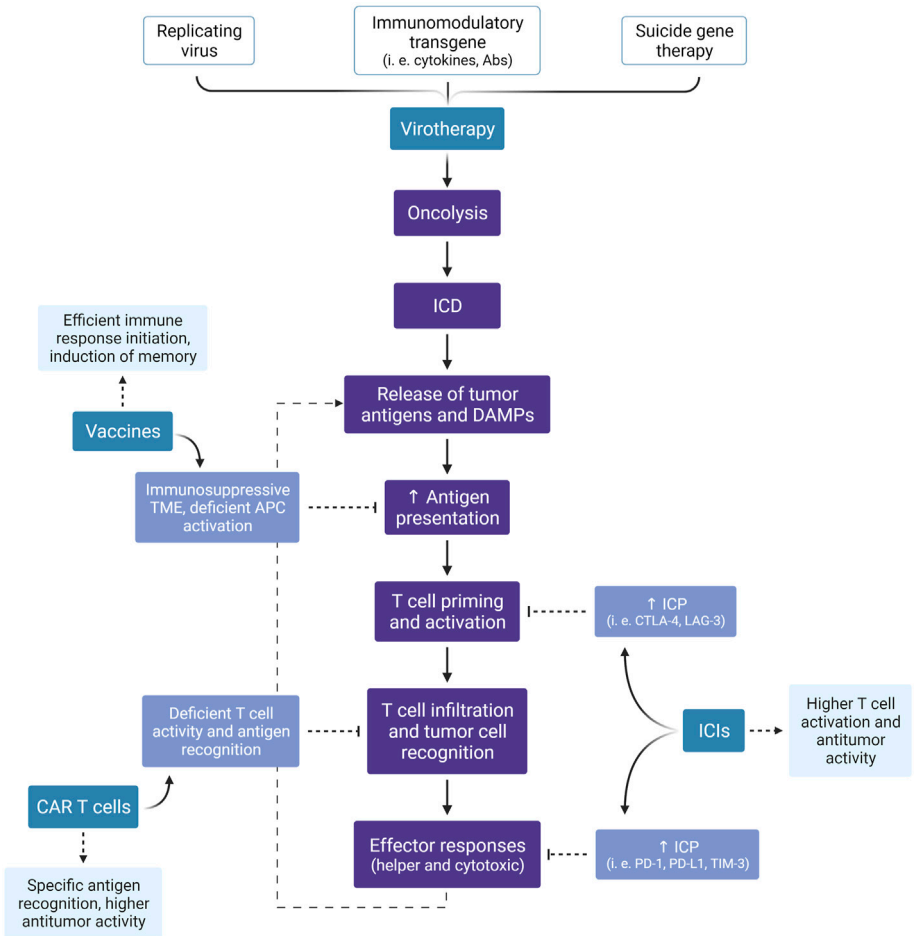

FIGURE 5 | Expected benefits of combined immunotherapies. Besides the direct antitumor effect of virotherapy, especially by the lytic effect of oncolytic viruses, this treatment modality can induce an anti-cancer immune response through the promotion of ICD. Here, we summarize the events illustrated in **Figures 3**, **4** and highlight the expected advantage when combining virotherapy with other immunotherapy strategies. ICD, Immunogenic Cell Death; DAMPs, Damage-Associated Molecular Patterns; TME, Tumor Microenvironment; APC, Antigen-Presenting Cell; ICP, Immune Checkpoint; ICI, Immune Checkpoint Inhibitor; CTLA-4, Cytotoxic T-Lymphocyte-Associated protein 4; LAG-3, Lymphocyte-Activation Gene 3; PD-1, Programmed cell Death protein 1; PD-L1, Programmed cell Death Ligand 1; TIM-3, T cell Immunoglobulin and Mucin domain-containing protein 3; CAR, Chimeric Antigen Receptor. Created with BioRender.com.

immunotherapies that target these points, especially ICI, a wide range of combination therapies is possible, as discussed here and summarized in **Figures 4**, **5**.

In the same way that combinations can enhance therapeutic efficacy, they can also be counterproductive or even intensify unwanted side effects. Therefore, clinical trials are essential to provide critical correlative data that can support the combined use of these new therapeutic options. Despite the importance of pre-clinical assays for the initial proof of concept, the animal models used in this stage are limited, since the main potential is precisely the performance of the human immune system (Bareham et al., 2021; Patton et al., 2021). Macedo and collaborators published a review that provides a current overview of virotherapy clinical trials, as well as the resulting combinations (Macedo et al., 2020). According to what they observed, the majority of clinical trials (62.9%) published between 2000 and 2020 investigated only the action of oncolytic viruses used as monotherapy, while 37.1% where OV was administered in combination with at least one other anti-cancer treatment or medication. Of these combinations, the most frequent were cytotoxic chemotherapy agents, chemotherapy prodrugs and radiotherapy. Only a small fraction (5%) of all clinical tests with oncolytic viruses investigated the combination with other immunotherapies such as ICIs or cytokines (Macedo et al., 2020).

Inhibitors of Immune Checkpoints

As previously mentioned, melanomas have a high mutational load, which contributes to the generation of neoantigens which can be targeted by the patients' T cells, but their function is often impeded due to upregulation of PD-1. There is also evidence that OV-based therapies increase infiltration of T cells in the tumor (Ribas et al., 2017). Thus, the combination of these immunotherapies is an interesting option (Chiu et al., 2020; Hwang et al., 2020). In randomized, open-label phase I and II

studies, T-VEC combined with ipilimumab (anti-CTLA4) showed significantly greater efficacy compared to ipilimumab alone (Puzanov et al., 2016; Chesney et al., 2018; Trager et al., 2020). Likewise, another phase 1b study using T VEC plus pembrolizumab (anti-PD-1) in advanced melanoma showed that this combination was well tolerated, although some patients had mild side effects such as chills, fatigue and pyrexia. Phase III clinical study is already being conducted to expand clinical information regarding efficacy (Long et al., 2016). The patients who responded to the combination therapy showed an increase in the infiltrated $CD8^+$ T cells, as well as an increase in the expression of PD-L1 protein, and thus providing mechanistic evidence for the improvement in the effectiveness of pembrolizumab therapy. In addition, greater expression of IFN-γ was detected in the tumor microenvironment in different cell subpopulations, contributing to a less immunosuppressive context (Ribas et al., 2017).

Several approaches using a variety of OVs and ICIs are in pre-clinical and clinical development. For example using a mouse model of metastatic pulmonary melanoma, it was demonstrated that virotherapy using influenza A viruses (IAVs) combined with ICI resulted in a sustained antitumor efficacy caused by the significant increase in the oncolytic effect (Sitnik et al., 2020). In other lines of evidence, Vijayakumar et al., applied Newcastle disease virus (NDV) in combination with radiotherapy plus checkpoint inhibitors (PD1 or CTLA4 targeted mAbs) induces an abscopal effect in immunocompetent B16-F10 murine melanoma model. These authors also show that recombinant NDV a single-chain variable fragment (scFv) anti-CTLA4 plus radiation was as effective as virus, radiation and systemic anti-CTLA4 in terms of survival benefit (Vijayakumar et al., 2019). An important limitation of the use of ICI therapy is that the majority of patients still fail to respond over time (Sharma et al., 2017; Grote et al., 2020). In this context, Liu and collaborators used therapy with oncolytic virus derived from a strain of alphavirus, M1, and were able to demonstrate refractory tumors were sensitized to subsequent checkpoint blockade by boosting T-cell recruitment and upregulating the expression of PD-L1 (Liu et al., 2020). Another important factor to be considered in cancer therapy is whether the effect of local treatment may also include untreated distant metastases. Often referred to as the abscopal effect, this could be an indication that systemic antitumor immune responses are being activated. Evidence in the literature indicates that this effect was observed experimentally in animal models when Kuryk et al. used oncolytic adenoviruses carrying GM-CSF (ONCOS-102) plus ICI therapy (Kuryk et al., 2019). In this scenario of cooperation and synergy, we could also imagine different combinations with other immune players.

Cytokines

A significant obstacle to successful immunotherapeutic interventions is the modulation of the TME, which, in addition to supporting tumor growth and dissemination, favors the evasion of antitumor immune responses. Dysfunctional interaction of tumor and stromal cellular components leads to a predominantly anti-inflammatory cytokine profile with interleukin-10 (IL-10) (Seo et al., 2001), transforming growth factor (TGF)-beta and other cytokines (Strauss et al., 2007), produced by immunosuppressive cells, for example, regulatory T cells (Tregs) (Knol et al., 2011). The administration of IL-2 was one of the first reproducible effective human cancer immunotherapies against metastatic melanoma (Rosenberg, 2014). Likewise, IFN-α also showed antitumor activity in animal models, in addition to its antiviral activity initially described (Gresser and Bourali, 1970). After clinical trials, both IL-2 and IFN-α demonstrated only mild clinical benefit when used as monotherapy and are approved by the FDA for use in melanoma (Atkins et al., 1999). However, due to the short half-life of most cytokines, high-dose IL-2 and IFN-α administration may be necessary, a situation that increases the incidence of adverse effects, which can make continued treatment unfeasible (Komenaka et al., 2004; Berraondo et al., 2019).

Viruses can be loco-regional adjuvants if applied intratumorally. In addition to their immunostimulatory properties associated with the release of DAMPs and PAMPs, OVs act by inducing acute localized inflammation, they can disturb the tumor niche through the production of inflammatory cytokines in infected/transduced cells. This implies a change in the pattern of cytokines present in the tumor microenvironment in a way that favors the breakdown of immunological tolerance. Few clinical trials have explored the combination of oncologic viruses and cytokine, IL-2 (Voit et al., 2003) and IFN-α (Macedo et al., 2020). However, instead of administering soluble cytokines directly, several approaches function for the design of recombinant virus oncolytic armed with immune modulators, such as cytokines and chemokines. Once the target cell is transduced, it starts to express the carried genes locally, reducing the systemic adverse effects (De Graaf et al., 2018). A classic example is the T-Vec, which locally induces the expression of GM-CSF (Andtbacka et al., 2015). For this purpose, several approaches, with different viral vectors, were used to express cytokines such as IL-2 (Carew et al., 2001; Bai et al., 2014), IL-12 (Varghese et al., 2006), IL-15 (Niu et al., 2015), IFN-γ (Vigil et al., 2007), IFN-β (Durham et al., 2017; Cerqueira et al., 2020; David et al., 2020), reinforces the potential of combining OV and cytokines for immunotherapy for melanomas (De Graaf et al., 2018).

Oncolytic Vaccines Use TSA/TAA in Combination With OV

Assuming that TSA/TAA are the main targets of the adaptive immune system, some strategies seek to incorporate these tumor antigens in OVs, referred to as "oncolytic vaccines", designed to potentiate antitumor immune responses, especially cytotoxic T lymphocytes (Elsedawy and Russell, 2013; Holay et al., 2017). Mulryan and coworkers used engineered vaccinia virus expressing TAA 5T4 (an oncofetal antigen), in animal models of melanoma and showed significant melanoma tumor retardation compared with mice vaccinated with respective controls. Although it is a self-antigen, in this work no autoimmune effects inherent to the treatment were detected (Mulryan et al., 2002). In another work, using the B16-ova

TABLE 2 | Clinical trials using CAR T-cells for the treatment of melanoma and other solid tumors.

Title	Target Antigen	Cancer	Status	ClinicalTrials.gov Identifier
Autologous CAR-T/TCR-T Cell Immunotherapy for Malignancies	CAR-T/TCR-T cells multi-target including CD19, CD22, CD33, BCMA, CD38, NY-ESO-1, DR5, C-met, EGFR V III, Mesothelin	B-cell Acute Lymphoblastic Leukemia, Lymphoma, Myeloid Leukemia, Multiple Myeloma, Hepatoma, Gastric Cancer, Pancreatic Cancer, Mesothelioma, Colorectal Cancer, Esophagus Cancer, Lung Cancer, Glioma, Melanoma, Synovial Sarcoma, Ovarian Cancer, Renal Carcinoma	Recruiting	NCT03638206
B7H3 CAR T Cell Immunotherapy for Recurrent/Refractory Solid Tumors in Children and Young Adults	B7H3	Pediatric Solid Tumor, Germ Cell Tumor, Retinoblastoma, Hepatoblastoma, Wilms Tumor, Rhabdoid Tumor, Osteosarcoma, Ewing Sarcoma, Rhabdomyosarcoma, Synovial Sarcoma, Clear Cell Sarcoma, Malignant Peripheral Nerve Sheath Tumors, Desmoplastic Small Round Cell Tumor, Soft Tissue Sarcoma, Neuroblastoma, Melanoma	Recruiting	NCT04483778
Gene Modified Immune Cells (IL13Ralpha2 CAR T Cells) After Conditioning Regimen for the Treatment of Stage IIIC or IV Melanoma	IL13Ralpha2	Stage IIIC or IV Melanoma	Recruiting	NCT04119024
MB-CART20.1 Melanoma	CD20	Melanoma (Skin)	Unknown	NCT03893019
CAR T Cell Receptor Immunotherapy Targeting VEGFR2 for Patients With Metastatic Cancer	VEGFR2	Metastatic Melanoma Renal Cancer	Terminated	NCT01218867
A Phase I Trial of T Cells Expressing an Anti-GD2 Chimeric Antigen Receptor in Children and Young Adults With GD2+ Solid Tumors	GD2	melanoma, sarcoma, osteosarcoma, neuroblastoma	Completed	NCT02107963
Administering Peripheral Blood Lymphocytes Transduced With a CD70-Binding Chimeric Antigen Receptor to People With CD70 Expressing Cancers	CD70	Melanoma, Pancreatic, Renal, Ovarian and Breast Cancer	Suspended	NCT02830724
B7-H3-Specific Chimeric Antigen Receptor Autologous T-Cell Therapy for Pediatric Patients With Solid Tumors (3 CAR)	B7-H3	Pediatric Solid Tumor, Osteosarcoma, Rhabdomyosarcoma, Neuroblastoma, Ewing Sarcoma, Wilms Tumor, Adrenocortical Cancer, Desmoplastic Small Round Cell Tumor, Germ Cell Cancer, Rhabdoid Tumor, Clear Cell Sarcoma, Hepatoblastoma, Melanoma, Carcinoma, Malignant Peripheral Nerve Sheath Tumors, Soft Tissue Sarcoma	Not yet recruiting	NCT04897321
Treatment of Malignant Melanoma With GPA-TriMAR-T Cell Therapy	GPA-TriMAR	Melanoma	Recruiting	NCT03649529
C7R-GD2.CART Cells for Patients With Relapsed or Refractory Neuroblastoma and Other GD2 Positive Cancers (GAIL-N)	C7R-GD2	Neuroblastoma, Osteosarcoma, Ewing Sarcoma, Rhabdomyosarcoma, Uveal Melanoma, Phyllodes Breast Tumor	Recruiting	NCT03635632
Autologous T Cells Expressing MET scFv CAR (RNA CART-cMET)	MET scFv	Malignant Melanoma, Breast Cancer	Terminated	NCT03060356

mosuse melanoma model, Diaz and coworkers verified an increase in the activation of ova-specific T cells after treatment with vesicular stomatitis virus (VSV) delivering ovalbumin (ova) (Diaz et al., 2007). Other studies went further and studied melanoma cDNA library delivery by the oncolytic viral vector VSV. After using these constructs to treat pre-established melanomas in animal models, remission was observed, which is associated with the ability of mouse lymphoid cells to mount a tumor-specific $CD4^+$ interleukin (IL)-17 dependent response (Pulido et al., 2012). Collectively, these findings corroborate the principles of personalization of cancer treatment, since the gamut of potential TAA/TSA epitopes will be inherent in the evolutionary history of tumors (Holay et al., 2017). This strategy will be quite valuable in the not-too-distant future, where tumor genome and transcriptome sequencing data will be increasingly available, and thus, likely to be coupled with viral therapies (Finck et al., 2020).

Perspectives for Combining OV and CAR-T Cell Therapy for Melanoma

Adoptive transfer of chimeric antigen receptor (CAR)-modified T cells has demonstrated remarkable rates of long-lasting complete remission in patients with hematological tumors (Guedan and Alemany, 2018). The approval by the US Food and Drug Administration (FDA) of Kymriah (tisagenlecleucel)

for acute lymphoblastic leukemia (ALL) (Maude et al., 2018) and Yescarta (axicabtagene ciloleucel) designed to treat large B-cell lymphoma (Neelapu et al., 2017), opened unprecedented perspectives for cancer treatment. In general, the CAR-T cell approach involves the *ex vivo* modification of the patients' own T cells using lentiviral and retroviral vectors to deliver the CAR sequence, followed by expansion and reinfusion in the patient (Simon and Uslu, 2018; Chicaybam et al., 2020). Other applications for the use of CAR T have been approved by the FDA as Abecma (idecabtagene vicleucel) for the treatment of multiple myeloma and its use for autoimmune diseases is already being discussed (Hong et al., 2020).

Despite impressive results reported for hematological malignancies, CAR-T cell therapy in solid tumors has failed to meet expectations (Newick et al., 2017; Dana et al., 2021; Marofi et al., 2021). Unlike hematological malignancies, melanomas, like many solid tumors, do not have well-defined targets for the design of a CAR since the available antigens are often expressed in normal cells, thus promoting off-target cell killing. Adding to that difficulty, expression of possible candidate antigens can vary as tumor immunoediting is a continuous process and may give the targeted cells a selective advantage that results in their escape from the CAR-T cells (Dunn et al., 2002; Poggi et al., 2014). Even so, many studies are underway to use the CAR-T cell approach in melanoma (**Table 2**), a topic that has been reviewed recently (Soltantoyeh et al., 2021; Uslu, 2021). Currently, data from these clinical trials are not available. The immunosuppressive TME is another barrier that must be overcome if CAR-T cell therapies are to be successful. Melanoma, like other solid tumors, is composed of a complex network containing different cell types, such as fibroblasts, endothelial cells, adipocytes and several cells of the immune system immersed in the extracellular matrix (ECM) (Villanueva and Herlyn, 2008; Simiczyjew et al., 2020). Acting together, they comprise an immunosuppressive microenvironment that promotes evasion of antitumor responses, especially of T effector/cytotoxic lymphocytes (Ruiter et al., 2002; Maibach et al., 2020; Simiczyjew et al., 2020). This also directly affects the recruitment and activity of CAR-T cells that may have reached the tumor sites, suggesting that therapy with CAR-T cells alone will not be sufficient to induce complete responses in melanoma. From this perspective, virotherapy, with its ability to revert immunosuppression and promote infiltration of T cells, is expected to fill in some necessary gaps for the effectiveness of CAR-T cell therapy in solid tumors.

In this context, Wing and coworkers used an oncolytic adenovirus to deliver a Bispecific T-cell Engager (BiTE) targeting a second tumor antigen in order to augment tumor cell recognition by CAR-T cells. Surprisingly, this combination was able to activate new populations of antitumor T cells in addition to the CAR-T cells (Wing et al., 2018). Since these assays were conducted in immunocompromised mice (NSG, NOD/SCID/IL2rγ$^{-/-}$), we hypothesize that in immunocompetent individuals, the chance of generating new cytotoxic T cell clones against neoantigens during oncolysis will be increased. Clinical trials will be needed to evaluate this hypothesis, as well as safety. Even though melanoma was not studied, this work opened perspectives for a strategy for other solid tumors. Recently, Jong and collaborators used the same strategy with different targets, sialylated CD43 × CD3 bispecific T cell engager, and demonstrated that it was not only able to bind to cultured patient-derived melanoma samples, but also reduced tumor outgrowth in grafted mice (De Jong et al., 2021). Other studies have pointed out that the use of OV armed with PD-L1 blocking mini-antibody (Tanoue et al., 2017) or IL12p70 and PD-L1 (Rosewell Shaw et al., 2017), combined with CAR-T cell therapy is more effective for tumor control and prolonged survival when compared to each agent as monotherapy. Another interesting evidence in the literature points to the use of oncolytic viral vectors armed with IL-2 and TNF-α to curtail the progression of the primary tumor, but not its metastases. Intriguingly, combining these viral vectors with CAR-T was able to control the primary tumor, as well as its metastases (Guedan and Alemany, 2018; Watanabe et al., 2018).

An important limitation of the use of OV is the incidence of immunity against viral components, including cells that present these viral antigens. Thus, antibodies may neutralize and inactivate viral particles before they reach their target, a limitation of particular concern for repeated administration of the OV. In an innovative approach, VanSeggelen et al. utilized CAR-T cells to protect the oncolytic virus from the immune system, delivering it only to the tumor niche. Thus, viral oncolysis could attract not only more CAR-T cells in a positive feedback loop, but also other immune cells contributing to antitumor responses (Vanseggelen et al., 2015). Collectively these studies point to the potential of these combinations, which need appropriate clinical trials (Guedan and Alemany, 2018).

CONCLUSION

Despite numerous advances in therapies for metastatic melanoma, many patients become refractory and succumb to the disease since they are left without treatment options (Keller and Bell, 2016). In this scenario, the search for innovative therapeutic interventions is urgent. Therapies employing oncolytic viruses, replicative or not, are gaining attention. Some successful examples include orphan drug designation for Pexa-Vec (Breitbach et al., 2015; Park et al., 2015) and Telomelysin (Trager et al., 2020) as well as the approval of T-VEC (Fukuhara et al., 2016) and Oncorine (Liang, 2018) by national regulatory agencies. At a time when millions of people are receiving anti-SARS-CoV2 vaccines based on recombinant viral vectors with few/low side effects reported (Yadav et al., 2021), it reinforces safety and reliability with regard to viral vectors as viewed by regulatory agencies around the world.

The use of virotherapy, including gene transfer with non-replicating viral vectors, has been shown to change the profile of TME acting as an adjuvant (Keller and Bell, 2016). As we can see in the diagram in **Figure 3**, the use of virotherapy induces oncolysis, at this moment by the direct action of the viral particles. In this first round of cell death, due to the release of DAMPs, such as ATP, HMGB1, type I IFNs and exposure of calreticulin, it is characterized as ICD. At the same time, the

release of tumor antigens (TAA and TSA) also occurs along with PAMPs associated with the virus. This is a favorable scenario for the recruitment of APCs that capture these tumor antigens and prime T cells, stimulating the formation of adaptive cellular responses against tumor cells. Likewise, the cytokine profile in the tumor microenvironment tends to change from an immunosuppressive to an inflammatory profile, favoring the recruitment of effector T cells. Thereafter, a second wave of oncolysis begins, but this time due to cytotoxicity of T cell antitumor clones, which can even act in distant metastatic sites with the application of viral therapy.

As novel therapies emerge, rational combinations will need to be overcome tumor resistance and adaptations of tumor cells and their cellular partners in the tumor microenvironment (Raja et al., 2018). Taking advantage of the fact that virotherapy can attract T cells to the tumor niche, ICIs can have their effectiveness enhanced if used together (**Figures 4**, **5**). Clinical trial has already been carried out to envision this combination (Long et al., 2016; Puzanov et al., 2016; Chesney et al., 2018; Trager et al., 2020). By the same reasoning, the use of CAR T cells against solid tumors such as melanoma is expected to be more effective when combined with virotherapy. However, as there is still no registered CAR-T cell therapy for melanoma, clinical trials will be necessary to verify this hypothesis.

Since the tumor microenvironment is abundant in anti-inflammatory cytokines such as IL-10 and TGF-β, the use of armed viral vectors can reverse this profile and restrict the expression of appropriately chosen cytokines, to favor a less immunosuppressive context. Unlike the systemic application of cytokines, which brings together a series of collateral effects, intratumoral expression by viral vectors tends to increase their availability in this microenvironment with a reduction in side effects. In summary, improvements in the design, delivery and targeting of oncolytic viral vectors will provide increasing potential as immunotherapies against melanoma. Allied to this, is the fact that combinations with different immunotherapy modalities can cooperate to increase therapeutic efficacy.

AUTHOR CONTRIBUTIONS

OLDC: Organized, wrote and edited the text, tables and figures; NGA and ACD: Wrote and edited the text, tables, designed and elaborated figures; FA, ECC, MACS, and NGT: wrote and edited the text; BES: Edited the text, secured funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.777775/full#supplementary-material

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METHODS
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A qPCR-Based Method for Quantification of RCA Contaminants in Oncolytic Adenovirus Products

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Oncolytic adenovirus is one of the most promising treatments against cancer and is widely evaluated clinically. During high titer production, "Wild-type-" like replication-competent adenovirus (RCA) contaminants can be generated through recombination events due to the DNA sequence similarity between oncolytic virus and host cells. These RCA contaminants raise various safety concerns in clinics. Cell culture-based methods have been developed to detect RCA contaminants in replication-deficient adenovirus vectors. These methods were based on that only RCA contaminants, but not the vectors, are able to grow in and lyse the test cell line. However, these methods are not suitable for distinguishing RCA contaminants from the oncolytic adenovirus products because both can replicate in test cell lines. Herein, we reported a qPCR-based method to quantify RCA contaminants quickly and reliably in E1B-deleted oncolytic adenovirus products. This method is based on specific detection of the E1B gene, which can be acquired during production *via* recombination events between viral and host cell DNA. The assay is sensitive with the limit of detection at 10 VP of the RCA contaminants and the limit of quantification at 75 VP of the RCA contaminants in each 40 µL qPCR reaction. We have also validated the method on virus batches produced in the non-GMP and GMP conditions. Our results showed that this qPCR-based method was reliable and robust for detecting and quantifying RCA contaminants in oncolytic adenovirus products. The method may also be adapted for other oncolytic adenoviruses products by switching primer sets.

Keywords: replication-competent adenovirus, conditionally replicating adenovirus, quantification, clinical production, qPCR, RCA contaminants

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INTRODUCTION

Oncolytic adenoviruses (OAdVs) are advanced medical products with increasing applications within cancer therapy. OAdVs are genetically engineered to replicate in tumor cells but not in normal cells (Everts and Van Der Poel, 2005). OAdVs are usually either constructed with tumor-/tissue-specific promoters controlling E1 expression (e.g., AdVince) (Yu et al., 2017) or through deletion of some part of the E1 gene (e.g., DNX-2401 and ONYX-15) (Wold and Toth, 2013; Heise et al., 1997). In contrast to oncolytic adenovirus, recombinant adenoviral vectors are often replication-incompetent, and their essential viral E1 sequence is usually replaced by therapeutic genes (Yu et al., 2017). They are also widely used in clinical gene therapy (Yu et al., 2017).

Many adenoviral products, including oncolytic adenovirus and recombinant adenovirus vectors, are produced in HEK293 cells. The adenoviral genome 1-4344 (Louis et al., 1997) presented in this

cell line provides the complementary E1-function for E1-deleted adenovirus. Similarly, another adenovirus-producing cell line 911 contains the 79-5789 adenoviral genome (Fallaux et al., 1996) to compensate for E1 function. Therefore, the presence of the E1 gene in producer cells may cause undesirable generation of "wild-type-" like replication-competent adenovirus (RCA) contaminants through recombination between viral and host cell DNA (Lochmüller et al., 1994). Herein, we use the term RCA to strictly refer to the RCA contaminants. These RCA contaminants constitute a risk of unintended viral spread and host inflammation response when the viral products are used clinically (U.S. Department of Health and Human Services et al., 2001). RCA contaminants can be avoided using cell lines containing a minimized adenoviral sequence (e.g., Per.C6 with adenoviral genome 459-3510) (Fallaux et al., 1998) or completely lacking the E1 gene (e.g., A549) to abolish RCA contaminants formation. Note that E1-free cell lines such as A549 can only be utilized for oncolytic adenoviruses, which retain replication capacity in cancer cells. However, to warrant safety, it is crucial to determine the number of RCA contaminants in each batch of oncolytic adenoviruses intended for clinical use (Fallaux et al., 1999). Replication-deficient adenoviral vectors can reliably be tested for RCA contaminants through cell-based assays, such as the cell culture/cytopathic effect (CPE) assay (Zhu et al., 1999), based on the out-growth of RCA contaminants. However, such methods cannot distinguish RCA contaminants from the actual oncolytic virus due to the lack of cell lines that only support the growth of RCA contaminants. Therefore, it is emerging to develop an accurate, sensitive, and robust method to examine the level of RCA contaminants in clinical batches of oncolytic adenovirus products.

In this study, we designed a real-time quantitative PCR (qPCR)-based assay that can be used to detect and quantify RCA contaminants in batches of E1B-deleted oncolytic adenoviruses. Recombination between the viral and the producer cell line genomes will produce a recombinant DNA template that can be detected. The assay utilizes a primer set that specifically binds to the E1B-region absent in the oncolytic virus but present in the producer cell genome and RCA contaminants. Thus, only the RCA contaminants are detected in the purified product. Our assay has a low limit of detection (LLOD) of 10 VP and a low limit of quantification (LLOQ) (95% confidence) of 75 VP in each 40 µL qPCR, presenting a high sensitivity and accuracy for monitoring potential RCA contaminants in clinical products. In addition, using this method, we also evaluate potential RCA contaminants for our GMP products intended for clinical usage.

MATERIALS AND EQUIPMENT

- Cell lines for virus production: human embryonic retinoblasts cell line 911 (a kind gift from Crucell, Netherlands) and human lung carcinoma epithelial cell line A549 (purchased from ATCC).
- Wild-type adenovirus 5 (Ad5wt) (purchased from ATCC).
- E1B-deleted oncolytic adenovirus virus (Ad5dE1B) derived from 911 (Ad5dE1B_911) or A549 (Ad5dE1B_A549) cell line.
- Oncolytic adenovirus produced under GMP condition (Ad5dE1B_GMP).
- 10 mM Tris-HCl: prepared by 1 m Tris-HCl, pH 8.0 (Invitrogen, AM9855G), and nuclease-free water (Invitrogen, AM9939).
- Lysis buffer: prepared by Proteinase K (Thermo Scientific, EO0491) and 10 mM Tris-HCl.
- Primer sets for RCA contaminants quantification: Fwd-AdE1B55K 5′- GCCGAGGTGGAGATAGATA-3′ and Rev-AdE1B55K 5′-CGTGTAGGATAAGGTTGGTATT-3′ (target region 2072-2240); Fwd-AdE1B19K 5′-TTCTGC TGTGCGTAACTTG-3′ and Rev-AdE1B19K 5′-TCTTGA TGACCTTCTCTTGGA-3′ (target region 1202-1392).
- SYBR Green PCR Master Mix (Applied Biosystems, 4309155).
- Thermocycler with fluorophore detector for qPCR (BioRad CFX96 system).

METHODS

The method is generally based on qPCR detection of RCA contaminants in E1B-deleted oncolytic adenoviruses. To minimize DNA loss and maximize detection of RCA contaminants, viral DNA was released using proteinase digestion of viral capsid without further purification steps. The digested product was directly subjected to qPCR analysis. Serial dilutions of wild-type Ad5 (Ad5wt) were used to generate the standard curve for quantification. A detailed protocol is described in the following.

Cell Culture

Human embryonic retinoblasts cell line 911 (Crucell, Netherlands) was maintained in DMEM (Gibco) with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin-streptomycin (PEST) (Gibco), and 1 mM sodium pyruvate (NaPyr) (Gibco). Human lung carcinoma epithelial cell line A549 (ATCC) was maintained in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco), 100 U/ml PEST (Gibco) and 1 mM NaPyr (Gibco). All cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Production and Titration of Adenovirus

Wild-type (WT) adenovirus, designated as Ad5wt, was propagated in A549 cells. The genetically engineered oncolytic adenovirus Ad5dE1B was produced in 911 or A549 cell lines, designated as Ad5dE1B_911 and Ad5dE1B_A549. These viruses were produced in non-GMP conditions and purified by CsCl density-gradient centrifugation (Yu et al., 2011). Ad5dE1B was also produced in GMP condition using the A549 producer cell line and designated Ad5dE1B_GMP. Virus titer (viral particles, VP) (**Table 1**) was determined by measuring absorbance at 260 nm as described (Maizel et al., 1968).

TABLE 1 | Titer (VP) of different batches of viruses used in the study.

Virus batch	Producer cell line	Titer (OD_{260}) (VP/μL)	Production condition
Ad5wt	A549	6.6×10^9	Lab batch
Ad5dE1B_A549	A549	8.5×10^9	Lab batch
Ad5dE1B_911	911	6.7×10^9	Lab batch
Ad5dE1B_GMP	A549	5.17×10^9	GMP

Real-Time Quantitative PCR

Lysis buffer was prepared by diluting Proteinase K in 10 mM Tris-HCl to a final concentration of 1 mg/ml (>30 U/ml). Here, proteinase K is overloaded to achieve maximum release of viral DNA. The concentration of proteinase K could be titrated down to fit the optimal condition. The serially diluted Ad5wt (for standard curve) or undiluted test samples were directly added to lysis buffer (total 18 μL containing 2 μL sample plus 16 μL lysis buffer) and incubated at 37°C for 16 h to release viral genomic DNA. Each sample was then heated at 100°C for 10 min to inactivate the proteinase. Primers (1 μL of each with a final concentration at 5 μM) and 20 μL of 2× SYBR Green PCR Master Mix were added to each sample (a total of 40 μL per reaction). qPCR was performed using cycling conditions: denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The signal was read at the end of each cycle. The melting curve was generated by increasing the temperature from 65°C to 95°C with an increment of 0.5°C per 5 s.

Statistical Analyses

For further analysis, a linear regression curve was fitted to the Ct values and the log-transformed virus particle quantity. As the no template control (blank water sample) gives no detectable signal, the lower limit of detection (LLOD) was set to the lowest dilution point tested in the assay. The lower limit of quantification (LLOQ) was then calculated by the formula LLOQ = LLOD + $3.2 \times SD_{LLOD}$.

RESULTS

Determine the Low Limit of Detection and Low Limit of Quantification

Two primer sets, Fwd-AdE1B55K/Rev-AdE1B55K and Fwd-AdE1B19K/Rev-AdE1B19K, were designed targeting either E1B 55K or 19K region as indicated in **Figure 1A**. Both primer sets target an absent region in our E1B-deleted oncolytic virus Ad5dE1B but will be present in the RCA contaminants. When evaluated by standard PCR, non-specific amplification was observed in neither of the two primer pairs (**Figure 1B**), verifying the specificity of these primer pairs. We thus selected Fwd-AdE1B19K/Rev-AdE1B19K to continue the method development. Ad5wt was serially diluted in 10 mM Tris-HCl and used to mimic RCA contaminants in the following qPCR assay. We plotted the Ct values against the log-transformed virus particle quantity to obtain a well linearized standard curve when analyzing serially diluted samples containing wild-type adenovirus (**Figure 1C**). The goodness-of-fit is usually $R^2 > 0.98$, indicating the robustness of the assay. Because no amplification signal was detected in the negative sample, we set the lower limit of detection (LLOD) at the lowest dilution point tested in this assay (10 VP per 40 μL qPCR), which gives the lower limit of quantification (LLOQ) at 75 VP per 40 μL qPCR with 95% confidence (**Figure 1C**). Additionally, a single peak in the melting curve also verified the specificity of the primer set Fwd-AdE1B19K/Rev-AdE1B19K (**Figure 1D**).

Detection of Replication-Competent Adenovirus Contaminants Present in Lab-Batch E1B-Deleted Oncolytic Adenovirus

Next, we detected and quantified the RCA contaminants in E1B-deleted oncolytic adenovirus virus (Ad5dE1B) produced in either 911 (Ad5dE1B_911) or A549 (Ad5dE1B_A549) cells. Ad5dE1B_A549 did not show any amplification signal after 45 cycles, indicating the RCA contaminants were below our detection limitation (**Figures 2A,B**). In clear contrast, Ad5dE1B_911 showed a Ct-value around 30, indicating 1.630×10^4 VP of RCA contaminants in 1.34×10^{10} VP (in 2 μL tested sample) of the virus produced in the 911 cell line (**Figure 2A**).

Detection of Ad5wt (Mimetic of Replication-Competent Adenovirus Contaminants) Spiked in Different Batches of E1B-Deleted Oncolytic Adenovirus Shows the Robustness of the Method

To test the robustness of the assay and evaluate whether background adenoviral genome DNA can affect the sensitivity, different amounts of Ad5wt (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6), serving as mimetic of RCA contaminants, were spiked into either 8.5×10^9 VP/μL of Ad5dE1B_A549 or 6.7×10^9 VP/μL of Ad5dE1B_911. Ad5wt spiked into Ad5dE1B_A549 showed a similar Ct value as Ad5wt diluted in 10 mm Tris-HCl, indicating the existence of excessive other adenoviral DNA sequences does not interfere with the detection

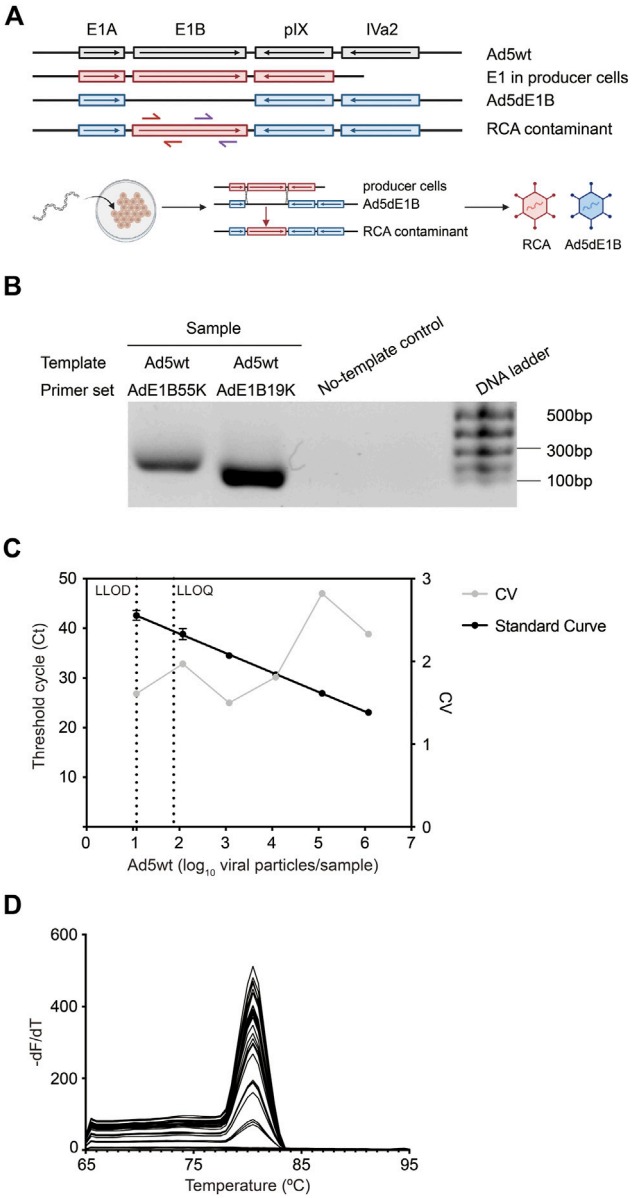
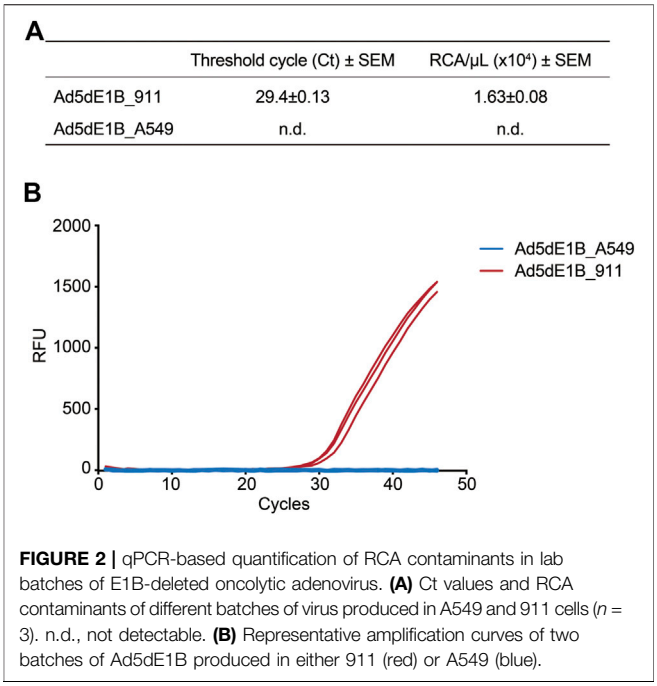


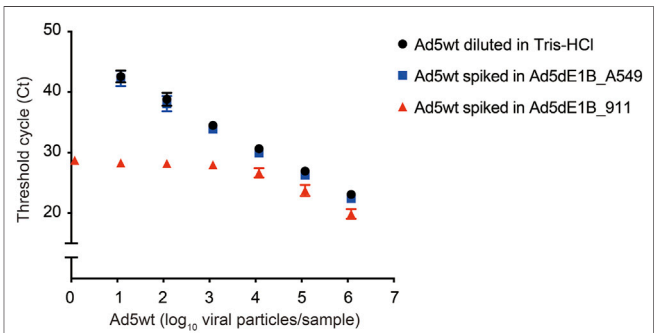
FIGURE 1 | Sensitivity and accuracy of the established qPCR. **(A)** Schematic overview of the 5′-end of adenoviral DNA templates. PCR primer sets Fwd-AdE1B19K/Rev-AdE1B19K (red arrows) and Fwd-AdE1B55K/Rev-AdE1B55K (purple arrows) are indicated at RCA contaminants. An illustration of the generation of RCA contaminants through recombination is shown in the lower panel. Ad5wt, wild-type adenovirus 5; Ad5dE1B, E1B-deleted adenovirus; RCA, replication-competent adenovirus. **(B)** Verification of the primer specificity by gel electrophoresis. Primer sets Fwd-AdE1B19K/Rev-AdE1B19K and Fwd-AdE1B55K/Rev-AdE1B55K were used to amplify Ad5wt and the amplicons were resolved in DNA gel electrophoresis. **(C)** Representative standard curve for the detection assay, showing the mean Ct value ± SD (n = 6) (left Y-axis) against $\log_{10}$-transformed VP of Ad5wt. The inter-assay coefficients of variability (CV, right Y-axis) are shown in gray. The average PCR efficiency of different repeats is 90.9 ± 4.8%. LLOD, the lower limit of detection. LLOQ, the lower limit of quantification. **(D)** Amplicon melting curves of final qPCR products generated using primer set Fwd-AdE1B19K/Rev-AdE1B19K in serial diluted Ad5wt samples.



A

	Threshold cycle (Ct) ± SEM	RCA/μL (x10⁴) ± SEM
Ad5dE1B_911	29.4±0.13	1.63±0.08
Ad5dE1B_A549	n.d.	n.d.

FIGURE 2 | qPCR-based quantification of RCA contaminants in lab batches of E1B-deleted oncolytic adenovirus. **(A)** Ct values and RCA contaminants of different batches of virus produced in A549 and 911 cells (n = 3). n.d., not detectable. **(B)** Representative amplification curves of two batches of Ad5dE1B produced in either 911 (red) or A549 (blue).



CV (%)	2.33	2.82	1.81	1.50	1.92	1.61
CV (%)	2.98	3.36	1.18	0.76	0.89	1.18
CV (%)	1.36	1.22	1.76	2.98	3.92	4.13

FIGURE 3 | qPCR quantification of replication-competent Ad5 spiked in E1B-deleted adenovirus produced in 911 or A549 cells. Different amounts of Ad5wt (as RCA contaminants mimetic) were spiked into E1B-deleted adenovirus produced in A549 or 911. Threshold cycle (Ct) was determined by qPCR (n = 6 for Ad5wt diluted in 10 mM Tris-HCl and spiked in Ad5dE1B_A549 samples; n = 2 for Ad5wt spiked in Ad5dE1B_911 samples; N indicates biological experiment repeats with triplicates samples in each experiment). The inter-assay coefficients of variability (CV) are indicated below.

(**Figure 3**). This warrants primer specificity. On the contrary, in the Ad5dE1B_911 spiked sample, we observed stabilization of the Ct values when the spiked Ad5wt was below 10^4 VP (**Figure 3**), further confirming the presence of RCA contaminants in the virus batch produced in 911 cells (**Figures 2A,B**).

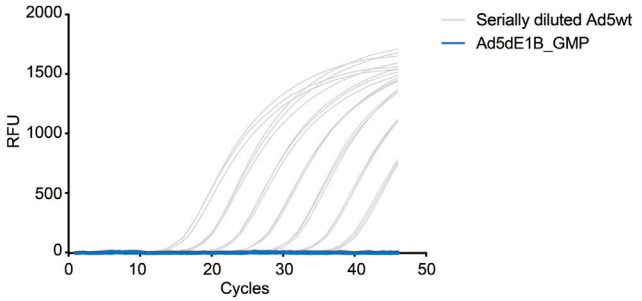


FIGURE 4 | qPCR-based quantification of RCA contaminants in GMP-grade E1B-deleted oncolytic adenovirus. Representative amplification curves of serially diluted Ad5wt in 10 mM Tris-HCl for the standard curve (gray) and a batch of GMP-grade E1B-deleted oncolytic adenovirus (blue) produced in A549 cells (5.17×10^9 VP/μL). The experiments were performed independently by two operators with three repeats on different days. Triplicate samples were used in each test.

Detection of Replication-Competent Adenovirus Contaminants Present in a GMP-Grade Batch of E1B Deletion Oncolytic Adenovirus

Based on the previous evaluation, the method was proven accurate and reliable in detecting RCA contaminants in highly concentrated adenoviral products. Next, we use this assay to evaluate the RCA contaminant level of an E1B-deleted adenovirus (Ad5dE1B_GMP) produced in A549 cells at the GMP facility at Baylor College of Medicine. Serially diluted Ad5wt virus was used as standard (**Figure 4**). As expected, no amplification signal could be detected in the clinical GMP-batch of the virus (**Figure 4**), confirming less than 10 VP of RCA contaminants in the oncolytic virus produced in A549 cells in 2 μL tested sample (equivalent to 1.03 VP × 10^{10} VP).

DISCUSSION

Oncolytic adenoviruses are currently developed to fight cancer due to their tumor selectivity, safety, and capability to deliver transgenes and stimulate immune responses against tumor cells (Tripodi et al., 2021). Several clinical trials evaluate oncolytic adenovirus as either a single therapy or in combination with conventional cancer therapies and immune checkpoint inhibitors (Mondal et al., 2020).

Currently, HEK293 is still the primary producer cell line for producing both recombinant adenoviral vectors and oncolytic adenoviruses. Due to the presence of E1 gene DNA in its genome, HEK293 cells are prone to generate RCA contaminants during production. Thus, quantification of the presence of RCA contaminants is critical for warranting the safety of clinical viral products. Assays based on cell culture and cytopathic effect (CPE) after viral infection have generally been used for RCA contaminants quantification for adenoviral vector products. The presence of RCA contaminants is judged manually by microscopic observation, and thus the results may not always be accurate and quantitative (Marzio et al., 2007). Moreover, researchers also showed that the cell-culture-based method could be combined with qPCR to improve detection sensitivity (Ishii-Watabe et al., 2003; Schalk et al., 2007). However, the oncolytic adenovirus presents an additional challenge as its proliferation capacity is retained and cannot be distinguished using a cell-culture-based CPE assay. Therefore, it is emerging to develop an accurate and sensitive assay to detect and quantify RCA contaminants for oncolytic adenoviruses.

Since E1B-deleted oncolytic adenoviruses are evaluated in several clinical trials (Habib et al., 2002; Mulvihill et al., 2001; Lu et al., 2004) (Mulvihill et al., 2001; Habib et al., 2002; Lu et al., 2004), our qPCR-based assay takes advantage of the sequence difference between the actual virus product and RCA contaminants. This difference allows us to design premiers targeting E1B specifically (**Figures 1A,B**) and thus distinguish between RCA contaminants and the E1B-deleted oncolytic virus, which is fundamental in the assay design. Based on the sensitivity of PCR, we achieved LLOD of 10 VP of RCA contaminants presented in each reaction.

When developing an assay for RCA contaminants quantification, one challenge is detecting a very low number of RCA contaminants among high concentrated viral particles. Therefore, we also validate our method using wild-type virus spiked samples to mimic this scenario, aiming to examine the specificity and sensitivity. Encouragingly, the regression curves show no difference between Ad5wt diluted in 10 mM Tris-HCl and Ad5wt spiked in Ad5dE1B_A549, indicating the method's robustness and affirming that the method can be applied to clinical oncolytic adenoviral products. The signal detected in Ad5dE1B_911 further confirmed the presence of RCA contaminants in the virus batch produced in the 911 cell line.

Others have also reported methods for detecting and quantifying RCA contaminants in oncolytic viruses by performing differential amplification steps (Walker et al., 2003), wherein the test virus was passaged sequentially on a normal human fibroblast cell line. RCA contaminants are then determined by combinational assessment of the cytopathic effects, total viral productivity, increasing potency of killing normal cells, and restriction endonuclease digest analysis of aberrant vector genome structure. This method can be reliable and objective, but the whole procedure is quite complex and time-consuming, which might involve human errors. Our method is a one-step qPCR-based method, which is sensitive, accurate, and robust. Similar methods can be developed specifically for each different oncolytic virus product by switching the specific corresponding primer sets. The method can also be applied to the detection of RCA events in patients treated with E1B-deleted oncolytic virus as, in the case of infection, wild-type viral genome present in the patients could lead to the generation of RCA

contaminants *via* recombination. In this case, primer sets should be optimized and designed to distinguish the RCA events from the wild-type viruses.

Conclusively, we report a time-saving qPCR-based method specifically for quantifying RCA contaminants from conditionally replicating oncolytic adenovirus, with high sensitivity and robustness.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MG, EY, CJ, and DY conceived and designed the experiments. MG and EY performed the experiments and analyzed the data. All authors wrote the paper together and approved the final version.

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BRIEF RESEARCH REPORT
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Inconsistencies in Modeling the Efficacy of the Oncolytic Virus HSV1716 Reveal Potential Predictive Biomarkers for Tolerability

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Treatment with HSV1716 *via* intralesional administration has proven successful for melanoma patients with the hope that oncolytic virotherapy would become another weapon in the systemic anticancer therapy (SACT) arsenal. In addition to challenges surrounding the systemic delivery of oncolytic viruses (OVs), problems associated with its *in vivo* modeling have resulted in low predictive power, contributing to the observed disappointing clinical efficacy. As OV's efficacy is elicited through interaction with the immune system, syngeneic orthotopic mouse models offer the opportunity to study these with high reproducibility and at a lower cost; however, inbred animals display specific immune characteristics which may confound results. The systemic delivery of HSV1716 was, therefore, assessed in multiple murine models of breast cancer. Tolerability to the virus was strain-dependent with C57/Bl6, the most tolerant and Balb/c experiencing lethal side effects, when delivered intravenously. Maximum tolerated doses were not enough to demonstrate efficacy against tumor growth rates or survival of Balb/c and FVB mouse models; therefore; the most susceptible strain (Balb/c mice) was treated with immunomodulators prior to virus administration in an attempt to reduce side effects. These studies demonstrate the number of variables to consider when modeling the efficacy of OVs and the complexities involved in their interpretation for translational purposes. By reporting these observations, we have potentially revealed a role for T-cell helper polarization in viral tolerability. Importantly, these findings were translated to human studies, whereby a Th1 cytokine profile was expressed in pleural effusions of patients that responded to HSV1716 treatment for malignant pleural mesothelioma with minimal side effects, warranting further investigation as a biomarker for predictive response.

Keywords: oncolytic virotherapy, preclinical modeling, T helper cells, tolerability, biomarker

INTRODUCTION

The advent of immunotherapies combined with or without chemotherapy has become an alternative first-line or subsequent treatment for several cancers (Herbst et al., 2013; Powles et al., 2014). In contrast, chemotherapies pose the risk of resistance mechanisms, destruction of healthy tissue, and unwanted side effects; immunotherapies [e.g., immune checkpoint inhibitors and oncolytic viruses (OVs)] represent attractive alternative therapies that utilize the body's own immune system to attack cancer cells, thereby leaving healthy tissues/organs unharmed. Indeed, investment in immunotherapies, as a leading treatment modality, is evidenced by over 70 immunotherapy drugs in the clinical pipeline and more than 1,000 clinical trials underway across the United States.

OVs are particularly promising for solid malignancies including breast cancer that are intrinsically resistant to other immunotherapies due to their highly immunosuppressive tumor microenvironment (TME) exhibited by decreased mutational load and neoantigen expression (Galon and Bruni, 2019). Reprogramming of the TME by OVs stimulate antitumor responses with efficacy demonstrated in a number of preclinical and early-phase clinical studies including breast cancer (Andtbacka et al., 2015; Bourgeois-Daigneault et al., 2018; Samson et al., 2018). Even though OVs constitute a wide range of viruses, Herpes simplex type-1 virus (HSV-1) is particularly attractive due to its well-characterized pathogenesis of natural infection and clinically proven antivirals, providing a "safety net" to clinical toxicity. HSV-1 in comparison to HSV-2 has also shown significantly higher levels of danger-associated molecular patterns (DAMPs) attributed to coordinating a $CD8^+$ T-cell response (Workenhe et al., 2014) which is thought to be critical for the control of tumor growth (Fridman et al., 2012). HSV1716 is a conditionally replication-competent virus derived from HSV-1 strain 17 that fails to replicate in normal non-cancer cells due to a deletion in the RL1 genes encoding ICP34.5. The first FDA-approved OV for melanoma (Johnson et al., 2015) has, since, demonstrated minimal systemic toxicity in over 100 phase I/II trials for patients with solid malignancies (Mace et al., 2008; Andtbacka et al., 2016; Streby et al., 2017); however, its early promising success has stalled as investigators attempt to reconcile the heterogeneity in the clinical response against both solid and disseminated tumors (Kaufman et al., 2022). Whilst, they are a miracle for some; they fail to work for all patients with overall response rates between 15% and 20% (Macedo et al., 2020).

This heterogeneity not only depends on whether a high enough concentration has been delivered to the target cells [which presents another set of challenges reviewed here (Howard and Muthana, 2020)] but also on a number of factors that influences viral infection; and therefore, OV-mediated antitumor therapeutic responses including; 1) the type of virus used and pre-existing immunity (Chen et al., 2000; Ricca et al., 2018); 2) the type of cancer being targeted (their immune phenotype and genomic mutation profile) (Maleki Vareki, 2018; Bonaventura et al., 2019); and 3) metabolic, nutritional, and microbiome status (Harper et al., 2020; Sumbria et al., 2020). Preclinical modeling of this milieu of interactions is crucial if we are to see OVs reach their full potential, yet immune-oncology modeling is arguably the most challenging problem translational scientist's face (Bareham et al., 2021).

Preclinical assessments for the therapeutic potential of oncolytic herpesviruses are heavily reliant on immunocompromised mouse models (Speranza et al., 2016). Xenograft models involving immunocompromised mice–bearing human tumor cell lines or whole tissue [patient-derived xenograft (PDX) models] offer high reproducibility and improved preservation of the biological and histopathological features of the original tumors, respectively (Derose et al., 2011). However, the former has demonstrated poor correlation with clinical results (Kerbel, 2003) [particularly subcutaneous models that are not orthotopic (Killion et al., 1998)] due to the differences between human and mouse biology (Mestas and Hughes, 2004), and the latter is costly due to low engraftment success rates and long establishment times. Additionally, lymphocyte-mediated responses to the tumor will be lost when using immunocompromised mice, whereby nude mice lose certain T-cell responses and SCID mice lose both their T- and B-cell responses (Belizário, 2009). To overcome this, humanized PDX models have been utilized to model the efficacy of CAR-T therapies by co-engraftment of a human fetal thymus to mimic a human functional immune system (Mhaidly and Verhoeyen, 2020); however; these are highly complex and expensive.

Syngeneic immunocompetent models allow for low-cost longitudinal study of the paradoxical role of immune cells in both tumor progression and elimination as well as safety and toxicity of OVs, but the mouse strain in both syngeneic and xenograft models will contribute to the immunophenotype and hence response to OV treatment. A summary of immunocompetent models for the study of oncolytic herpesviruses by Speranza et al. (2016) demonstrates the range of responses seen with efficacy predominantly relying on either intratumoral inoculation or combination therapy in comparison with immunocompromised studies. The validation of results in multiple models is often regarded as the best practice but inconsistencies between models as described can hinder the interpretation of clinically relevant data versus technical artifacts. Here, we present a series of conflicting interventional efficacy studies using HSV1716 for the treatment of breast cancer in syngeneic orthotopic mouse models. These immunocompetent models are required to understand the mechanistic biology of OVs, but in an attempt to recapitulate our previous success (Howard et al., 2022), we have uncovered valuable determinants of viral toxicity, and the heterogenic immune responses seen.

MATERIALS AND METHODS

Ethics Statement

Animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 with approval from the

UK Home Office approval (PP1099883), the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines, and the University of Sheffield Animal Welfare Ethical Review Body (AWERB).

Cell Lines

Mouse mammary cancer cells EO771 (obtained from Dr. Jessalyin Ubellacker, Harvard University, United States), 4T1-Luc-BR (obtained from Prof. Sanjay Srivastava, University of Texas, United States), and PyMT-TS1 (American Type Culture Collection, ATCC) were cultured in a DMEM growth medium supplemented with 10% v/v fetal bovine serum (FBS, Gibco, Invitrogen, Paisley, United Kingdom), in a humidified incubator under 5% v/v CO_2 conditions. E0771 and 4T1 cells were stably transfected to express luciferase cultured in DMEM +10% FCS (Gibco, Invitrogen, Paisley, United Kingdom). The identities of all cell lines were regularly confirmed using microsatellite analysis and were tested to be free of mycoplasma.

Viruses

HSV1716 and GFP expressing HSV1716 were obtained from Virtuu Biologics Ltd. in stocks of 1×10^8 particle-forming units (PFU) in compound sodium lactate (Hartmann's solution) with 10% v/v glycerol. HSV1716 is derived from HSV strain 17+ with deletions of both copies of the RL1 gene encoding for the neurovirulence factor ICP34.5 (HSV1716). HSV1716-GFP has a green fluorescent protein (GFP) added to the RL1 gene locus and is driven by the phosphoglycerate kinase (PGK) promoter (Conner et al., 2008). All vials were stored at −80°C and freshly thawed on ice in 0.1 ml aliquots immediately before each experiment.

In Vivo Studies

Female C57Bl/6, FVB, or Balb/c mice were obtained from Charles River Laboratory (Kent, United Kingdom) at 6–8 weeks and acclimatized in the Biological Services Laboratory for 7 days prior to experimentation. The animals were maintained on a 12:12 h light/dark cycle with free access to food and water. The animals were anesthetized using 3%–4% v/v isoflurane in 70%: 30% v/v N_2O:O_2.

Experimental Design

For tumor growth in female immunocompetent mice (n = 3–9/group), 3×10^5 mLUC-E0771, 3×10^5 PyMT-TS1, and 1×10^5 mLUC-4T1 cells were injected into the inguinal mammary fat pads of C57Bl/6, FVB ,and Balb/c mice, respectively, in 50% matrigel: 50% PBS. Mammary tumor growth was assessed by digital caliper measurement every 2–3 days, and when tumors reached ~100 mm^3, mice were randomly divided into groups and treated with either PBS or HSV1716 (concentration range 1×10^5–1×10^7 PFU/mouse). Further experimental details pertaining to each model are described as schematics in the appropriate figures. Of note, the animals implanted with luciferase-expressing cell lines were imaged using a luminescence *in vivo* imaging system (IVIS Lumina II imaging, Caliper Life Sciences) following the intraperitoneal injection of D-luciferin (150 mg/kg, Invitrogen). This was to track any metastatic burden. The assessment of the condition of mice following OV administration was attributed to the following health score. A score of five indicated a healthy mouse. A point was deducted for displaying each of the following symptoms: pallor, respiratory distress, piloerection, reduced mobility, and swelling.

Clinical Chemistry

The systemic toxicity of the virus was assessed in plasma samples using a Roche Cobas 8000 analyzer at the Department of Clinical Chemistry, Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Trust. Alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were measured as increases in the concentrations of these liver function tests indicating liver or muscle damage. We also measured intracellular fluids including potassium, phosphate, and uric acid which are associated with the rapid release and metabolism of intracellular nucleic acids as a marker of tumor lysis syndrome.

Tissue Analysis

Tissue (tumors, spleen, and liver) was harvested from mice after being killed with half of the tissue being embedded in an OCT freezing medium, and half was snap-frozen for flow cytometry. Immunofluorescence of tumors was carried out on 4-μm tumor cryosections. The sections were blocked with 1% w/v BSA and 5% v/v goat serum for 30 min and incubated, at room temperature, with primary conjugated antibodies against CD3 (1:200 dilution, BD Pharmingen), CD4 (1:50 dilution, BioLegend), CD8 (1:100 dilution, BioLegend), F4/80 (1:100 dilution, BioLegend), and GFP (1:100 dilution). After 1-h, the sections were counterstained with 50 ng/ml DAPI solution and mounted with ProLong™ Antifade (Thermo Fisher Scientific). Images were captured with a Life Technologies EVOS FL Auto at ×20 magnification with DAPI, GFP, RFP, and Cy5 light cubes. Five fields were captured per slide, and the number of positive cells was expressed as an average per field of view.

Cytokine Bead Array

Serum samples and tumor tissue lysates underwent cytokine bead array (CBA) analysis to assess the expression levels of a series of cytokines. Mouse flex sets were obtained from BD Biosciences and included IL-4, IL-12, IFN-Y, TNF, and GM-CSF. Each BD™ CBA Flex Set contained two vials of standard and one vial each of capture bead and PE detection reagent. The formulization of the capture bead and PE detection reagent components was carried out to a 50× concentration to confirm product performance when multiplexed. An Attune autosampler was used to read the samples.

Flow Cytometry

In brief, tumors, spleens, and livers were dispersed by enzymatic digestion after first dicing into pieces approximately 1 mm^3. Tissue pieces were incubated for 30 min at 37°C in serum-free IMDM (VWR International, PA, United States) supplemented with 2 mg/ml dispase, 0.2 mg/ml collagenase IV (Sigma Aldrich, St. Louis, MO, United States), and 100 U/ml DNase (Merck Millipore,

Burlington, MA, United States). Dispersed tissues were passed through 70-μm nylon filters (Becton Dickinson, Franklin Lakes, NJ, United States), permeabilized *via* the FOXP3 Fixation/Permeabilization kit (eBioscience), and analyzed for the expression of different markers: pro-inflammatory monocytes ($CD14^+/CD16^+$), immunosuppressive monocytes ($CD14^+/CD163^+$), T_{Helper} ($CD3^+/CD4^+$), T_{Reg} ($CD3^+/CD4^+/FOXP3^+$), and cytotoxic T cells ($CD3^+/CD8^+$). All antibodies were sourced from BioLegend and used at a concentration of 2 μl per test. The membrane-impermeant, fixable, amine-reactive dye Zombie UV™ Fixable (BioLegend) was used to discriminate between live and dead cells. Flow cytometry was performed using an LSRII flow cytometer (BD Biosciences), and data were analyzed by FlowJo software.

Human Pleural Effusion Samples

Human samples were obtained from a phase I/IIa trial of intrapleural administration of HSV1716 for the treatment of mesothelioma (NCT01721018). The participants and study design are published in Danson et al. (2020). The samples from patients (n = 4) given four doses of HSV1716 were chosen to reflect the multiple dosing performed in the murine studies. The cell populations from pleural effusions were analyzed by flow cytometry using the same markers, as described earlier but with antihuman antibodies (all antibodies were sourced from BioLegend and used at a concentration of 2 μl per test). The cell viability of 2/4 samples was significantly affected by long-term storage; therefore flow cytometry data represent n = 2. The following NanoString nCounter™ gene expression analysis was performed with data from two samples described and reported. Amplification-free gene expression profiling of pleural effusions using a NanoString nCounter™ FLEX platform and the nCounter™ PanCancer Immune Profiling Panel, which consist of 750 immune-related genes and 20 housekeeping genes (NanoString Technologies Inc.), was undertaken. For this, total mRNA was extracted using the RNeasy™ Mini Kit (QIAGEN) and quality controlled using a NanoDrop™ 8000 spectrophotometer. For gene expression profiling, 150 ng of total RNA from each sample was used for NanoString probe hybridization which was undertaken overnight (20 h) at 65°C in a PCR machine with a heated lid [each reaction mixture contains 5 μl of RNA solution (150 ng), 8 μl of reporter probe, and 2 μl of capture probe]. After overnight hybridization, excess probes were removed using the NanoString nCounter™ Prep Station and magnetic beads; the hybridized mRNA/probe was immobilized on a streptavidin-coated cartridge. The processed cartridge was subsequently scanned, and raw data were generated at high-resolution (555 fields of view, fov) using a NanoString nCounter™ digital analyzer platform and processed using nSolver™ data analysis software (V.4.0). Imaging quality control (QC), mRNA positive control QC, and normalization QC were checked, and all the samples were in line with the quality parameters of NanoString gene expression assays. Differential expression was performed using the nSolver™ Advanced Analysis Module v.2.0.115. Data normalization was performed using the geNorm algorithm for the selection of the best housekeeping genes.

Statistical Analysis

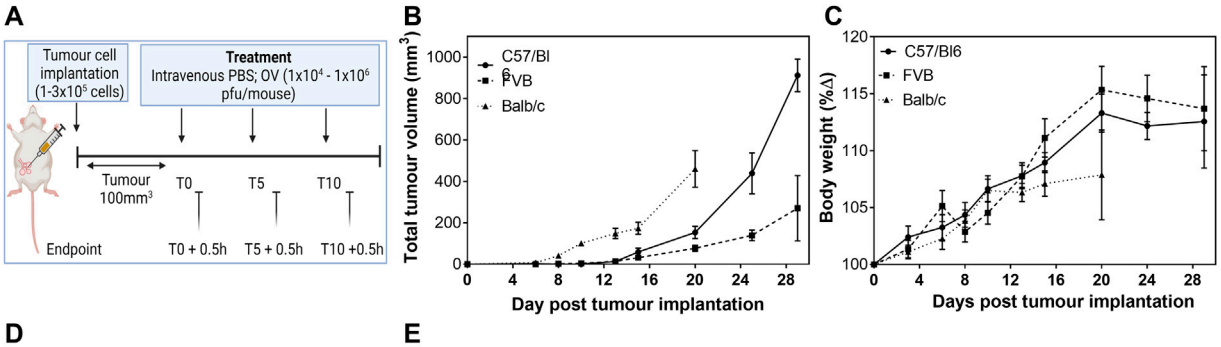
Group-wise comparisons were carried out using one-way independent ANOVA with Tukey's multiple comparison test (unless otherwise stated in the figure legends) by GraphPad Prism software version 9.0. Data are expressed as means ± SD, and statistical significance was defined as $p \leq 0.05$.

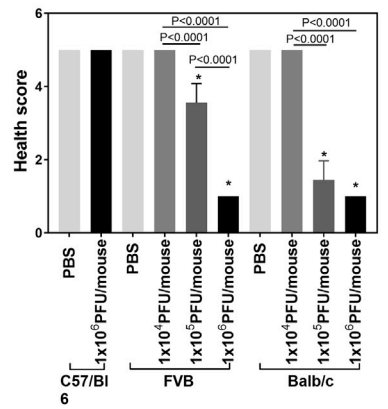
RESULTS

Tolerability to Oncolytic Viruses is Dependent on the Strain of the Mouse Model

We have recently demonstrated that magnetization of the oncolytic virus HSV1716 enhances tumor targeting resulting in increased tumor elimination and a 50% survival advantage in a C57/Bl6 model of E0771 TNBC (Howard et al., 2022), as well as the ability to steer magnetic macrophages *via* magnetic resonance imaging (Muthana et al., 2015). Whilst these studies overcome some of the limitations associated with systemic delivery of OVs, we have uncovered some interesting differences in response when repeated in other syngeneic, orthotopic models of triple-negative breast cancer (TNBC). The treatment protocol from Howard et al. (2022) was replicated in a Balb/c mouse model using 4T1-Luc-BR cells (**Supplementary Figure S1A**). Tumor growth was rapid as detected by IVIS imaging of luciferase-expressing 4T1 cells (**Supplementary Figure S1B**) and caliper measurements (**Supplementary Figure S1C**). At a mean tumor volume of 100 mm^3 (day 7 post-implantation), mice received three doses of HSV1716 (1×10^6 pfu/mouse) by intravenous injection 5 days apart. In stark comparison to C57/Bl6 tumor-bearing mice in our previous study, Balb/c demonstrated significant tolerability issues at identical concentrations of HSV1716. This manifested as subacute (approx. 20 min post-administration) respiratory distress, pallor, and reduced activity, resulting in their cull. The mice which did not reach their severity limit were administered with log lower concentrations of HSV1716, but ultimately their health deteriorated above untreated controls by day 10 (**Supplementary Figure S1D**). From day 20 post-implantation, the body weight of control mice started to decrease (**Supplementary Figure S1E**), and upon post-mortem, it was noted that primary tumors had invaded the body cavity, demonstrating the aggressiveness of this model. No metastases were evident by IVIS.

Due to the striking difference in response to the virus between the two syngeneic mouse models we undertook a tolerability study using inbred mouse strains well known for their immunological characteristics related to cell-mediated immunity. C57/Bl6 mice (implanted with E0771 cells) and Balb/c mice (implanted with 4T1 cells) display prototypical T-cell subset polarizations with C57/Bl6 mice showing predominant Th1-like immune responses and Balb/c mice





E

Strain	Treatment (pfu/mouse)	Adverse effects (AE)	Outcome
Balb/c	$1x10^6$	Severe; Hunched, pallor, swollen, piloerection, reduced activity, decreased respiration	Euthanised immediately.
	$1x10^5$	Severe; Hunched, pallor, swollen, reduced activity	Euthanised immediately.
	$1x10^4$	None	Tolerated.
FVB	$1x10^6$	Severe; Hunched, pallor, swollen, piloerection, reduced activity	Euthanised immediately.
	$1x10^5$	Moderate; Piloerection, reduced activity	Recovery ~20 minutes.
	$1x10^4$	None	Tolerated.
C57/Bl6	$1x10^6$	None	Tolerated.

FIGURE 1 | Maximum tolerated dose of HSV1716 is mouse strain-dependent. C57/Bl6, FVB, and Balb/c mice were implanted with mLuc-E0771, PyMT-TS1, and mLuc-4T1-Br breast cancer cells, respectively [**(A)**, created using BioRender], and received a range of OV treatment (1×10^4–1×10^6 pfu/mouse) once average tumor volume had reached 100 mm^3 to determine the maximum tolerated dose (MTD) **(B)**. Mice receiving their MTD were culled 30 min post-treatment at each timepoint for analysis ($n = 3$/group per timepoint). Health was monitored by measuring body weight **(C)**, and a final health score was calculated **(D)** using severity and duration of adverse effects seen during 30 min observation window **(E)**. Data are shown as mean ± SD. Statistical significance was determined by one-way ANOVA where * = $p <$ 0.05 versus PBS group.

predominant Th2 responses (Pinchuk and Filipov, 2008; Radaelli et al., 2018). FVB mice (implanted with PyMT-TS1 cells) represent a balanced profile. A treatment regime of three intravenous injections of HSV1716 at concentrations ranging 1×10^4–1×10^6 pfu/ml was performed once tumors had reached an average volume of 100 mm^3 (**Figure 1A**), although due to the difference in tumor growth rate (**Figure 1B**) the date was determined on a strain by strain basis. Prior to the treatment, body weight measurements suggest that all mice were in comparably good health despite the more aggressive growth of 4T1 tumors (**Figure 1C**). Following the treatment, the animals were monitored for adverse effects, and cohorts were culled 30 min post-administration as the timepoint at which previous studies succumbed to treatment side effects. E0771-bearing C57/Bl6 mice were unaffected by the highest concentration of virus used in this study (1×10^6 PFU/mouse). This is congruent with our previously published studies (Howard et al., 2022); therefore, lower doses in this mouse strain were not tested. The spleens of all tumor-bearing mice were noticeably larger than those of normal mice, regardless of the strain. In both Balb/c and FVB mice, a linear decline in their health score (**Figure 1D**) was seen in relation to increasing concentrations of virus ($p < 0.0001$). Adverse events included decreased respiration, pallor, piloerection, and reduced activity (**Figure 1E**). There was a log difference in the maximum tolerated dose by C57/Bl6, FVB, and Balb/c mice of 1×10^6 pfu/mouse, 1×10^5 pfu/mouse, and 1×10^4 pfu/mouse, respectively.

The subacute timing of the effects observed together with their anaphylactic-type presentation suggests that this could not be attributed to the preparation of the virus itself but in reaction to the stimulation of immune pathways generating a cytokine storm. A clinical chemistry panel was, therefore, performed to assess the classical biochemical features of lysis of tumor cells. The analysis of plasma was hindered by hemolysis and limited signal detection most likely due to difficulty sampling sick mice and timing of collection (0.5 h post- viral administration), respectively. We attempted to measure tumor lysis syndrome from tumor lysates (**Supplementary Figure S2**), and whilst data demonstrated differences between the strains of mice (alkaline phosphatase concentration in particular), there was no evidence of cell lysis at this early timepoint despite the presence of HSV1716+ cells in tumor tissue sections (**Figures 2A,B**). The timing of sampling may be responsible for the lack of changes in the clinical chemistry although this was deemed to be the most appropriate timepoint at which recoverable animals displayed the severest symptoms. Using immunofluorescence, immune populations were characterized to assess the T-helper status within tumors. $CD3^+$ T cells were present in tumors of C57/

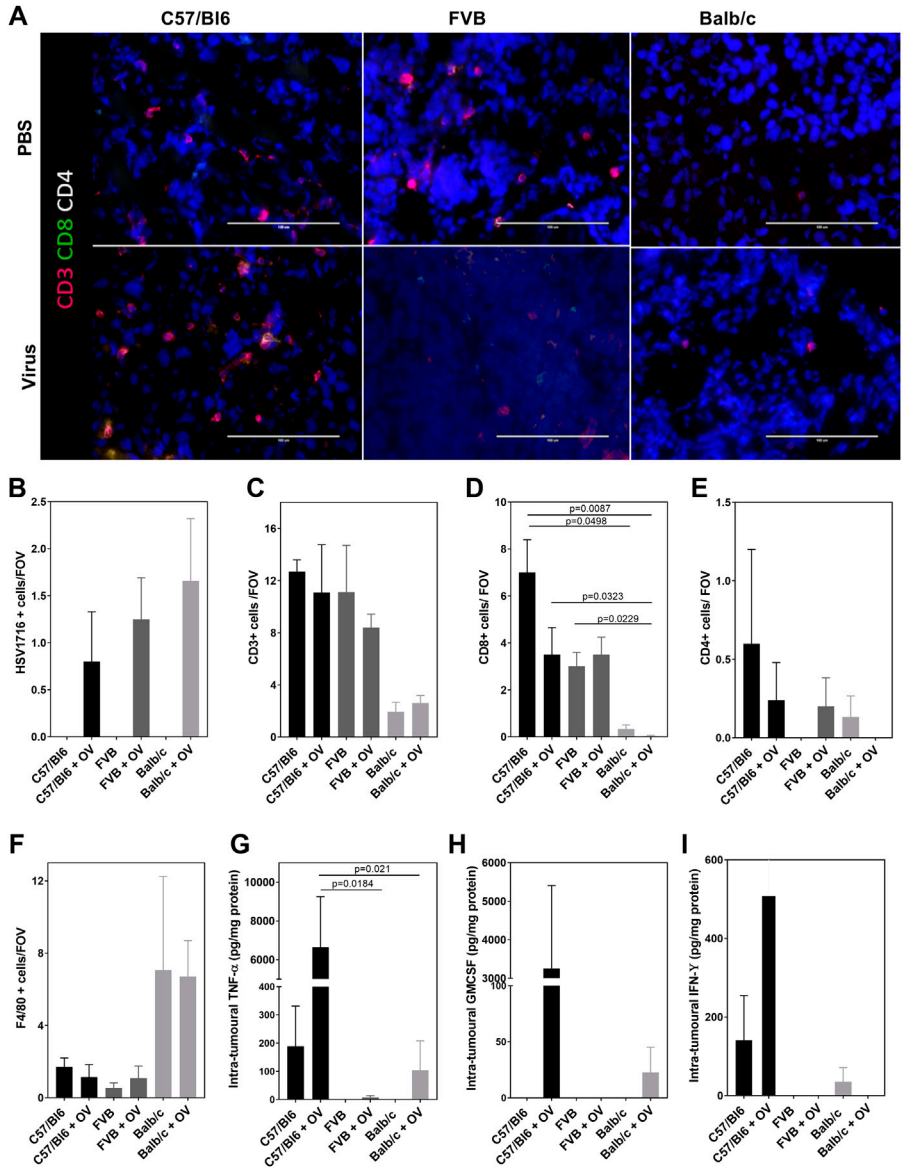


FIGURE 2 | Model dependent T-cell activation. Representative images of tumor sections examined by terminal immunofluorescence staining **(A)** and their quantification of signals for HSV1716 + cells **(B),** $CD3^+$ T cells **(C)**, $CD8^+$ T cells **(D)**, $CD4^+$ T cells **(E)**, and F4/80 + macrophages **(F)**. Intratumoral concentrations of TNF-α **(G)**, GM-CSF **(H)**, and IFN-ϒ **(I)** were detectable using the CBA assay. Data are shown as mean ± SD. Statistical significance was determined by one-way ANOVA with a Tukey *post hoc* test.

Bl6 and FVB mice in comparison to Balb/c mice (**Figures 2A,C**). Additionally, these cells displayed a higher proportion of CD8 staining over CD4 (**Figures 2A,D,E**) with both markers demonstrating a decreasing trend with polarization toward a Th2 phenotype. The pattern of CD8 T-cell activation in tumors of C57/Bl6 mice was substantiated by the presence of intratumoral cytokines known to mediate their effector functions with an increase in GM-CSF, IFN-Y, and TNF-α (**Figures 2A,F–H**) over other mouse strains. Macrophages were the dominant immune cell present in the tumors of Balb/c mice in the notable absence of T-cell activation (**Figures 2A,I**).

With the maximum tolerated doses per mouse strain determined, we repeated the efficacy study in PyMT-TS1-bearing FVB mice. A treatment protocol identical to that described in **Supplementary Figure S1** was performed (**Supplementary Figure S3A**), and survival was monitored (**Supplementary Figure S3B**). Although treatment was tolerated by the mice both immediately post-administration at the reduced concentration and throughout the duration of the study, as determined by body weight (**Supplementary Figure S3C**), there was no effect on tumor progression (**Supplementary Figure S3D**) or survival benefits in comparison to PBS-treated controls. This suggests that virotherapy

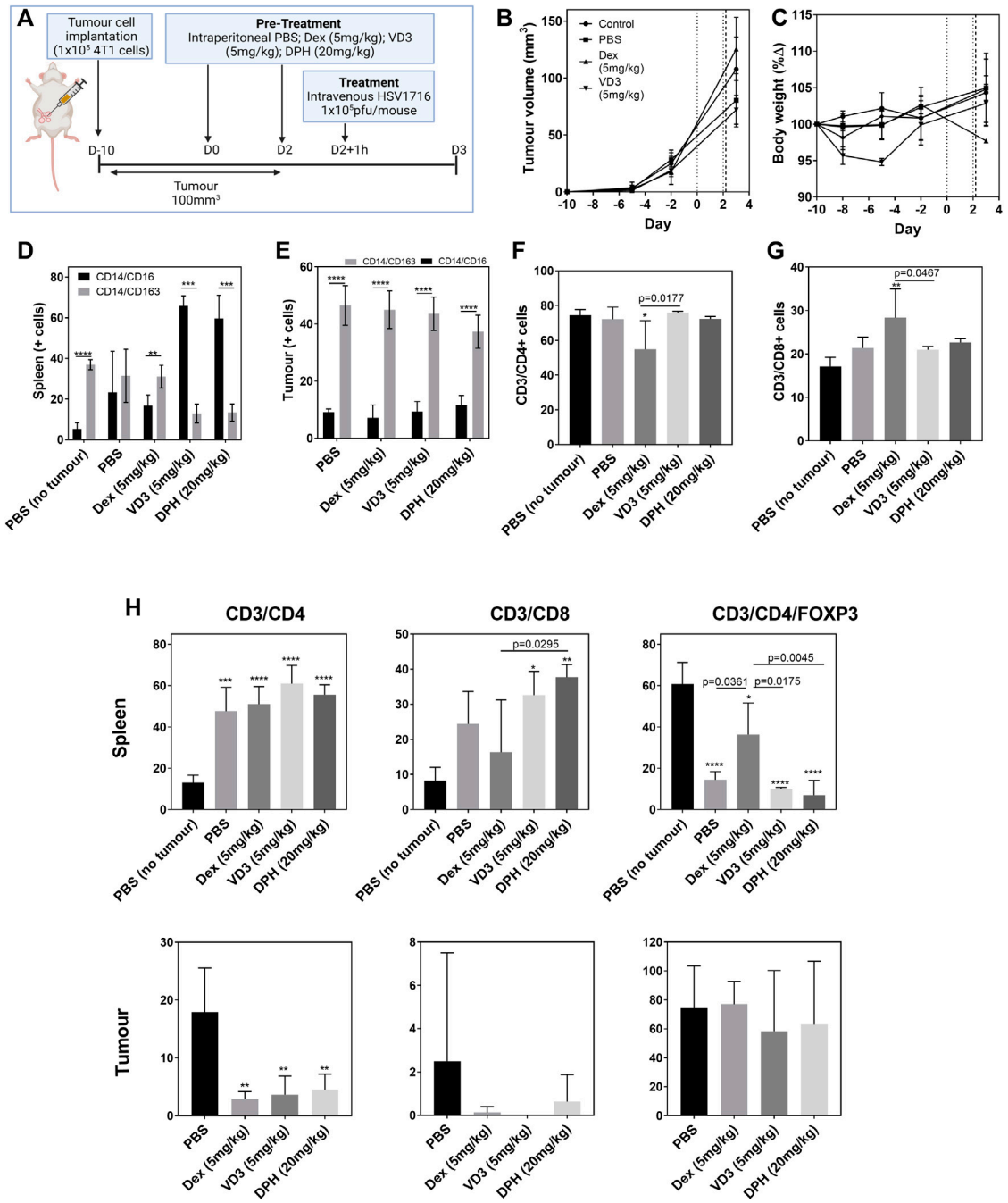


FIGURE 3 | Prophylactic immunomodulation to enhance OV efficacy. Tumor-bearing Balb/c mice (n = 4/group) were pre-treated intraperitoneally with different immunomodulators (vertical dotted lines) prior to intravenous OV administration (vertical dashed line) [**(A)**, created using BioRender] in an effort to alter the immune microenvironment for enhanced efficacy and tolerability. Tumor volume **(B)** and body weight **(C)** were measured prior to killing 24 h post OV treatment. Dissociated cell populations from spleen **(D)** and tumor **(E)** samples were analyzed by flow cytometry for pro-inflammatory ($CD14^+/CD16^+$) or immunosuppressive markers ($CD14^+/CD163^+$). Lymphocytes harvested from blood samples were positively selected for $CD4^+$ **(F)** and $CD8^+$ **(G)** T-cell markers. T-cell analysis from cell populations within spleen and tumor samples was also quantified **(H)**. Data are shown as mean ± SD. Statistical significance was determined by one-way ANOVA with a Tukey *post hoc* test where * = $p < 0.05$, ** = $p < 0.001$, and **** = $p < 0.0001$ versus PBS no tumor.

requires a concentration above a threshold dose to elicit an effect (either through saturation of the body with high concentrations or by more targeted delivery).

Modification of the Immune Phenotype

In an effort to treat our mouse models with concentrations of virus above a threshold dose while avoiding side effects, we

attempted to modify the immune environment prior to the administration by pre-treatment with a number of drugs. Vitamin D3 (VD3) is a fat-soluble steroid predominantly known to help maintain the bone health (Chapuy et al., 1992); however, it is also thought to play a role in the adaptive immune system, particularly T-lymphocyte regulation *via* upregulation of Th2 cytokines associated with an anti-inflammatory response (Boonstra et al., 2001). The corticosteroid dexamethasone (Dex) targets inflammation and prevents extension of the cytokine storm, thus preventing the persistence and maintenance of the immune system (Elenkov, 2004). Antihistamines such as diphenhydramine (DPH) are used to inhibit histamine production through alteration to the Th1/Th2 balance in basophils and T cells by increasing the stimulation of Th1 cells and release of IL-2 and IFN-Y while inhibiting Th2 activation (Kato et al., 1999).

The Balb/c model was chosen to study the prophylactic modification of the immune profile due to the sensitivity to HSV1716 described earlier. 1×10^5 4T1 cells were allowed to grow to an average volume of 100 cm^3 prior to virus treatment. During this time, two doses of PBS, Dex (5 mg/kg), VD3 (5 mg/kg), and DPH (20 mg/kg) were administered intraperitoneally 48 h apart with the second dose 1 h prior to virus treatment (**Figures 3A,B**). OVs were tolerated at a concentration of 1×10^5 pfu/mouse by all groups, and mice were culled 24 h later in order to evaluate T helper cell activation following treatment. Our previous studies have shown that the cessation of treatment results in tumor regrowth (Howard et al., 2022); therefore, the timepoint was selected to ensure immunological changes following a single virus dose were detected. No adverse effects as a result of treatment with immunomodulators were observed, and body weights remained stable throughout the study (**Figure 3C**). The changes in clinical chemistry as an evidence of tumor lysis syndrome were undetectable in terminal serum samples (**Supplementary Figures S4A–H**).

The overall inflammatory status was measured by flow cytometry from dissociated tumor, spleen, and liver cell populations (**Figures 3D–F**). Within 4T1 tumors, the population of immunosuppressive $CD14^+CD163^+$ cells was 4-fold greater than that of pro-inflammatory $CD14^+CD16^+$ cells ($p < 0.0001$, **Figure 3D**). Pre-treatment with the immunomodulators did not change these ratios at the tumor level. The analysis of splenocytes indicates a dominant immunosuppressive population in non-tumor–bearing Balb/c mice ($p < 0.0001$, **Figure 3E**) with dexamethasone also displaying immunosuppressive properties within the spleen as expected. Interestingly, both VD3 and DPH stimulated a pro-inflammatory response with a 5.4-fold increase in $CD14^+CD16^+$ splenocytes ($p < 0.0001$). T-cell populations within these tissues were quantified from viable lymphocytes as the parent population and antibodies were used to select for $CD4^+$ T cells ($CD3^+/CD4^+$), $CD8^+$ cytotoxic T cells ($CD3^+/CD8^+$), and Tregs ($CD3^+/CD4^+/FOXP3^+$) (**Figure 3G**). $CD4^+$ T cells were the dominant population within the blood of both tumor-bearing and non-bearing mice treated with PBS (**Figure 3H**). Treatment with Dex induced a significant decrease in $CD4^+$ T cells ($p < 0.05$ vs. PBS no tumor) and a significant concomitant increase in $CD8^+$ T cells ($p < 0.01$). The presence of tumors stimulated a large $CD4^+$ T-cell response in the spleen when compared to non-tumor–bearing mice ($p < 0.0001$); this was unaltered by the use of the immunomodulators. There was also a trend for increasing the presence of $CD8^+$ T cells within the spleen in tumor-bearing mice with the administration of VD3 and DPH also contributing, resulting in a significant increase (**Figure 3H**). Therefore, 60% of $CD4^+$ T cells in spleens of untreated non-tumor–bearing mice expressed FOXP3 as a marker for Tregs ($p < 0.0001$). FOXP3 expression decreased in tumor-bearing mice demonstrating a switch to activate $CD4^+$ T cells. Treatment with Dex significantly increased the proportion of Tregs within the spleen compared to PBS-treated tumor-bearing mice ($p = 0.0361$). Within the tumor itself, the majority of $CD3^+$ cells were identified as Tregs regardless of immunomodulation. $CD4^+$ T cells were more prominent compared to $CD8^+$ T cells as we have seen previously with this model (**Figure 2**). A significant decrease was induced by all immunomodulators ($p < 0.01$) (**Figure 3H**).

Human Efficacy is Driven by Th1 Response

Inconsistencies in the efficacy data from the mouse models described make it difficult to define clinically relevant information. However, these "negative" data describe a pattern of effects that correlates with the host's immunophenotype (**Figure 4A**). Viral tolerability in our studies correlated with T helper cell polarization displayed by inbred strains of mice. C57/Bl6 mice present with a Th1 dominance allowed the tolerability of concentrations ~1×10^6 pfu/mouse safely and perhaps even higher. Conversely, 1×10^4 pfu/mouse was determined as the maximum tolerated dose (MTD) by Th2-type Balb/c mice. Interestingly, the MTD of an inbred mouse strain that represents a balanced immunophenotype (FVB) lay between its polarized counterparts at 1×10^5 pfu/mouse. We investigated the clinical relevance of this finding using pleural effusion samples from a phase I/IIa trial of intrapleural administration of HSV1716 which reported good tolerability among patients as well as an antitumor immune response (Danson et al., 2020). Here, we reported a 5–10 fold increase in IFN-ϒ, IL-2, and TNF-α cytokine levels in pleural fluid from 8/11 patients following HSV1716 treatment, representing robust Th1 responses. The differential expression of transcription factors involved in the pathways for cytokine production supports the concentrations of Th1 cytokines measured (**Figure 4B**). An increase in IL-12 signaling *via* its receptor activates Stat4, which upregulates IFN-Y transcription. IFN-Y proceeds to activate Stat1 which upregulates T-bet, further enhancing IFN-Y production. Although Th2 signaling is mediated by IL-4 receptor activating Stat6 (as seen in **Figure 4B**), the activation of Stat4, Stat1, and T-bet inhibits GATA3 required for IL-5 and IL-13 production; hence we observed a downregulation of Th2 genes compared to Th1. The differential gene expression of T-cell markers (**Figure 4C**) showed a higher proportion of $CD4^+$ over $CD8^+$ cells; and whilst overall a balanced immune response was noted (**Figure 4D**), flow cytometry analysis of T-cell populations clearly showed a dominant $CD8^+$ cell presence immediately following HSV1716 treatment which slowly decreased with

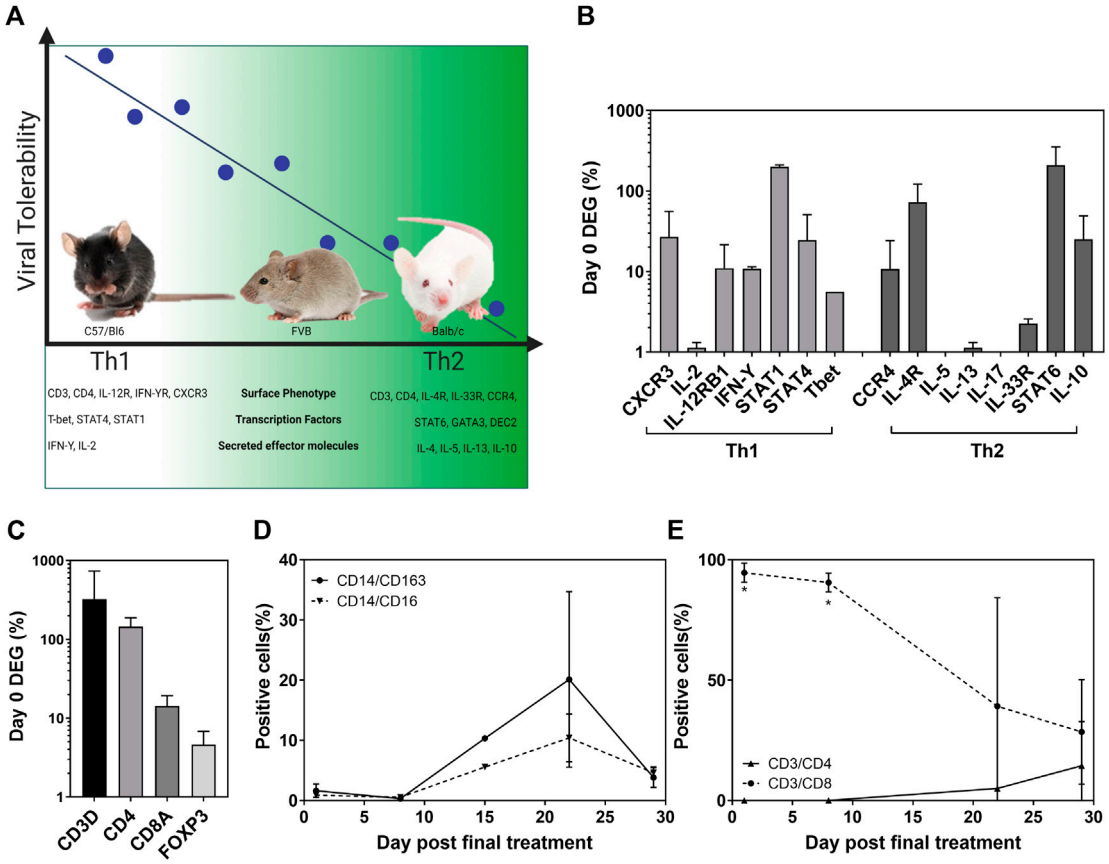


FIGURE 4 | Viral tolerability correlates with host Th bias in both mice and humans. Murine tolerability of HSV1716 correlated with T helper cell polarization associated with prototypical strains of inbred mice [**(A)**, created using BioRender]. Pleural effusion samples taken from MPM patients (n = 2), having received four doses of HSV1716, revealed a Th1-dominant cytokine pattern **(B)** and T-cell activation **(C)**, following the analysis of differentially expressed genes (DEG) by NanoString. Flow cytometry of cell populations demonstrated an overall balanced immune system in the weeks following treatment **(D)** with a significant $CD8^+$ T-cell–mediated response which declined over time **(E)**. Data are shown as mean ± SD. Statistical significance was determined by Students t-test where ∗ = p< 0.001.

time along with a concomitant increase in $CD4^+$ cells (**Figure 4E**). Despite the small sample size, the human data correspond with our C57/Bl6 mouse model that a Th1 immunophenotype confers tolerability to viral treatment. Moreover, a robust $CD8^+$ cell response may mediate the antitumor response seen in both these studies.

DISCUSSION

These studies aimed to investigate the efficacy of HSV1716 as a SACT for triple-negative breast cancer in three different cell lines: E0771, 4T1, and PyMT-TS1. Due to the selective growth of these cells, each required a different inbred mouse strain: C57/Bl6, Balb/c, and FVB mice. We have previously demonstrated *in vitro* efficacy against all 3 cell lines as well as *in vivo* efficacy in E0771 tumor-bearing C57/Bl6 mice (Howard et al., 2022). Here, we observed severe side effects during recapitulation of this experiment in Balb/c and FVB models which limited the concentration of administrable HSV1716, resulting in poor efficacy. We have shown that the adoption of a more targeted approach for systemic delivery of OVs can increase their concentration at the target tumor through magnetic targeting (Howard et al., 2022) and cell delivery (Muthana et al., 2011; Iscaro et al., 2022) for breast and prostate cancers. Further nano-enabled formulations have shielded OVs from immunosurveillance with or without additional ligands for targeted recognition (Iscaro et al., 2019). Through these strategies, saturating concentrations of virus may be avoided and hence improve tolerability. However, in order to study the progression of metastatic breast cancer and the effects of these immunotherapies on immune cells, *in vivo* models representative of the complex interactions between the different cell types are required. Although preclinical studies have generated promising data, many have not translated to humans. Therefore, models (or combination of models) with greater predictive potential are required, together with a willingness to attempt even the most challenging models. Whilst, the two syngeneic mouse models presented here conflicts with our previous success of magnetized-HSV1716 in C57/Bl6 mice; these responses better represent the heterogeneic human population. Therefore, we sought to understand the drivers behind these reactions.

Our comparison of response to HSV1716 by different inbred mouse strains is striking in the log increments of tolerated doses correlating with host T-cell polarization across the strains. This phenomenon is not seen in non-tumor–bearing mice (unpublished data from our laboratory); therefore, the presence of the tumor must play a role in the activation of the immune system in response to the virus. The subacute appearance of adverse effects experienced by the mice supports this theory that the initiation of an antitumor immune response at the tumor site may cause an early release of neoantigens and induction of a cytokine storm. We hypothesized that how the host responds to the cytokine storm will depend on their Th status (**Figure 4A**) resulting in the stratification of symptoms seen here in the different mouse strains and doses of HSV1716. However, our attempts at validating this theory by clinical chemistry analysis were not statistically significant at either 30 min or 24 h post-treatment, tumor lysis syndrome (TLS) was also fatal in a Balb/c model of plasmacytoma following the intravenous administration of vesicular stomatitis virus (VSV) although progression was much slower with euthanasia 5–8 days post-treatment and evidence of TLS collected at day 4 (Zhang et al., 2016),demonstrating another variable to consider when designing these experiments. Another study investigating the lytic activity of VSV in a syngeneic flank model of lung cancer reported no tolerability issues following both intratumoral and intravenous treatment although, importantly, these were performed in C57/Bl6 mice (Schreiber et al., 2019).

In order to increase the dose of HSV1716 to achieve efficacy, we pre-treated Balb/c mice (i.e., the most susceptible strain) to investigate the alleviation of the tolerability issues. Subsets of patients undergoing immunotherapy have received systemic corticosteroids, vitamin D, and antihistamines either prior to the initiation of immunotherapy or throughout their treatment protocol to manage drug-induced adverse effects (Harmankaya et al., 2011; Grover et al., 2020). Indeed, prophylactic immunomodulation of our tumor-bearing mice was asymptomatic, following intravenous HSV1716. Also, following the prohibitory nature of these side effects on effectively studying HSV1716 treatment in Balb/c models, it is believed that an allergy-type reaction promotes immune evasion and resistance to immunotherapy, suggesting that this treatment is doomed to fail in these models regardless of the concentration achieved. Our data are in accord with this theory, yet rather than negatively selecting these types of models, we need to work with them as a more accurate reflection of the immune heterogeneity within humans. Additionally, while we did not measure efficacy in our immunomodulatory study, recently published articles have demonstrated that cancer patients who took antihistamines during immunotherapy treatment had significantly improved survival (Fritz et al., 2021; Li et al., 2022) and, therefore, may provide a dual purpose in our inflammatory mouse models. In mice, a contrasting impact of corticosteroids on anti-PD1 immunotherapy has been reported (Maxwell et al., 2018), and CTLA-4 blockade restored T-cell numbers exposed to dexamethasone in a model of intracranial glioma (Giles et al., 2018), highlighting the immunosuppressive properties of such immunomodulators. Again, both these studies were performed in C57/Bl6 mice.

Unfortunately, the inoculum used in the immunomodulatory study was not high enough to demonstrate any observable benefits in terms of reduction of the side effects we saw previously. As with all models of infectious organisms, reproducibility can be problematic even if inoculums are prepared from the same stock as calculations are based on titers at the point they were made and frozen. Long-term storage and freeze-thawing can impact the actual titer, but this is not known until after the infection. The lack of expected recoverable symptoms in the PBS group suggests that the titer was less than anticipated; therefore, we could not fully evaluate whether the immunomodulators enhanced tolerability to the virus. However, this study did provide evidence for the therapeutic manipulation of immune subsets to promote a more pro-inflammatory response including the enhancement of antitumor $CD8^+$ T cell levels. This is dependent on both the composition of intratumoral immune infiltrates (Fridman et al., 2012) and $CD8^+$ T-cell levels in peripheral blood (Workenhe et al., 2014) as we have shown in samples taken from our C57/Bl6 mice and could explain why this strain is preferable for modeling OVs. Importantly, dexamethasone demonstrated a significant increase in peripheral $CD8^+$ T cells, which warrants further investigation. The differences in immunological responses of inbred mouse strains allow for the assessment of responses to pathogens. Factors that determine response to OVs include the tumor microenvironment and immune tumor infiltrates, but these studies also suggest that a genetic predisposition toward a particular Th phenotype may also play a role in the systemic response to OVs. These genetically programed biases in Th1 and Th2 immune responses have been shown to modulate atherogenesis (Schulte et al., 2008). Additionally, it has been reported that natural genetic variation in Th cell bias may also precede clinical disease in humans (Olson et al., 2013). A comprehensive review of the literature provides evidence for these Th biases and how they have influenced the outcome of viral infections, including age, ethnicity, and co-morbidities. Altogether, this suggests that predisposition to a particular Th status is measurable and may indicate which patients will tolerate or even respond best to oncolytic viruses. Indeed, we have shown that HSV1716 was well tolerated by MPM patients (Danson et al., 2020) and that cytokine analysis of pleural fluid demonstrated a Th1 response in a phase I/IIa clinical trial. Although administration was *via* an intrapleural catheter and the sample size was small, these findings corroborate our mouse studies that a Th1 bias is associated with both tolerability and enhanced efficacy. This efficacy was driven by a $CD8^+$ T cell-mediated response seen here in both human and C57/Bl6 samples and thought to be critical for the control of tumor growth (Fridman et al., 2012). A study of C57BL/6 mice–bearing syngeneic GL-261 gliomas also demonstrated a survival advantage when an HSV virus–expressing mIL-12 initiated a Th1 response and $CD8^+$ cell influx compared with the parent virus (Parker et al., 2000). The modification of OVs for the co-expression of immunostimulatory transgenes is one way to skew toward a desired Th response. Here, we used immunomodulatory drugs in an effort to readdress immune homeostasis in prototypical Th2 Balb/c mice. $CD8^+$ T cells

were noticeably absent in 4T1 tumors of Balb/c mice in comparison to the other strains investigated but following VD3 and DPH administration an increase in these cells within the spleen was observed and may have eventually contributed to the intratumoral immune infiltrates had this particular study continued. Th bias may, therefore, act as a predictive biomarker for both HSV1716 patient tolerability and response. The correlation between Th polarization and tolerability may be used to stratify dose concentrations, while the identification of Th2 dominant hosts may benefit from co-administration with immunomodulators, OVs expressing Th1 transgenes, or an alternative treatment altogether. This is particularly pertinent while current immunotherapies are a miracle for some, not all patients respond and the lack of biomarkers for their identification is costly for both patients and healthcare providers.

It should be noted that while we have utilized three different inbred mouse strains in our investigations, we have only studied their response to HSV1716. If taken alone, it could be argued that FVB and Balb/c models are not appropriate for such efficacy studies or that OVs should be administered only as an intratumoral therapeutic. However, contextualizing HSV1716 response by comparison to other OVs is confounded by a lack of consensus over the experimental design of immunological studies. As stated by Hensel et al. (2019), "variations in experimental variables such as mouse strain, animal physiology, age, gender, drug combinations, time-points, dose, treatment strategies, tumor sub-types, and tumor inoculation methods can create infinite confounders that influence the immune parameters and need to be considered even for a study with a single agent." Very few studies (if any) confirm OV efficacy in different immunocompetent mouse strains, yet here, these "inconsistencies" build a bigger picture arguably more applicable to heterogenic human populations. Ultimately, embracing these struggles and reporting the spectrum of responses may be the key to improving translational oncolytic virotherapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the UK Gene Therapy Advisory Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the UK Home Office.

AUTHOR CONTRIBUTIONS

FH: conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft, and visualization. JC: resources, clinical trial conceptualization, validation, investigation, and data curation. SD: clinical trial conceptualization, methodology, formal analysis, investigation, resources, and data curation. MM: investigation, data curation, and writing—review and editing.

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REVIEW
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Targeting Triple Negative Breast Cancer With Oncolytic Adenoviruses

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Breast cancer (BC) is the most common cancer globally, accounting for 685,000 deaths in 2020. Triple-negative breast cancers (TNBC) lack oestrogen (ER) and progesterone (PR) hormone receptor expression and HER2 overexpression. TNBC represent 10–15% of all BC with high incidence in women under 50-years old that have *BRCA* mutations, and have a dismal prognosis. African American and Hispanic women are at higher risk partly due to the common occurrence of *BRCA* mutations. The standard treatment for TNBC includes surgery, radiotherapy, and chemotherapy although, resistance to all standard-of-care therapies eventually develops. It is crucial to identify and develop more efficacious therapeutics with different mechanisms of action to improve on survival in these women. Recent findings with oncolytic adenoviruses (OAds) may generate a new strategy to improve on the outcomes for women afflicted by TNBC and other types of BC. OAds are genetically engineered to selectively lyse, eliminate and recruit the host antitumour immune responses, leaving normal cells unharmed. The most common modifications are deletions in the early gene products including the E1B55 KDa protein, specific regions of the E1A protein, or insertion of tumour-specific promoters. Clinical trials using OAds for various adenocarcinomas have not yet been sufficiently evaluated in BC patients. Preclinical studies demonstrated efficacy in BC cell lines, including TNBC cells, with promising novel adenoviral mutants. Here we review the results reported for the most promising OAds in preclinical studies and clinical trials administered alone and in combination with current standard of care or with novel therapeutics. Combinations of OAds with small molecule drugs targeting the epidermal growth factor receptor (EGFR), androgen receptor (AR), and DNA damage repair by the novel PARP inhibitors are currently under investigation with reported enhanced efficacy. The combination of the PARP-inhibitor Olaparib with OAds showed an impressive anti-tumour effect. The most promising findings to date are with OAds in combination with antibodies towards the immune checkpoints or expression of cytokines from the viral backbone. Although safety and efficacy have been demonstrated in numerous clinical trials and preclinical studies with cancer-selective OAds, further developments are needed to eliminate metastatic lesions, increase immune activation and intratumoural viral spread. We discuss shortcomings of the OAds and potential solutions for improving on patient outcomes.

Keywords: tumour-selective, novel therapies, lysis, immune activation, metastatic breast cancer, OAd

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INTRODUCTION

Breast cancer (BC) is the most prevalent female cancer worldwide with over 2.1 million women diagnosed and over 620,000 BC-related deaths in 2018 (Sharma, 2021). BC is commonly divided into three groups, 1) luminal BC that express the oestrogen (ER) and progesterone (PR) receptors, 2) basal BC that overexpress human epidermal growth factor receptor 2 (HER2), and 3) basal triple-negative BC (TNBC) that does not express any of the three receptors (**Figure 1**) (Goldhirsch et al., 2011; Uscanga-Perales et al., 2019). The TNBC subtype is the most aggressive and has the poorest prognosis of all BC subtypes although, it is the least common, constituting only 10–15% of cases (Pal et al., 2014). In fact, 5% of all-cancer-related deaths are characterised as TNBC every year (Adel, 2021). Current therapeutics for BC have limited efficacy in TNBC patients since hormonal therapies have no effect and resistance to cytotoxic drugs rapidly develops.

Younger premenopausal women that have *BRCA* mutations and those of African or Hispanic descent are at the highest risk of developing TNBC (Yao et al., 2017). African-American women are twice as likely and Hispanic women are 1.3 times more likely to develop TNBC than white and non-Hispanic women, respectively (Howlader et al., 2014). The increased frequency in these ethnic groups is associated with obesity and, in the Mexican-American population particularly, there is a high prevalence of *BRCA* mutations (25%) (Kwan et al., 2009; Gaudet et al., 2011; Weitzel et al., 2013). It has been demonstrated that all women with *BRCA1* mutations have a higher risk of developing TNBC (57%) (Atchley et al., 2008). In addition to *BRCA* mutations, dysregulation of additional signalling pathways and transcription factors have been indicated to play a role in the development of TNBC (**Table 1**).

CURRENT TREATMENTS FOR TNBC

The current therapeutic approaches to treat TNBC are surgery, radiotherapy, chemotherapy, and a combination of these (Wahba and El-Hadaad, 2015). Surgery and radiotherapy are typical treatments in early stages (I-III) of the disease while chemotherapy is the choice at late-stage (IV) disease, when the cancer has already metastasised. However, aside from surgical resection of the primary tumour, current therapeutics are rarely curative and poor outcomes with rapid progression of the cancer result in high morbidity, mortality and early deaths. Therefore, novel therapeutic strategies are urgently needed (**Figure 2**). One promising strategy is the development of oncolytic adenoviruses (OAds). OAds have proven safety and efficacy in several clinical trials targeting solid cancers including BC and cancers with similar pathway alterations as seen in TNBC patients (Cody and Hurst, 2015; Nattress and Halldén, 2018; Bazan-Peregrino et al., 2021).

ONCOLYTIC ADENOVIRUSES

Human adenoviruses (HAdV) are small DNA viruses with a linear double stranded DNA (dsDNA; 35–40 kb) encapsulated by a protein coat (**Figures 3A,B**). The HAdV family is well characterised with seven serotypes and over 100 genotypes (Baker et al., 2018). In the majority of clinical trials, viruses of serotype C subtype 5 (HAdV5) have been employed due to the ease of genetic engineering of the viral genome, feasibility of large scale GMP production and proven safety in cancer patients, as well as their innate tropism for adenocarcinomas. The HAdV5 genome and its functions are well understood and can easily be engineered to replicate and kill cancer cells selectively with no toxicity to surrounding healthy tissue. Numerous OAd mutants have been engineered and evaluated in early phase clinical trials and were reported to have promising efficacy with only self-limiting side-effects. The main strategies by which HAdV5 are engineered to selectively replicate in tumour cells are: 1) deletion of genes that are necessary for viral replication in normal cells and are expendable in cancer cells with already deregulated cell cycle and signalling pathways, 2) insertion of tumour specific promoters such as hormone response elements, and 3) a combination of these approaches and/or insertion of cytotoxic transgenes to enhance anti-cancer efficacy (McConnell and Imperiale, 2004; Baker et al., 2018; Nattress and Halldén, 2018) (**Table 2**).

The first step in HAdV-infection is binding of the fibre knob to the Coxsackie and Adenovirus Receptor (CAR) on the epithelial

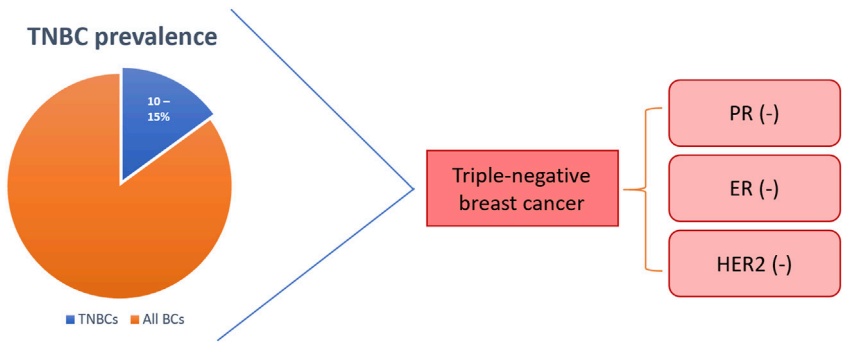

FIGURE 1 | Triple-negative breast cancer (TNBC) is hormone receptor-negative with poor or no HER2-expression. TNBC represents 10–15% of all breast cancers. BCs: breast cancers; PR: progesterone receptor; ER: oestrogen receptor; HER2: human epidermal growth factor receptor 2.

TABLE 1 | Dysregulated pathways and factors associated with TNBC.

Altered pathway/factor	Function	References
EGFR	Overexpression contributes to deregulated cell proliferation	Williams et al. (2015)
MAPK signalling pathway	Overactivation promotes uncontrolled cell proliferation and resistance to cell death	Qi et al. (2019)
PI3K/AKT/mTOR signalling pathway	Overexpression of mTOR (40–70%) and PIK3CA mutations (~22%) deregulates cancer cell proliferation	(Costa et al., 2018; Qi et al., 2019)
BRCA1 gene	Mutations render the protein defective in DNA damage repair in the majority of TNBC	Atchley et al. (2008)
Cancer-associated transcription factors (TFs)	Dysregulation of TMPRSS2, ETS, KLF4 and KLF5 promotes uncontrolled cell proliferation	Qi et al. (2019)

EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinas;, PIK3CA, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha; AKT, protein kinase B; mTOR, mammalian target of rapamycin; BRCA1, Breast cancer gene 1; TMPRSS2, transmembrane serine protease 2; ETS, E26 transformation-specific; KLF4, Krüppel-like factor 4; KLF5, Krüppel-like factor 5.

cell surface (**Figure 3C**) (Baker et al., 2018). This leads to binding of the viral penton protein to αvß3-and αvß5-integrins followed by clathrin-dependent endocytosis and transport of the viral DNA to the nucleus along microtubules (**Figure 3C**). The first gene to be expressed is E1A that is an absolute requirement for expression of other viral genes and for viral replication. E1A forces the cell into S-phase by E1ACR2-binding to pRb leading to the release of E2F followed by S-phase entry and expression of additional early viral genes (Öberg et al., 2010; Baker et al., 2018). Subsequently, the E1B55K and E1B19K genes are expressed to inhibit the G1/S checkpoint activation in order to protect the infected cell from premature apoptosis (Leitner et al., 2009; Öberg et al., 2010). The E3 genes are expressed to avoid immune-mediated cell killing and E4 proteins prevent activation of the DNA damage repair (Wang et al., 2003; Cherubini et al., 2011).

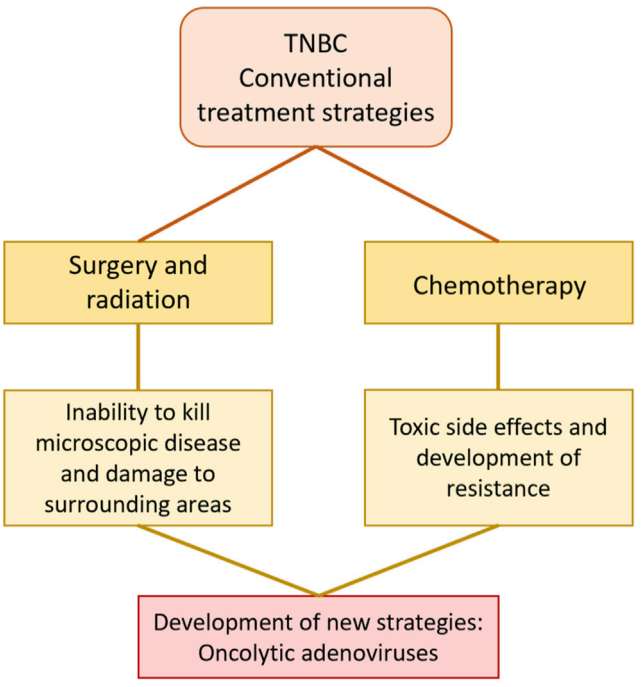

FIGURE 2 | Conventional treatment strategies for TNBC include surgery, radiation, and chemotherapy but have limited efficacy. The poor outcomes of current therapeutics highlight the need for the development of novel strategies. Oncolytic adenoviruses are one of the most promising novel therapeutics for solid cancers including TNBC.

There are a variety of underlying mechanisms, both direct and indirect, in which OAds can lead to anti-tumour efficacy in patients. Following initial infection, OAds naturally lead to oncolysis of tumour cells following sustained cancer-selective replication; this oncolytic process is vital in the replication cycle of OAds as it allows the release and spread of progeny virions to neighbouring cells throughout the tumour microenvironment (TME) (Mathis et al., 2005). Aside from direct oncolysis, OAds can also kill cancer cells *via* immunogenic cell death, which is an attractive property of OAd cancer therapy when considering the naturally immunosuppressive TME (Dunn et al., 2002). Aside from the release of virions, lysed cancer cells also release immunogenic factors including tumour-associated antigens (TAAs) and pathogen- and damage-associated molecular patterns to resident antigen presenting cells (APCs) of the TME. Following migration of APCs to draining lymph nodes and the successful cross-presentation of antigens, activation of anti-tumour immune responses secondary to OAd oncolysis can ensue, particularly by $CD8^+$ T cells (Nattress and Halldén, 2018). It has recently been shown that OAds can decrease tumour-infiltrating $CD8^+$ T cell exhaustion in patients with metastatic cancer following systemic OAd treatment. This reduction in exhaustion was also associated with improved overall survival of the patients (Liikanen et al., 2022). In one preclinical model, OAds were also shown to induce tumour-specific memory $CD8^+$ T cell responses, meaning that patients receiving OAd therapy may benefit from anti-tumour immune responses to future recurrent disease (Chen et al., 2021). The most studied and clinically promising OAds to date are listed in **Table 2** and described below.

CLINICAL TRIALS WITH ONCOLYTIC ADENOVIRUSES INCLUDING BC PATIENTS

Deletion of the p53-Binding E1B55 KDa Protein in Onyx-015

The first OAd to enter clinical trials was Onyx-015 which contains deletions in the E1B55K gene and in the E3B domain (Khuri et al., 2000; Kirn, 2001). Expression of E1B55K, driven by E1A, inhibits cellular p53 to prevent premature apoptosis and to

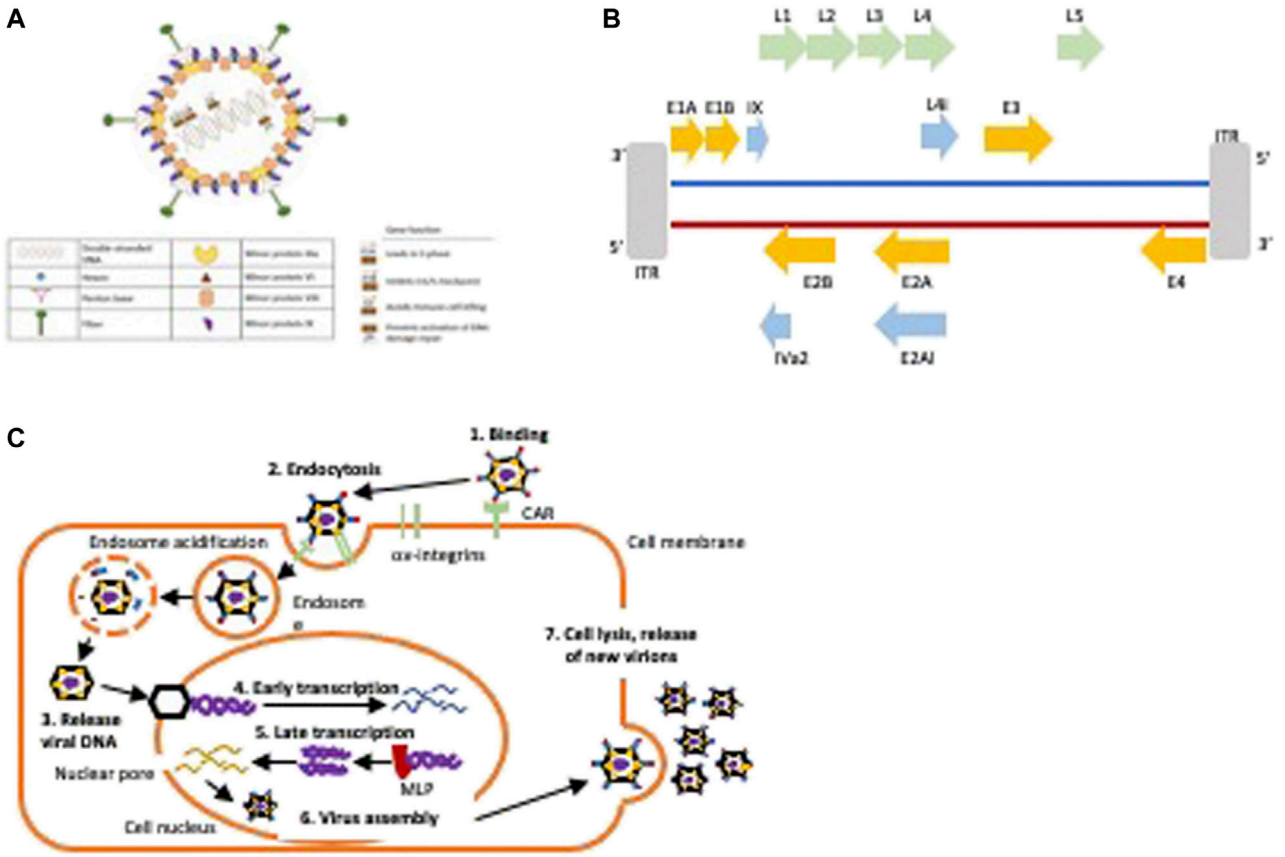

FIGURE 3 | Structure and life cycle of HAdV5. **(A)** Hexon is the major coat protein, the fiber protein binds to the Coxsackie and adenovirus receptor (CAR), the penton base is essential for viral uptake through the endosome by binding to cellular αvβ3-and αvβ5-integrins, the minor proteins are essential for capsid stabilisation and in viral genome stability. Gene functions for early expressed viral genes (E1-E4) are indicated. **(B)** Schematic of the viral genome. The first protein to be expressed is E1A that leads to S-phase induction by binding pRb and releasing E2F. E1A initiates the transcription of other early genes including the E1B55K that inhibit premature apoptosis by binding to p53, the E1B19K that prevents death receptor mediated apoptosis, the E3 genes that prevent immune-mediated cell killing of infected cells, and the E4 genes that prevent activation of DNA damage repair responses. The E2A and E2B proteins are essential for viral DNA synthesis and amplification. The late genes (L1-5) are transcribed after activation of the major late promoter (MLP) to enable assembly of new virions. Strategies to engineer OAds include deletion of early viral genes or specific gene regions that are essential for viral propagation in normal cells, for example, the pRb-binding E1ACR2-domain or the p53-binding E1B55K gene or insertion of tumour-specific promoters to drive early E1 gene expression. E3 gene regions are frequently deleted to enable insertion of transgenes. **(C)** Illustration of the HAdV5 life cycle.

enable S-phase entry and viral propagation (Nemunaitis et al., 2000). Mutants deleted in E1B55K cannot complete a productive lifecycle in normal healthy cells while in cancer cells with dysregulated p53 pathway viral genome amplification, gene expression and assembly of virions will proceed. The immunomodulatory E3B genes were also deleted in Onyx-015 as an added safety feature in these early clinical trials (Heise et al., 1997; Nemunaitis et al., 2000). The Onyx-015 mutant was evaluated in numerous early phase clinical trials targeting solid tumours and was reported to be safe but had limited efficacy as a single agent while efficacy was improved in combination with chemotherapeutic drugs (Larson et al., 2015). In one of these

TABLE 2 | Common deletions in engineered Oncolytic Adenoviruses (OAds).

Mutation and function	Virus	References
Deletion of E1B55 KDa protein to prevent p53-binding in normal cells	Onyx-015	(Nemunaitis et al., 2007) (Heise et al., 1997)
Deletion of E1ACR2 domain to prevent pRb-binding and S-phase entry in normal cells	Δ24	Fueyo et al. (2000)
	*d*l922-947	Heise et al. (2000)
	DNX-2401	Lang et al. (2018)
	ORCA-010	Dong et al. (2014)
Replacing the E1A constitutive promoter with tumour specific promoters	OBP-301 (Telomelysin)	Nemunaitis et al. (2010)
Deletion of E3gp19K immunomodulatory genes to allow MHC class I antigen presentation	ORCA-010	Dong et al. (2014)

trials Onyx-015 was combined with Etanercept (Enbrel) that act as an anti-inflammatory drug and inhibitor of tumour-necrosis factor (TNF) (Nemunaitis et al., 2007). Nine patients with different types of cancer including two patients with metastatic BC were enrolled and administered Onyx-015 intravenously on days 1, 8 and 15 of a 3-weeks cycle at different dose levels (1×10^{10}, 1×10^{11}, and 1×10^{12} viral particles (vp) per injection). Etanercept was administered before and during the first viral cycle. All nine patients developed grade 1/2 fever 24 h after administration of the virus with no significant adverse events demonstrating clinical safety. Both BC patients showed progressive disease (PD).

More recent findings demonstrated that E1B55K is also essential for viral mRNA export and mutants lacking this protein therefore had decreased viral fitness explaining the disappointing efficacy in the clinic with Onyx-015 (O'Shea et al., 2004; O'Shea et al., 2005). Furthermore, the deletion of the immunoregulatory genes in the E3B domain were also found to decrease viral spread in tumours in patients due to rapid viral removal by macrophages (Wang et al., 2003). These early studies clearly demonstrated the safety of OAds and that more selective gene-deletions were necessary in future developments of cancer-specific mutants to enhance viral potency.

Deletion of the pRb-Binding E1ACR2 Domain

Because of the disappointing clinical outcomes with Onyx-015 and other E1B55K-deleted mutants the focus has since been on improving the efficacy of OAds by deleting the E1ACR2 domain (24 amino acids). Deletion of E1ACR2 prevents replication in normal healthy cells but is redundant in cancer cells with deregulated cell cycle and growth control. In contrast to the E1B55K-deleted mutants the E1ACR2-deleted variants retain high viral potency (Heise et al., 2000). To date several OAds with E1ACR2-deletions have been evaluated in clinical trials and shown to be safe and significantly more efficacious than the E1B55K-deleted mutants (Fueyo et al., 2000; Lang et al., 2018). One of these promising mutants is ICOVIR-7 that also has a modified E2F1-promotor replacing the E1A-promoter in addition to an RGD-4C motif in the HI-loop of the fibre domain to enhance tumour selectivity, integrin-binding and entry into tumour cells (Nokisalmi et al., 2010). ICOVIR-7 was evaluated in a phase I trial targeting solid tumours in 21 patients including three BC patients with the virus delivered intratumourally at escalating doses from 2×10^{10} to 1×10^{12} vp/administration. Only self-limiting grade 1/2 side effects occurred such as fever, fatigue, elevated liver transaminases and anaemia without serious adverse effects demonstrating the safety of ICOVIR-7 at high doses. Interestingly, one of the BC, one prostate cancer and one ovarian-cancer patient, showed a decrease or stabilization in tumour markers suggesting anti-tumour efficacy (Nokisalmi et al., 2010). An improved mutant based on ICOVIR-7 is VCN-1 that was evaluated in clinical trials targeting pancreatic cancer patients and reported to have direct antitumour effects including the tumour stroma (Bazan-Peregrino et al., 2021). VCN-1 expresses Hyaluronidase that contributes to the degradation of the tumour stroma to facilitate the dense TME but has not yet been evaluated in BC patients.

Tumour-Specific Promoter-Driven OAds

Replacement of the E1A-promoter with tumour specific promoters is another approach used alone or in combination with specific gene deletions. OBP-301 (Telomelysin) is an HAdV5-based OAd with the human telomerase reverse transcriptase promoter (hTERTp) replacing the E1A-promoter (Nemunaitis et al., 2010; Yano et al., 2015). The hTERTp is highly active in cancer cells, but not in normal cells. Additional modifications in OBP-301 are replacement of the E1B genes with the internal ribosomal entry site (IRES) to improve specificity (Nemunaitis et al., 2010). OBP-301 was evaluated in a phase I clinical trial including 16 patients of which one was a BC patient (Nemunaitis et al., 2010). Administration of 1×10^{10}, 1×10^{11}, and 1×10^{12} vp once/treatment group resulted in only grade 1/2 side effects such as fever, fatigue, and chills, demonstrating safety in all patients. Viral replicative activity was observed in a subset of patients, including the BC patient, who was given a dose of 1×10^{12}vp intratumourally. In ongoing Phase I/II trials a second intratumoural or intravenous injection is administered in combination with radiation, chemotherapy and/or immunotherapy (NCT04685499; NCT03921021; NCT04391049). However, a challenge to this strategy is to find the right balance between the antiviral response and the antitumour immunity (Sato-Dahlman et al., 2020). Examples of additional tumour-specific promoter-driven OAds are shown in **Table 3** and clinical trials with reported outcomes in BC patients are listed in **Table 4**.

PRECLINICAL STUDIES IN MODELS OF BC

rAd-sTRII

The rAd-sTRII OAd is an HAdV5 mutant with two deletions in the E1A gene and expression of a soluble 159-amino-acid sequence of transforming growth factor-β type II receptor (sTGFβRII) in the E3B domain (**Table 5**) (Wang et al., 2006). The E1A deletions include amino acid sequences 4–25 and 111–123 that are essential for binding to the p300/CREB-binding protein (CBP) and to pRb proteins, respectively. The rAd-sTRII was demonstrated to be highly cytotoxic in a dose-dependent manner in the TNBC cell line MDA-MB-231 (Wang et al., 2006). Cells infected with rAd-sTRII showed inhibition of TGF-β signalling by producing sTGFβRII without affecting the replication potential of the virus.

rAd.DCN

The rAd.DCN mutant has the hTERT promoter replacing the E1A promoter, the same amino acid deletions as described in rAd-sTRII above, as well as expression of the decorin gene (**Table 5**) (Zhao et al., 2019). It was suggested that decorin (DCN) inhibits TGF-β signalling and inhibit metastasis, angiogenesis, and other pathophysiological processes (Isaka et al., 1996). The rAd.DCN OAd was administered

TABLE 3 | Tumour-specific promoter-driven oncolytic adenoviruses (OAds) used in preclinical and clinical trials.

OAd	Promoter	Cancer Type	Clinical Trial status	Clinical Trial NCT number
ICOVIR-5	E2F1 promoter	Solid tumours	Completed Recruiting Not yet recruiting	NCT01844661 NCT01864759 NCT04758533 NCT05047276
VCN-01		Advanced solid tumours	Completed Recruiting Not yet recruiting	NCT02045602 NCT02045589 NCT03799744 NCT05057715 NCT03284268
CG0070		Bladder cancer	Completed Recruiting	NCT02365818 NCT04452591 NCT04387461 NCT04610671
SynOV1.1	AFP promoter	Hepatocellular Carcinoma	Not yet recruiting	NCT04612504
OBP-301	hTERT-promoter	Carcinomas, melanomas and advanced solid tumours	Recruiting Active	NCT04391049 NCT03921021 NCT04685499 NCT03172819 NCT03213054 NCT03190824 NCT02293850
CRAd-S-pk7	Survivin-promoter	Recurrent High-Grade Gliomas	Not yet recruiting	NCT05139056
AdVince	CgA-promoter	Neuroendocrine Tumours	Recruiting	NCT02749331

TABLE 4 | Oncolytic adenoviruses (OAds) in clinical trials including breast cancer patients.

OAds	Genetic modification	Clinical Trial results	References
Onyx-015	Deletion: E1B55K and E3B	Two patients: one PD on day 125, one discontinued	Nemunaitis et al. (2007)
ICOVIR-7	E2F-1 promoter E1ACR2 deletion RGD-4C motif in fibre	Three patients: one with stabilized tumour markers	Nokisalmi et al. (2010)
OBP-301 (Telomelysin)	hTERTp replacing E1A promoter IRES replacing E1B genes	One patient: detectable virus replication	Nemunaitis et al. (2010)

TABLE 5 | Oncolytic adenoviral mutants in promising preclinical studies in TNBC models.

OAds	Genetic modification	Preclinical results	References
rAd-sTRII	Two E1A deletions prevent binding to CBP and pRb proteins sTGFβRII insertion in E3B	Inhibits TGFβ signalling and cancer cell growth	Wang et al. (2006)
rAd.DCN	hTERT promoter driving E1A GM-CSF and IRES replacing E1B19K	Inhibits tumour growth Prevents lung metastasis	Zhao et al. (2019)
SG400-E2F/IL-15	E2F-1 driving E1A IL-15 insertion in E3	Selective cancer cell killing	Yan et al. (2019)
Ad5-10miR145T	10 tandem repeats of miR-145 binding sites downstream of E1A Insertion of IRES and deletion of E3	E1A expression prevented by miR-145 Selective cancer cell killing	Shayestehpour et al. (2017)

GM-CSF, Granulocyte-macrophage colony-stimulating factor; IRES, internal ribosome entry site.

intratumourally and intravenously to mice with TNBC xenografts on two occasions (Zhao et al., 2019). The authors reported significant inhibition of tumour growth for both modes of administration. Furthermore, after intravenous administration development of lung metastasis was prevented. It was reported that rAd.DCN inhibited tumour growth and metastasis as a consequence of decorin interfering with wnt/β-catenin, vascular endothelial growth factor (VEGF), the Met pathways and by modulating anti-tumour inflammatory and immune responses (Zhao et al., 2019).

SG400-E2F/IL15

SG400-E2F/IL15 is based on HAdV5 with the E2F-1-promoter replacing the E1A-promoter and interleukin-15 (IL-15) coding sequence inserted into the E3 region for selectivity and efficacy, respectively (**Table 5**) (Yan et al., 2015; Yan et al., 2019). The transcription factor E2F-1 is frequently overexpressed in BC cells and predicts poor prognosis (Johnson et al., 2016). IL-15 is an immune regulator that prevents cancer cell proliferation by activating natural killer (NK) cells and $CD8^+$ memory T-cells (Gillgrass et al., 2014). SG400-E2F/IL-15 was demonstrated to

strongly inhibit tumour growth both in the cultured TNBC MDA-MB-231 cells and in mice with MDA-MB-231 xenografts (Yan et al., 2019).

Ad5-10miR145T

A different approach was used when constructing Ad5-10miR145T by inserting several binding sites for a tumour suppressor miRNA (miRNA-145; miR-145) to regulate E1A expression (**Table 5**) (Bader et al., 2010; Ji et al., 2017; Shayestehpour et al., 2017). It has been demonstrated that miR-145 acts as a tumour suppressor and is frequently downregulated in BC (Eades et al., 2015; Zhao et al., 2016; Ding et al., 2017; Shayestehpour et al., 2017). The Ad5-10miR145T mutant carries 10 binding sites for miR-145 downstream of E1A that do not affect viral replication when miR-145T is absent. After infection with Ad5-10miR145T in TNBC and normal epithelial breast cells (HMEpCs), the E1A gene expression was reduced in HMEpCs since they express high levels of miR-145 and consequently, viral replication was prevented. In contrast, Ad5-10miR145T potently replicated and killed all tested BC cells including MDA-MB-453, MCF-7, and BT-20 that do not express miR-145 (Shayestehpour et al., 2017). Considering that MDA-MB-453 and BT-20 cells are derived from TNBC and that miR-145 has been reported as downregulated in TNBC (Eades et al., 2015), Ad5-10miR145T may be a safe and promising therapeutic for TNBC.

Deletions of Immunomodulatory Viral Genes

Cancer cell killing by OAds is mediated by both virus-induced cell lysis and recruitment and activation of the host anti-tumour immune responses (Shaw and Suzuki, 2019). The massive lysis of cancer cells causes release of novel TAAs that in turn attract and activate host APCs, dendritic cells (DC) and cytotoxic T-cells. The viral immunomodulatory E3gp19K protein prevents MHC class I presentation of antigens and deletion of this protein promotes T-cell activation and has therefore been deleted in numerous OAds (Halldén et al., 2003; Wang et al., 2003; Man et al., 2018). For example, ORCA-010 has a single-base mutation (T29183) in E3gp19K resulting in increased membrane permeabilisation and enhanced release of progeny virions and TAAs from infected cells (Dong et al., 2014). ORCA-010 is E1ACR2-deleted with an inserted RGD-4C motif in the fibre for improved integrin binding and has demonstrated efficacy in prostate, lung and ovarian cancer models.

PROMISING COMBINATIONS OF OADS WITH CURRENT CLINICAL THERAPEUTICS

PARP Inhibitors

The enzyme poly (ADP-ribose) polymerase (PARP) one binds to single-strand DNA breaks (SSBs) and catalyses the formation of linear and branched poly (ADP-ribose) (PAR) chains that in turn recruit additional DNA repair proteins (Zaremba and Curtin, 2007; Underhill et al., 2011). Since 2005, PARP inhibitors (PARPi) have been extensively developed to treat BRCA deficient cancers, including TNBC (D'Andrea, 2018; Farmer et al., 2005; Bryant et al., 2005). The efficacy of PARPis in BRCA-deficient BC is because unrepaired SSBs lead to double strand breaks (DSB) that normally would be repaired by homologous recombination (HR) that is not functional in BRCA deficient cancers. In BRCA1/2 deficient cells, non-homologous end joining (NHEJ) or single-strand annealing (SSA) may occur that are both error-prone, leading to complex chromatid rearrangements and eventually, to apoptosis (D'Andrea, 2018) (Underhill et al., 2011). In addition, PARPis inhibitors function as chemosensitisers by enhancing the responses to chemotherapy by preventing repair of damaged DNA (Dréan et al., 2016).

In 2018, Olaparib became the first PARPi approved by the FDA for the treatment of metastatic BC and later the same year Talazoparib was approved by both the FDA and European Medicines Agency (EMA) to treat germline BRCA mutated HER2-negative locally advanced or metastatic BC (Robson et al., 2017; Ettl et al., 2018; Litton et al., 2018; Cortesi et al., 2021). When compared with standard chemotherapy, Olaparib showed longer progression-free survival (PFS) (7 vs. 4.2 months), better response rate (59.9 vs. 28.8%), less toxicity (36.6 vs. 50.5% of grade 3 or higher adverse events), and fewer patients stopped treatment because of toxicity (4.9 vs. 7.7%; (Robson et al., 2017). Talazoparib, when compared with standard chemotherapy, showed longer PFS (8.6 vs. 5.6 months), higher objective response rate (62.6 vs. 27.2%), significant overall improvements, and significant delays for clinically meaningful deterioration (Litton et al., 2018).

PARP Inhibitors Combined With Oncolytic Viruses

In a preclinical model of anaplastic thyroid carcinoma, the OAd *dl*922-947 (deleted in E1ACR2 and E3B) was combined with Olaparib (Passaro et al., 2015). It was demonstrated that virus-induced DNA damage was not repaired leading to increased cell death and potent *dl*922-947 replication and oncolytic activity suggesting the feasibility of PARPis and OAds as an improved therapy that warrants further investigation.

EGFR as a Therapeutic Target

The epidermal growth factor receptor 1 (EGFR1; ErbB) is overexpressed in up to 70% of TNBC (Ueno and Zhang, 2011; Maennling et al., 2019). It has been established that inhibition of BRCA1 leads to upregulation of EGFR in mammary epithelial cells (MECs) (Burga et al., 2011). Because BRCA1 is frequently non-functional in TNBC, this likely contributes to the upregulation of EGFR. It was demonstrated that administration of erlotinib, an EGFR inhibitor, prevented growth but did not eliminate BRCA1-related breast cancers (Burga et al., 2011). Currently the EGFR is therapeutically targeted by monoclonal antibodies (mAbs) against the

extracellular domain of EGFR, or by tyrosine kinase inhibitors (TKIs) that bind to the ATP pocket and therefore, prevent signal transduction (Guerrab et al., 2016; Nakai et al., 2016). However, outcomes from clinical trials using TKIs for TNBC have been surprisingly disappointing (Nakai et al., 2016; Shi et al., 2018). In a Phase II clinical trial, 115 patients with metastatic BC received cisplatin plus the EGFR inhibitor cetuximab and 58 patients received cisplatin alone (Ueno and Zhang, 2011; Baselga et al., 2013). The combination treated group showed better outcomes than with cetuximab alone, with an overall response rate of 20 vs. 10%, a PFS of 3.7 vs. 1.5 months, and an overall survival (OS) of 12.9 vs. 9.4 months.

EGFR Targeting in Combination With Oncolytic Viruses

The OAd ICOVIR15-cBiTe, armed with an EGFR-targeting bispecific T-cell engager, demonstrated good antitumour efficacy in human lung adenocarcinoma and epidermoid carcinoma cell lines (Fajardo et al., 2017; Barlabé et al., 2020). The bispecific T-cell engager (BiTE) was composed of two single chain antibodies (scFV) that targeted the tumour-specific EGFR and CD3 on the T-cell receptor to activate T-cells and bring the complex close to the tumour cell for improved elimination. The EGFR mutant EGFRvIII is frequently expressed in glioblastomas and is specifically expressed in the cancer cells. Targeting of the EGFRvIII was exploited to engineer an OAd to target the receptor and was reported to the show selective and potent anti-glioma effects (Piao et al., 2009). Although EGFRvIII expression in BC, specifically in TNBC, is less common, an EGFRvIII-retargeted OAd may be a therapeutic approach for TNBC cases with this mutation (Del Vecchio et al., 2012).

Androgen Receptor as a Therapeutic Target

The androgen receptor (AR) is a member of the nuclear steroid hormone receptor family. This family also includes ER and PR, and although the roles of these receptors are well established in BC, little is known about the biological functions of AR in TNBC (Rampurwala et al., 2016; Hwang et al., 2019). In a gene expression-profile analysis from 21 data sets containing 587 TNBC cases, six subtypes of TNBC were identified including the luminal AR subtype (LAR) (Lehmann et al., 2011). AR was also expressed in additional subtypes in up to 75% of all TNBCs (Hwang et al., 2019). However, the LAR subtype was found to be dependent on AR signalling with high levels of AR expression (Rampurwala et al., 2016). It was demonstrated that LAR cell lines were sensitive to the AR antagonist bicalutamide and even more sensitive to enzalutamide (Lehmann et al., 2011; Cochrane et al., 2014).

In a Phase II clinical trial using bicalutamide in patients with AR-positive and ER/PR-negative metastatic BC the most commonly reported adverse events were fatigue, hot flushes, limb oedema, and aspartate aminotransferase (AST) and alkaline aminotransferase (ALT) elevation (Gucalp et al., 2013). The PFS was 12 weeks, and a 6-months clinical benefit rate of 19% was reported for bicalutamide given at 150 mg/day. Enzalutamide was evaluated in a Phase II clinical trial in patients with AR-positive TNBC (Traina et al., 2018). The evaluable subgroup, which included patients with AR expression of ≥10% by IHC, showed an increase in OS of 4.9 months compared to the non-enzalutamide treated group (Traina et al., 2018).

A phase I trial with the selective CYP17 and AR inhibitor Seviteronel showed good tolerance to a once-daily dose of 450 mg (Bardia et al., 2018). AR targeting on its own have proven good tolerance and less toxicity than chemotherapy. However, further research is needed in order to gain a better understanding of efficacy of anti-androgens in TNBC.

AR Targeting in Combination With OAds

Numerous OAds with AR response elements (AREs) have been developed for evaluation in prostate cancer patients (Sweeney and Halldén, 2016). The AREs typically replace the E1A-promoter to drive tumour-specific viral replication. The most frequently used ARE is from the regulatory domain of the prostate-specific antigen (PSA). One of the first developed ARE-driven OAds CG7060, also known as CN706 or CV706, showed up to 7-fold increases in replication in the presence of androgens in AR-positive cell lines (Rodriguez et al., 1997). In a Phase I clinical trial with CV706 both safety and significant reduction of PSA levels (up to 65%) was reported in prostate cancer patients (DeWeese et al., 2001).

The AR mutant (C685Y) often expressed in late-stage cancer, is activated by both androgens and anti-androgens and was utilised to engineer a novel OAd (Johnson et al., 2013). The receptor coding region was fused to the viral E1A gene to increase specific viral oncolysis. This AR-driven mutant was reported to have significantly enhanced viral activity in the presence of bicalutamide both *in vitro* and *in vivo,* suggesting a promising novel therapy for both prostate cancer and AR-positive TNBC patients.

IMMUNOTHERAPY FOR TNBC

Novel immune therapeutics have been developed as promising treatments for numerous cancer types including TNBC (Lee and Marks, 2010; Han et al., 2020; Mediratta et al., 2020). Currently the most promising agents are the immune checkpoint inhibitors that prevent the inhibition of cytotoxic T-lymphocyte (CTL) activity and reactivate the host anti-tumour defences (Pardoll, 2012; Navarrete-Bernal et al., 2020).

PD-1/PD-L1 Antibodies

Programmed cell death 1 (PD-1; CD279) is a 288 amino acid transmembrane protein expressed on activated $CD4^+$ and $CD8^+$ T-cells, B-cells, NK-cells, monocytes, and dendritic cells (DCs) (Zajac et al., 1998; Bardhan et al., 2016; Kurachi, 2019; Seliger, 2019; Han et al., 2020). T-cell receptor activation is suppressed when PD-1 on T-cells binds to the ligands PD-L1 or PD-L2, negatively regulating the immune response (Zak et al., 2015; Vikas et al., 2018). PD-L1 is expressed on T- and B-cells, macrophages, DCs, bone marrow-derived mast cells, mesenchymal stem cells, and non-hematopoietic cells

(Yamazaki et al., 2002; Riella et al., 2012; Bardhan et al., 2016). It has been established that PD-L1 is overexpressed in numerous cancers such as glioblastoma, lymphoma, melanoma, ovarian, and BC, among others that result in inhibition of the immune response in the TME (Matsuzaki et al., 2010; Muenst et al., 2013; Sun et al., 2014; Hawkes et al., 2015). High expression levels of PD-L1 correlates with poor prognosis and targeting the PD-1/PD-L1 pathway is a promising therapeutic approach (Sakuishi et al., 2010; Sabatier et al., 2015).

Pembrolizumab is an anti-PD-1 antibody that was approved by the FDA in 2014 for patients with advanced melanoma and is one of the most researched immune-checkpoint-targeted therapies (Vikas et al., 2018). In a phase II clinical trial, pembrolizumab was administered alone intravenously in patients with metastatic TNBC resulting in an objective response rate (ORR) of 5.7%, including two patients with complete response (CR) and four patients with partial response (PR). Adverse events (AE) were observed in 60.6% of patients, of which 12.9% were grade 3 or 4 (Adams et al., 2019). Although the ORR was lower than single-agent chemotherapy, treatment with pembrolizumab had less toxicity and showed durable responses.

Another FDA approved anti-PD-L1 antibody, Atezolizumab, was approved in 2016 for patients with urothelial carcinoma and is now also approved for TNBC by FDA. In a phase III clinical trial, 902 patients with TNBC whereof 451 patients received atezolizumab plus nab-paclitaxel, and 451 patients received placebo plus nab-paclitaxel (Schmid et al., 2020). The OS for PD-L1-positive patients was 25 months for the atezolizumab group vs. 18 months for the nab-paclitaxel alone group suggesting a promising outcome for the combination with standard-of-care in TNBC patients.

Pembrolizumab in Combination With Oncolytic Viruses

Pembrolizumab has been combined with several oncolytic viruses, including OAds, in both preclinical studies and clinical trials targeting different cancer types (Chen et al., 2018). Pembrolizumab in combination with ONCOS-102 was reported to have synergistic antitumour effects in mouse models of malignant melanoma (Kuryk et al., 2018). DNX-2401 plus pembrolizumab treatment was reported to be safe in glioblastoma patients. Phase I and II ongoing clinical trials are evaluating OBP-301 OAds combined with pembrolizumab in treatments of patients with advanced solid tumours, and patients with inoperable, recurrent, and progressive squamous cell carcinoma of the head and neck (NCT03172819; NCT04685499).

CTLA-4 Antibodies

CTLA-4 is a CD28-homolog cell surface glycoprotein expressed on regulatory T-cells (Treg) and is upregulated on activated $CD4^+$ and $CD8^+$ T-cells. B7-1 and B7-2 bind to CTLA-4 and CD28 (Buchbinder and Desai, 2016; Mediratta et al., 2020). Binding of B7 to CD28 molecules leads to T-cell proliferation, differentiation and cell survival while binding of B7 to CTLA-4 leads to inhibitory signals. CTLA-4 is often upregulated in cancer patients and is therefore an attractive target to enhance T-cell antitumour activity (Navarrete-Bernal et al., 2020). Ipilimumab is an approved anti-CTLA-4 antibody for the treatment of several cancers in combination with nivolumab (anti-PD-1 antibody) (Mediratta et al., 2020). Currently, there are at least three ongoing clinical trials studying the use of ipilimumab in combination with different therapeutic agents for advanced malignancies, including TNBC (NCT03126110; NCT03752398; NCT03546686).

CTLA-4 Antibodies in Combination With Oncolytic Viruses

Treatments combining CTLA-4 antibodies and oncolytic viruses have been reported although, not yet for OAds (Engeland et al., 2014; Puzanov et al., 2016; Peters et al., 2019; Liu et al., 2021; Sugawara et al., 2021). In one interesting study, Maraba rhabdovirus was tested in TNBC-like murine models with virus administered twice intratumourally followed by five administrations of anti-CTLA-4 and anti-PD-1 antibodies intraperitoneally. A meaningful reduction in tumour growth was reported in animals treated with the combinations (Bourgeois-Daigneault et al., 2018).

BARRIERS TO THE SYSTEMIC DELIVERY OF OADS TO TNBC PATIENTS

Although intratumoural injection of OAds has been assessed and has been feasible in different solid tumour contexts, only intravenous systemic delivery of OAds will be able to reach distant metastatic disease. As up to 46% of TNBC patients have distant metastases, strategies to overcome the barriers to successful systemic delivery need to be overcome in order to improve efficacy across all sites of a patient's disease (Yin et al., 2020).

One of the immediate hurdles that OAds encounter upon entering the blood stream is the presence of neutralising antibodies (NAbs) that sequester the virus rendering them unable to reach tumour sites. Around 85% of the population harbour NAbs against Ad5 OAds owing to previous natural infections meaning that this is a pertinent factor to overcome (Mast et al., 2010). Aside from NAbs, red blood cells (erythrocytes) are also able to bind OAds via both CAR and complement receptor-1. NAb and erythrocyte binding will therefore reduce the bioavailablity of circulating virus although there are certain strategies that can overcome this including adenovirus nanocomplexes (Park et al., 2010), modification of the viral coat proteins with liposome complexes (Han et al., 2008), and the use of tumour-infiltrating lymphocytes (TIL) or mesenchymal stem cells as carriers (Barlabé et al., 2020; Santos et al., 2021).

Free OAd particles within the blood that avoid NAb and erythrocyte binding are, however, highly susceptible to clearance from the systemic circulation. A major contributor to this process is the hepatic reticuloendothelial system, containing phagocytic Kupffer cells that can eliminate up to 90% of administered OAds

within minutes (Parker et al., 2006; Carlisle et al., 2009; Parker et al., 2009). One strategy to help ablate the hepatic binding of OAds involves the modification of adenoviral hexon proteins, such as substitution with the adenoviral serotype 3 hexon (Short et al., 2010). A more recent strategy involves the insertion of specific amino acid peptides into the fibre knob protein of the OAd. This strategy involves two concepts: firstly, the native fibre knob is disrupted by the peptide insertion rendering it unable to bind to CAR, αvβ3/5 and coagulation factor 10 (FX), thus reducing its sequestration in the blood and non-tumour sites. Secondly, the peptides can be designed to target other tumour-specific binding sites such as αvβ6 (A20 peptide) (Uusi-Kerttula et al., 2018) and (Man et al., 2018).

Aside from vascular and hepatic sequestration, another consideration for systemic OAd therapy is the location of tumours and metastatic sites. Like many solid cancer types, the first sites of metastatic spread from primary TNBC tumour are the lungs (41%), liver (29%) and bone (24%). However, when factoring in total metastatic burden over the total disease course, 46% of patients were noted to have at least one lesion within the central nervous system (CNS) which is not the case across all solid tumour types (Lin et al., 2008). The relatively common occurrence of CNS metastases in TNBC is a significant challenge due to the fact that systemic therapies must cross the blood-brain barrier (BBB), which is particularly resistant to the transport of most small polar molecules, macromolecules and therapeutic agents (Tang et al., 2007). One strategy for metastatic CNS TNBC in mice, albeit using oncolytic herpes simplex virus (HSV), involved the intra-arterial infusion of hyperosmolar mannitol to transiently disrupt the BBB during the infusion of oncolytic HSV. The mannitol infusion allowed for extensive trafficking of the virus to intracerebral tumours compared to PBS infusion controls however this is a fairly invasive procedure (Liu et al., 2005). Alternatively, oncolytic recombinant poliovirus was delivered intrathecally to rats for the treatment of human glioblastoma multiforme, increasing survival without toxicity (Ochiai et al., 2006). An exciting new approach using an engineered oncolytic adenovirus (CRAd-S-pk7) in a phase 1 clinical trial for malignant glioma whereby neural stem cells (NSCs) were used as a vehicle to cross the BBB due to their natural tumour tropism. This trial involved intracranial delivery however systemic intravenous delivery has been efficacious in mouse models. This trial demonstrated safety and tolerability of NSC-OAd delivery even when combined with neurosurgical resections and chemoradiotherapies, and is an exciting potential candidate for treating metastatic CNS TNBC patients with systemic OAds (Barish et al., 2017; Fares et al., 2021).

DISCUSSION AND CONCLUDING REMARKS

TNBC tumours are characterised by the absence of the ER/PR hormone receptors and HER2, and short overall survival with rapid metastasis and recurrence (Collignon et al., 2016). New agents to target this type of cancer remains an important challenge since current standard-of-care chemotherapeutics are not curative. OAds have the potential to benefit TNBC patients due to their completely different mechanisms of action that can overcome drug resistance and provide long-lasting protection through activation of the host anti-tumour immune responses (Shaw and Suzuki, 2019).

Although clinical trials using OAds such as Onyx-015, ICOVIR-7 and OBP-301 have shown good efficacy in solid cancers, improved efficacy is necessary to eliminate BCs including TNBCs (Nemunaitis et al., 2007; Nemunaitis et al., 2010; Nokisalmi et al., 2010). Novel engineered OAds have shown promise for TNBC patients in preclinical studies. The SG400-E2F/IL-15 mutant selectively and effectively infected and killed BC cells (Yan et al., 2019). The Ad5-10miR145T mutant also infected and killed BC cells, leaving normal cells unharmed (Shayestehpour et al., 2017). Ad5-10miR145T proved highly efficacious *in vivo* in TNBC tumour xenografts in mice. Because miR-145 is frequently downregulated in TNBC the Ad5-10miR145T comprise a promising new agent to be evaluated in clinical TNBC.

Recently an OAd was developed that targets pancreatic cancer through αvβ6-integrin-mediated binding and uptake, Ad5-3Δ-A20T (Man et al., 2018). The highly potent mutant harbour deletions in the E1B19K and E1ACR2, the E3gp19K for optimal replication-selectivity and immune stimulation, respectively. Ad5-3Δ-A20T is highly selective for αvβ6-integrin-expressing pancreatic cancer cells that express the integrin in high levels and effectively eliminates the cells both as a single agent and in combination with the chemotherapy drug gemcitabine. Ad5-3Δ-A20T also has a higher efficacy after systemic delivery than the corresponding parental virus in mice with PDAC xenografts indicating a longer half-life in blood (Stella Man et al., 2019). Since αvβ6-integrin is expressed only in solid tumours, including TNBC cell lines and tumours, and not in healthy tissues (Whilding et al., 2017), we suggest that Ad5-3Δ-A20T has potential for translation into the clinic for treatment of TNBC. We recently demonstrated that the replication and anti-tumour efficacy of OAds including Ad5-3Δ-A20T can be augmented when combined with novel therapeutics such as histone deacetylase inhibitors (HDACis) (Rodríguez et al., 2022).

Small molecule anti-cancer drugs that target the dysregulated pathways in TNBC have been shown to improve efficacy in combination with oncolytic viruses. For example, inhibitors of EGFR and AR in combination with OAds are anticipated to have good responses in TNBC through synergistic effects on multiple cellular pathways (Piao et al., 2009; Johnson et al., 2013). Combining the small molecule PARP inhibitor Olaparib with the OAd *dl*922-947 resulted in significantly improved efficacy in anaplastic thyroid carcinoma models (Passaro et al., 2015). The main reason for the enhanced tumour elimination is likely due to synthetic lethality, with virus causing cellular DNA damage that cannot be repaired in the presence of a PARPi mimicking the efficacy with PARPi in BRCA1/2 mutated TNBC cells. Because of the frequent BRCA mutations in TNBC, this approach is anticipated to improve clinical outcomes in clinical TNBC. Other combinations including the novel immune checkpoint

inhibitors targeting PD-1/PD-L1 and CTLA-4 with OAds have shown good efficacy in several solid tumours (Engeland et al., 2014; Puzanov et al., 2016; Kuryk et al., 2018; Peters et al., 2019; Liu et al., 2021; Sugawara et al., 2021). This approach is expected to be highly efficacious due to the simultaneous release of novel cancer antigens by virus-mediated lysis and blocking of the immune checkpoints to reactivate the host anti-tumour defences.

Although great advancements are being made in the design of OAds with far more sophisticated mutants being generated, the current barriers of intravenous systemic delivery to all metastatic tumour sites cannot be ignored. Achieving sufficient bioavailability within the blood with sufficient trafficking and tumour penetrance will invariably be a bottleneck to efficacy for most OAds tested clinically. Coordinated efforts to investigate better strategies of delivering OAds to patients is equally important to their design, particularly in the context of hard-to-reach tumour sites such as those within the CNS.

To conclude, the safety of OAds have been demonstrated in thousands of patients administered various mutants using all modes of administration. Antitumour efficacy has been reported when virus was delivered intratumourally and in combination with cytotoxic drugs, small molecule inhibitors and immune checkpoint blockade. The use of OAds to treat TNBC is still in its infancy and further preclinical and clinical trials need to be performed however, promising results are to be expected.

AUTHOR CONTRIBUTIONS

GG-T initiated the work and produced figures and tables, researched the topics and wrote the first draft. CN revised the manuscript, added a significant amount of clinical data and edited the final manuscript. GH supervised the work and wrote the final manuscript.

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REVIEW
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Development of Molecular Mechanisms and Their Application on Oncolytic Newcastle Disease Virus in Cancer Therapy

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Cancer is caused by the destruction or mutation of cellular genetic materials induced by environmental or genetic factors. It is defined by uncontrolled cell proliferation and abnormality of the apoptotic pathways. The majority of human malignancies are characterized by distant metastasis and dissemination. Currently, the most common means of cancer treatment include surgery, radiotherapy, and chemotherapy, which usually damage healthy cells and cause toxicity in patients. Targeted therapy is an effective tumor treatment method with few side effects. At present, some targeted therapeutic drugs have achieved encouraging results in clinical studies, but finding an effective solution to improve the targeting and delivery efficiency of these drugs remains a challenge. In recent years, oncolytic viruses (OVs) have been used to direct the tumor-targeted therapy or immunotherapy. Newcastle disease virus (NDV) is a solid oncolytic agent capable of directly killing tumor cells and increasing tumor antigen exposure. Simultaneously, NDV can trigger the proliferation of tumor-specific immune cells and thus improve the therapeutic efficacy of NDV in cancer. Based on NDV's inherent oncolytic activity and the stimulation of antitumor immune responses, the combination of NDV and other tumor therapy approaches can improve the antitumor efficacy while reducing drug toxicity, indicating a broad application potential. We discussed the biological properties of NDV, the antitumor molecular mechanisms of oncolytic NDV, and its application in the field of tumor therapy in this review. Furthermore, we presented new insights into the challenges that NDV will confront and suggestions for increasing NDV's therapeutic efficacy in cancer.

Keywords: Newcastle disease virus, oncolytic virotherapy, tumor, apoptosis, antitumor immunity

INTRODUCTION

Cancer seriously threatens human health due to its high incidence and mortality and is the second cause of death globally, exceeded only by cardiovascular diseases (Moliner et al., 2019; Xia et al., 2022). In 2020, there were an estimated 19.3 million new cancer cases worldwide and nearly 10 million cancer-related deaths (Sung et al., 2021). Cancer's high mortality rate is mainly because patients with early cancer have no apparent symptoms and are already in the late stage or metastatic stage when diagnosed (Huang et al., 2017; Regel et al., 2020). Tumor cells evade immune system

surveillance and inhibit the immune response due to high mutagenicity (Park et al., 2020). At the same time, uncontrolled cancer cells invade the tissue, eventually leading to organ failure and even death (Fares et al., 2020). Currently, surgery, radiotherapy, and chemotherapy are the main methods for cancer treatment (Moo et al., 2018; Yahya and Alqadhi, 2021). Although surgery, radio-/chemotherapy, and targeted therapy can help some patients with early tumors, most therapy methods for individuals are terminated because of severe side effects (Boshuizen and Peeper, 2020). Cancer prognosis is still not optimistic; therefore, an essential question in cancer therapy as to how to improve cancer patients' survival rate effectively remains unexplored.

In recent years, several approaches have been developed for cancer therapy, such as immune checkpoint–based therapy (Chen et al., 2021), targeting circular RNAs (Chen et al., 2019), chimeric antigen receptor T (CAR-T) cell therapy (Adachi et al., 2018), and CRISPR/Cas9-based therapy (Zhen and Li, 2019). But all of these approaches have some limitations, which include off-target effects for targeted therapy, inefficiency of monotherapy, and unpredictable or predictable side effects (Zugazagoitia et al., 2016). An oncolytic virus (OV) is a promising cancer treatment strategy. OV is a useful therapeutic reagent that identifies and destroys malignant cells after a recurring viral infection (Martin and Bell, 2018; Raja et al., 2018; Leber et al., 2020). The lytic products after tumor dissolution can reverse the tumor microenvironment, promote the recruitment of immune cells, and further activate the antitumor immune response (Mahasa et al., 2017; Harrington et al., 2019). Several viruses, including the Newcastle disease virus (NDV), vaccinia virus, adenovirus, reovirus, herpes simplex virus, and measles virus, are being widely studied to treat various types of advanced cancer (Mondal et al., 2020; Goradel et al., 2021). Talimogene laherparepvec (T-VEC), a genetically engineered herpes simplex virus, is the first OV approved to treat advanced melanoma by the US FDA (Liu et al., 2003; Andtbacka et al., 2019). However, due to the heterogeneity of cancer tissue and the complexity of cancer cells, a single type of OV is not enough to destroy all cancer cells (Lawler et al., 2017; Martin and Bell, 2018). Some cancer cells and non-transformed supporting cells may be resistant to certain OVs (Alvarez-Breckenridge et al., 2013; Pol et al., 2016). Based on these, a single type of viral therapy may not be effective against all types of cancer (Kwan et al., 2021). Therefore, we believe that the combination of OV therapy and other cancer therapies will be significant for cancer patients (Dai et al., 2022).

NDV is a natural avian–derived virus (Sinkovics and Horvath, 2000), and its infection is a highly contagious disease that causes enormous economic losses to the poultry industry worldwide (Ganar et al., 2014; Susta et al., 2018). NDV has been developed as an oncolytic agent or a vaccination vector over the last 20 years due to its intrinsic oncolytic ability (Molouki and Peeters, 2017; Hu et al., 2020; Vannini et al., 2021). Compared with other OVs, oncolytic NDV has inherent antitumor advantages (Meng et al., 2021). Natural NDV strains exhibit an antitumor effect in human cancer cells and cause oncolysis without harming the normal cells (Yurchenko et al., 2019; Burman et al., 2020). In addition to causing direct damage to host cells through viral infection and replication, NDV activates multiple signaling pathways, triggering autophagy, inflammation, and apoptosis (Cuadrado-Castano et al., 2015; Li et al., 2019; Gong et al., 2021). It also activates antitumor immune responses, thus assisting viral replication (Wang et al., 2020; Zhan et al., 2020; Kan et al., 2021). With the constant maturity of the reverse genetic operating system of NDV (Peeters et al., 1999; Römer-Oberdörfer et al., 1999; Nettelbeck et al., 2021), an increasing number of transgenic NDVs are identified, which makes the application of NDV a new stage in cancer therapy. A genetically engineered NDV strain (NDV-F3aa) is effective in the experimental treatment of a gastric tumor peritoneal model without significant toxicity, and in some cases, it may completely cure gastric tumors (Song et al., 2010). Combining NDV therapy with other cancer therapies also provides new ideas for cancer treatment (Schirrmacher and Fournier, 2014; Xu et al., 2021). Hence, the NDV represents broad prospects for cancer treatment.

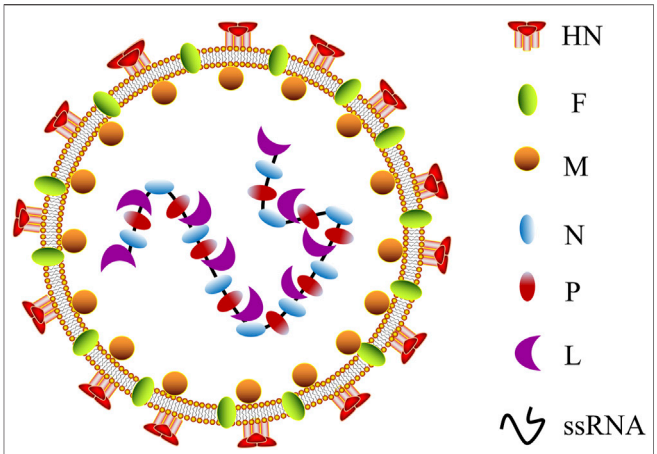

FIGURE 1 | Schematic representation of NDV morphology. NDV, Newcastle disease virus; HN, hemagglutinin–neuraminidase; F, fusion protein; M, matrix protein; N, nucleocapsid protein; P, phosphoprotein; L, RNA-dependent large polymerase protein; ssRNA, single-stranded RNA.

This review will concentrate on the biology, process of infection, and replication of NDV in cancer cells and the primary molecular mechanism of NDV oncolysis, and its preclinical and clinical applications in diverse cancers. In addition, we will highlight the limitations of NDV in clinical research and share our new insights into the use of NDV in cancer therapy.

NEWCASTLE DISEASE VIRUS BIOLOGY

Twelve different serotypes of avian paramyxoviruses (APMVs) have been reported up to date (Gogoi et al., 2017). NDV is the most characterized member of the genus *Avulavirus* in the family of Paramyxoviridae (APMV-1) (Kapczynski et al., 2013). It is an RNA virus with diameters ranging from 100 to 500 nm, enclosed by a viral lipid membrane (Kapczynski et al., 2013; Nagai et al., 1989). NDV was first identified as a valuable virus for virulence

studies in the 1970s (Gogoi et al., 2017; Cassel and Garrett, 1965). According to their pathogenicity and virulence in infected chickens, NDV strains are classified as lentogenic (avirulent), mesogenic, and velogenic (fully virulent) (Sinkovics and Horvath, 2000; Dimitrov et al., 2016). NDV contains a negative single-stranded RNA (ssRNA) genome of approximately 15.2 kb that consists of a leader (55 nucleotides) and trailer (114 nucleotides) terminal sequences (Nagai et al., 1989; Bello et al., 2020), which encode six different structural proteins: hemagglutinin–neuraminidase (HN), nucleocapsid (N) protein, fusion (F) protein, phosphoprotein (P) protein, matrix (M) protein, and RNA-dependent large polymerase (L) protein (**Figure 1**). V and W proteins are auxiliary and exist only in virus-infected cells. The V protein is an IFN antagonist and plays a vital role in the virulence of NDV (Alamares et al., 2010). Notably, in the NDV genome, each gene encodes a single protein and is characterized by a coding sequence flanked by highly conserved gene start (GS) and gene end (GE) transcriptional signals (Munir et al., 2012).

The viral N protein, P protein, and L protein bind to the viral RNA genome to form a ribonucleoprotein complex (RNP), essential for virus replication (Yusoff and Tan, 2001). The M protein is located in the layer below the virus lipid membrane and participates in virus assembly and budding (Nagai et al., 1989). HN and F proteins are located on the virus membrane's outer surface, where they join with the host cell's lipid bilayer membrane to form a viral shell. In addition, HN and F proteins jointly mediate viral attachment and fusion on the cell surface (Fournier et al., 2004). The fusion of virus and host cell must be completed through the F protein and HN protein participation, and the cleavage site of virus F protein (Fcs) is the critical factor (Peeters et al., 1999; Seal et al., 2000). Simultaneously, antibodies F and HN are the significant components that resulted in vaccine-inducing body protection, following vaccination of avian or non-avian species (Xiao et al., 2012; Dey et al., 2014), revealing the potential of NDV as a vaccine vector resistant to the animal and human disease. Currently, the LaSota and Hicher B1 vaccine strains have been widely used as a live NDV vaccine throughout the world (Carrasco et al., 2016; Dey et al., 2017). The strains are naturally occurring lentogenic strains that are highly expressed in embryonated chicken eggs and elicit a significant immune response (Ginting et al., 2017). One of the advantages of NDV as an oncolytic agent is that both lytic and non-lytic strains of NDV can fast-replicate in all species of avian and multiple human cancer cells (Lam et al., 2011; Zamarin and Palese, 2012), resulting in effective cell lysis and offering substantial protection from disease.

NEWCASTLE DISEASE VIRUS DISSOLVES TUMOR AND ACTIVATES AN ANTITUMOR IMMUNE RESPONSE

The NDV oncolytic properties originate from its capacity to proliferate in cancer cells (Shobana et al., 2013). Further research showed that it might be related to the deficiency of the interferon (IFN) system in tumors (Stojdl et al., 2000). H. Song et al. discovered that NDV enters the cell through a pH-independent direct fusion of its envelope to the host membrane *via* receptor-mediated endocytosis (Sánchez-Felipe et al., 2014). The process of NDV infection and replication in tumor cells is described as follows (Moliner et al., 2019). NDV binds to the sialic acid receptor on the surface of tumor cells through the HN protein, and then, protein F initiates the fusion of the viral and host cell membranes (Song et al., 2019; Xia et al., 2022). Viral RNA polymerase transcribes the viral negative single-stranded RNA into positive single-stranded RNA as a template for mRNA and protein synthesis (Burman et al., 2020; Sung et al., 2021). The rough endoplasmic reticulum processes surface proteins F and HN, assembled on the host cell membrane and mature to produce new virions that start a new round of tumor cell infection (Cuadrado-Castano et al., 2015). Importantly, virus-mediated direct oncolysis causes the release of tumor-associated antigens (TAAs), pathogen-associated molecular patterns (PAMPs), and danger-associated molecular patterns (DAMPs). These can activate antigen-presenting cells (APCs), including antigen-cross-presenting dendritic cells (DCs). Activated APCs then activate the immune cells, resulting in the generation of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells directed toward tumor and viral antigens (Burman et al., 2020; Schirrmacher and Fournier, 2014) (**Figure 2**). It is worth mentioning that NDV does not replicate in the normal cells of non-avian hosts (Fiola et al., 2006).

NDV is an effective oncolytic agent. The oncolytic properties of NDVs are also correlated with the pathogenic classification of NDV strains (lytic or non-lytic) (Dey et al., 2014). It has been found that mesogenic and velogenic NDVs are lytic while lentogenic NDV is non-lytic (Fournier et al., 2012; Ganar et al., 2014). Velogenic NDV kills cancer cells rapidly because they destroy the cytoplasmic membrane of infected cells (Wu et al., 2014). Lytic NDV exhibits multi-loop replication, whereas the non-lytic virus exhibits only single-loop replication (Fournier et al., 2012). In addition, the replication process of NDV takes place in the cytoplasm. This replication mode prevents the virus from integrating with the host genome or recombining with the human virus itself (Ganar et al., 2014). Therefore, NDV is non-pathogenic to humans and thus relatively safe with no side effects, which is a significant advantage of NDV as an oncolytic agent.

MULTIPLE ANTITUMOR MOLECULAR MECHANISMS OF NEWCASTLE DISEASE VIRUS

The induction of apoptosis, autophagy, necroptosis, and immunogenic death (ICD), as well as the stimulation of the immune system, are among NDV's oncolytic processes (Cuadrado-Castano et al., 2015; Zhang et al., 2018; Shao et al., 2019; Kan et al., 2021). The antitumor mechanism of NDV is briefly described in the following section.

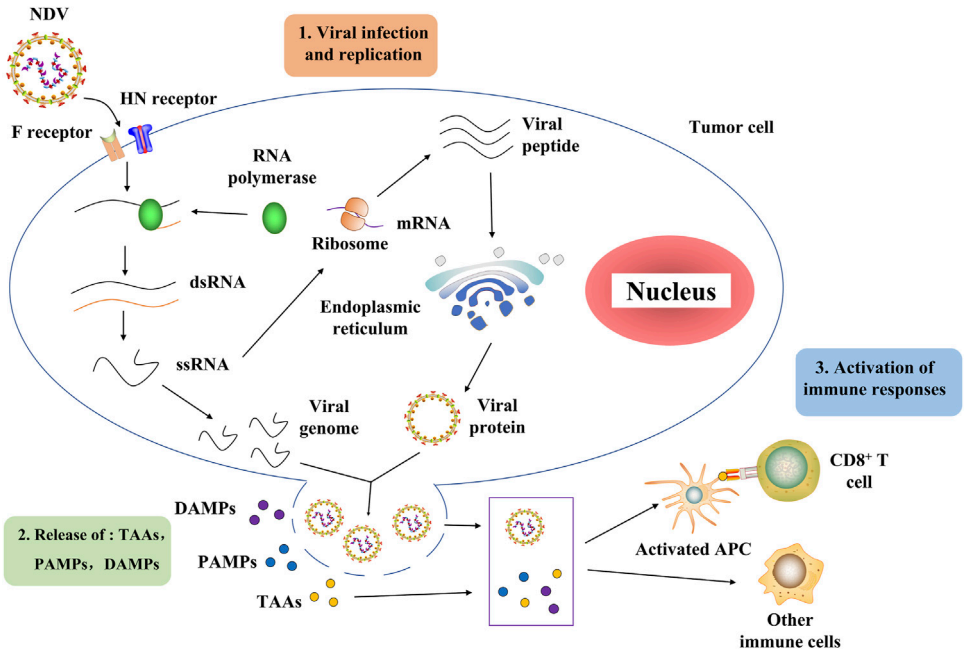

FIGURE 2 | Process of NDV through which it infects tumor cells and activates the host immune system. NDV exerts its antitumor effect mainly in two stages. In the first stage, the NDV binds to the sialic acid receptor on the surface of tumor cells through the HN protein, and then protein F initiates the fusion of the viral and host cell membranes. Then, the viral RNA polymerase transcribes the viral negative single-stranded RNA into positive single-stranded RNA as a template for mRNA and protein synthesis. The rough endoplasmic reticulum processes surface proteins F and HN, assembled on the host cell membrane and germinated to produce new virions that begin a new round of tumor cell infection. In the second stage, the virus-mediated direct oncolysis leads to the release of TAAs, PAMPs, and DAMPs that activate APCs, including dendritic cells capable of antigen cross-presentation. Activated APCs activate immune cells, resulting in the generation of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells directed toward tumor and viral antigens. NDV, Newcastle disease virus; HN, hemagglutinin–neuraminidase; F, fusion protein; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; TAAs, tumor-associated antigens, PAMPs, pathogen-associated molecular patterns, DAMPs, danger-associated molecular patterns; APCs, antigen-presenting cells.

Newcastle Disease Virus Activates the Immune Response

As mentioned earlier, NDV selectively infects tumor cells and rapidly replicates in tumor cells to directly dissolve tumors (Fiola et al., 2006). Significantly, NDV oncolysis reshapes the tumor microenvironment (TME), transforming cold tumors into hot tumors (Burman et al., 2020). This process is beneficial for immune cells to infiltrate tumors. On the one hand, NDV induces the release of the risk-related molecular model of strong antitumor immunity after oncolysis, such as TAAs, PAMPs, and DAMPs (**Figure 2**). These key risk–related molecular models can activate not only some innate immune cells (NK cells) but also tumor-specific T cells ($CD4^+$ and $CD8^+$ T cells) and recruit APCs into the tumor to initiate an immune response (Schild et al., 1989; Ricca et al., 2018). Remarkably, upregulation of many immune checkpoint molecules (CTLA-4 and PD-1) has been observed on $CD4^+$ and $CD8^+$ T cells in recent years (Zamarin et al., 2014; Nakao et al., 2020). This suggests the possibility of combining NDV and immune checkpoint inhibitors to break immune resistance. On the other hand, the activated non-specific immune cells kill and devour infected tumor cells that are not lysed or resistant to viral oncolysis (Fuertes et al., 2011); when the inflammatory response to NDV infection helps the immune system clear tumors, it also causes immune cells to clear NDV, limiting antitumor effects (Buijs et al., 2014). As a result, developing NDV-based cancer regimens necessitates striking a balance between appropriate viral replication, tumor lysis, and immune response activation.

Newcastle Disease Virus Mediates the Apoptosis Pathway

Apoptosis usually occurs as a defense mechanism, such as in the immune response or when cells are damaged by harmful substances (Norbury and Hickson, 2001); while NDV can induce apoptosis to dissolve tumors (**Figure 3**). The oncolytic selectivity of NDV on tumor cells depends on tumor cell resistance to apoptosis (Mansour et al., 2011). NDV infection induces the apoptosis of tumor cells mainly through the exogenous and the endogenous pathways (mitochondrial-related pathways) (Liao et al., 2017; Song et al., 2019). Tumor cells infected with NDV can cause the release of cytokines such as IFN-α, IFN-β, and TNF-α, which activates the NF-kB signaling pathway, which in turn stimulates the exogenous apoptotic pathway (Wilden et al., 2009; Elankumaran et al., 2010). Furthermore, compared with normal cells that can secrete both IFN-α and IFN-β, tumor cells infected with NDV strain AF2240 only release IFN-β (Ch'ng et al., 2013). A study by Ghrici et al. (2013)revealed that the mitochondrial-related pathway may

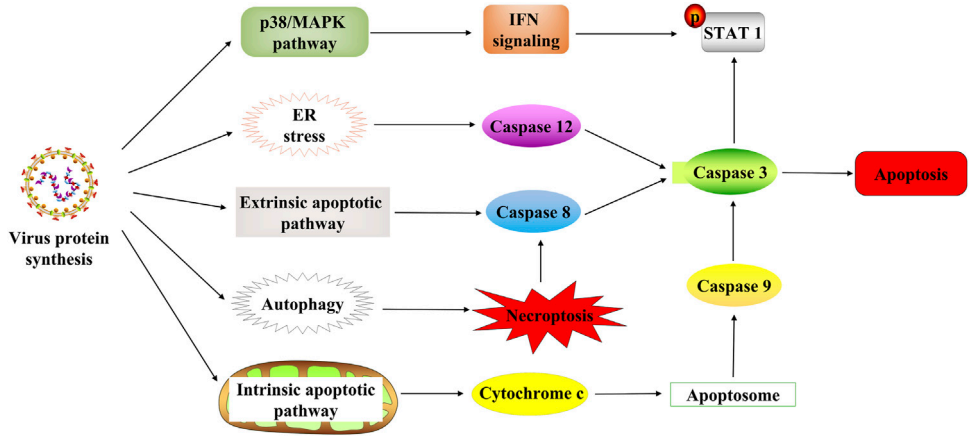

FIGURE 3 | NDV-induced cell death in tumor cells. NDV regulates cell death through multiple mechanisms after infection with tumor cells, including the p38/MAPK pathway, ER stress, apoptosis pathway, and autophagy pathway. STAT1, signal transducer and activator of transcription 1; ER, endoplasmic reticulum.

be the central activator in NDV-induced apoptosis. They found that AF2240 infected cells activated the opening of mitochondrial transition pores, resulting in the activation of caspase-8 and then the viral NP gene expression. Therefore, the apoptosis-inducing effect of NDV may be independent of virus replication and protein synthesis. In 2015, the p38/MAPK pathway was fully elucidated in NDV-mediated apoptosis (Ch'ng et al., 2015). In NDV-infected tumor cells, phosphorylation of p38 mitogen–activated protein kinase (MAPK) was increased by proinflammatory cytokines during infection. This cytoplasmic stimulation degrades the inhibitor of NF-κB, thus releasing NF-κB (Lawrence, 2009). Furthermore, NDV stimulates the immune system to produce cytokines such as IFN-λ (Bu et al., 2016a), which targets phosphor-STAT1 degradation to block IFN-I signaling (Qiu et al., 2016) and exerts an antitumor effect. Thus, the IFN responsiveness may provide a detection indicator for virotherapy (Pease and Kratzke, 2017). ER stress contributes to the antiviral response to NDV by inducing and increasing apoptosis (Bu et al., 2016b; Shokeen et al., 2021). ER stress reduces viral replication due to eIF2α phosphorylation and induces an alternative caspase 12-dependent programmed cell death response (Bu et al., 2016b; Yan et al., 2018).

Newcastle Disease Virus Regulates Autophagy

Autophagy is an evolutionarily conserved intracellular process that influences cellular immune responses (Su et al., 2015). At the same time, autophagy is associated with various diseases such as cancer (Smith and Macleod, 2019). The NDV infection also induces tumor-specific autophagy (**Figure 3**). Many recent studies have focused on the critical role of autophagy in the viral treatment of cancers (Huang et al., 2018; Mattoscio et al., 2018). In addition, NDV exploits the autophagic processes to facilitate their replication, enhancing oncolysis against tumor cells, often leading to tumor necroptosis (Cheng et al., 2016). Furthermore, NDV promotes viral replication *via* autophagy by inhibiting caspase-dependent apoptosis in cancer cells. Because NDV-induced apoptosis, host immune response, and autophagy affect NDV replication in cancers, it is reasonable to conclude that apoptosis and autophagy are mutually regulated (Pei et al., 2016; Ravegnini et al., 2017; Zhang et al., 2017). Previous research study has revealed that the oncolytic NDV strain NDV/FMW promotes apoptosis in lung cancer cells and facilitates oncolysis in resistant tumor cells suggesting a link between apoptosis and autophagy. Its effect is amplified by the pharmacological regulation of autophagy (Hu et al., 2015). Thus, NDV induces autophagy in apoptotic pathways through the regulation of autophagic activity (**Figure 3**). Then autophagy inhibits apoptosis and contributes to NDV infection in cancer cells, activating immunity responses *in vivo* and eventually killing the tumor cells.

PRECLINICAL APPLICATION OF NEWCASTLE DISEASE VIRUS IN VARIOUS CANCERS

NDV has been widely used in preclinical research as a novel anticancer drug for numerous solid tumors and resistant cancers (**Table 1**). Combining NDV therapy with various cancer medications may fully activate the innate and adaptive antitumor immunity based on the inherent oncolytic capabilities of NDV and its interaction with the immune system. This part summarizes NDV's use in the preclinical treatment of various cancers.

Gastric Cancer

Gastric cancer is one of the most common malignancies in the digestive system and the second leading cause of cancer-related deaths worldwide, with an approximately overall 5-year survival rate of 30% (Ma et al., 2019; Zhao et al., 2019). Although significant development has been achieved in the treatment of gastric cancer, the prognosis of most patients with

TABLE 1 | NDV strains in the treatment of different cancers in preclinical trials.

Cancer type	NDV strain	Combination	Outcome	Reference
Gastric cancer	NDV (F3aa)	—	There was no gross tumor in six (40%) NDV-treated mice, and the nodules were significantly smaller than untreated mice	Song et al. (2010)
	rL-hIFN-λ1	—	rL-hIFN-λ1 inhibited the growth of gastric cancer cell lines which contained the IFNλ-R1 receptors and accelerated cancer cell apoptosis	Bu et al. (2016a)
	NDV-D90	—	NDV-D90 induced gastric cancer cell apoptosis and reduced cell invasion in a dose-dependent manner in the highly differentiated gastric cancer cell line	Sui et al. (2017)
Liver cancer	rNDV-18HL	—	rNDV-18HL selectively replicated in orthotopic HCC xenografts, which induced tumor necrosis, reduced intrahepatic metastasis, and prolonged the survival in mice	Wei et al. (2015)
	NDV/Anh-IL-2	—	NDV/Anh-IL-2-treated animals exhibited significantly increased numbers of tumor-infiltrating lymphocytes	Wu et al. (2016)
	LaSota	Fludarabine	The combination of fludarabine with NDV significantly improved NDV-mediated antitumor immunity and prolonged survival in a mouse model of HCC	Meng et al. (2019)
	AF2240 and V4-UPM	5-Fluorouracil	The combination of NDV and 5-fluorouracil had greater antitumor efficacy than NDV or 5-FU alone	Assayaghi et al. (2019)
Lung cancer	rL-RVG	—	The growth of A549 cells in the rL-RVG group was inhibited more effectively than those infected with the wild-type NDV strain	Yan et al. (2015)
	NDV/FMW	Chloroquine	Treatment of spheroids with the autophagy inhibitor chloroquine increased NDV/FMW-induced cytotoxicity	Hu et al. (2015)
Breast cancer	AMHA1	—	NDV is replicated efficiently in cancer cells and spares normal cells and induces morphological changes and apoptosis in breast cancer cells	Al-Ziaydi et al. (2020a)
	AF2240	—	Breast cancer cells in allotransplanted mice treated with AF2240 showed a noticeable inhibition of tumor growth and induced apoptotic-related cytokines	Raihan et al. (2019)
	AMHA1	2-Deoxyglucose	The combination therapy group induced the highest rate of tumor growth inhibition (100%), followed by the NDV group (96.8%)	Zhao et al. (2008)
Cervical cancer	LaSota	—	NDV treatment significantly reduced the viability of cervical cancer cells and inhibited tumor growth by inducing ROS-mediated apoptosis	Keshavarz et al. (2020a)
	NDV HB1	—	Peritumoral injection of NDV oncolysate induces robust antitumor immune responses in the mouse model	Mozaffari Nejad et al. (2021)
Colorectal cancer	R2B Mukteshwar	—	Significant tumor lytic activity was evident when R2B Mukteshwar was injected *via* the intratumoral route	Sharma et al. (2017)
	rAF-IL12	—	rAF-IL12 regulated the immune system and increased the expression levels of apoptosis-related genes in HT29 tumor-bearing nude mice	Syed Najmuddin et al. (2020)
Prostate cancer	NDV/FMW	—	In nude mice bearing prostate tumors, the tumors injected with the supernatants of NDV/FMW-infected cells grew smaller than mock-treated tumors	Wang et al. (2020)
Glioblastoma	LaSota	Temozolomide	The combination of NDV-LaSota and temozolomide (TMZ) was effective in inducing apoptosis of glioma cells *in vitro* and *in vivo*	Bai et al. (2018)
	MTH-68/H	Mesenchymal stem cells	NDV induces dose-dependent cell death in glioma cells and a low level of apoptosis and inhibition of self-renewal in glioma stem cells	Kazimirsky et al. (2016)
Melanoma	NDV-NS1	Vanadyl sulfate	NDV, in combination with vanadyl sulfate, significantly increased the number of immune cells and resulted in rapid tumor regression in the B16-F10 mouse model	McAusland et al. (2021)
Clear cell renal cell carcinoma	AF2240	—	AF2240 induced the activation of the p38 MAPK/NF-κB/IκBα pathway in clear cell renal cell carcinoma, which resulted in cell death due to apoptosis	Ch'ng et al. (2015)
Orthotopic glioma	NDV HB1	—	NDV HB1 treatment significantly prolonged median survival (50%) and induced a long-term, tumor-specific immunological memory response	Koks et al. (2015)

[a]*NDV(F3aa): the mutant NDV strain with the F cleavage site is modified with three amino acids; rL-hIFN-λ1: the recombinant NDV strain LaSota containing human IFN-λ1 gene; NDV-D90: the NDV strain that was isolated from natural sources in China; rNDV-18HL: the recombinant NDV Italien expressing the chimeric HAb18 antibody; rNDV/Anh-IL-2: the recombinant NDV* Anhinga *strain expressing IL-2 cytokine; NDV/FMW: the oncolytic NDV strain FMW; NDV AMHA1: the attenuated strain AMHA1 of NDV; AF2240: the NDV strain AF2240 that was isolated by the Malaysian Veterinary Research Institute in 1960; NDV HB1: the avirulent, non-lytic Hitchner B1 strain of NDV; R2B Mukteshwar: the R2B Mukteshwar strain of NDV; rAF-IL12: the recombinant NDV-AF2240 strain expressing IL-12 cytokine; MTH-68/H: the live attenuated oncolytic viral strain of the NDV; NDV-NS1: the recombinant fusogenic NDV expressing the influenza virus NS1 protein.*

gastric cancer remains poor (Liu et al., 2020). Fortunately, the established NDV strains can effectively target and kill gastric cancer cells and activate immune responses (Ma et al., 2019; Liu et al., 2020; Wang et al., 2020), improving tumor treatment efficacy. To detect the oncolytic effect of NDV in gastric cancer, NDV–GFP was constructed by Wong et al. (2010)by inserting the enhanced green fluorescent protein (EGFP) gene, which is a reporter gene. Their study revealed that the GFP-expressing cells counterstained positive for the carcinoembryonic antigen expression in peritoneal lavage samples from gastric adenocarcinoma patients undergoing staging laparoscopy.

Furthermore, NDV–GFP may provide a more sensitive method than conventional cytology for detecting gastric cancer. Bu et al., 2019a and Bu et al., 2019breported that the recombinant LaSota strain expressing rL-RVG (rabies virus glycoprotein) suppressed nAChRs (nicotinic acetylcholine receptors) to reduce cell migration and EMT (epithelial to mesenchymal transition) in gastric cells. Moreover, rL-RVG suppressed the growth of gastric cancer subcutaneous tumor cells *in vivo* (Bu et al., 2019a).

As previously stated, NDV infection results in the release of multiple cytokines, including type I interferon (IFN), interleukin 1 (IL-1), and tumor necrosis factor-alpha (TNF-α) *in vitro* and *in vivo* (Shobana et al., 2013). Meanwhile, it has been confirmed that NDV strains armed with IFN or IL gene result in higher oncolytic efficacy in tumor cells (Mohamed Amin et al., 2019). For gastric cancer, the presence of various polymorphisms for genes coding IL-2, which is associated with poor prognosis in gastric cancer patients, might provide a therapeutic target to inhibit gastric cancer progression (Bai et al., 2014; Andersen et al., 2017). Thus, NDV has an excellent application prospect in immunotherapy for gastric cancer. In addition, it is reported that immune cells with improved survival and prognosis induce immunological memory in the gastric cancer cells and enhance tumor regression (Wong et al., 2010; Lam et al., 2011). In humans, the progression of gastric cancer is associated with the immune function of specific lymphocytes, such as NK cells (Liu et al., 2015; Subhash et al., 2015; Zhao et al., 2018). Cytokines are secreted after the NDV infection of tumor cells, which causes NK cells to become activated. The activated NK cells promote the cytokine release and the activation of other immune cell functions (Bie et al., 2016). To summarize, NDV has a severe toxic effect on gastric cancer cells. NDV-activated cytokines and immune cells, on the other hand, increase antitumor cytotoxic activity against gastric cancer cells and are subsequently predicted to cure gastric cancer.

Liver Cancer

Liver cancer is the fifth most frequent cancer worldwide and the fourth contributor to cancer-related death globally (Mokrane et al., 2020; Ioannou, 2021). Curative resection or liver transplantation is the primary treatment for individuals with liver cancer, but therapeutic success is still poor (Huge et al., 2020). Therefore, novel treatment strategies are urgently needed to eliminate cancer cells effectively. Chen et al. (2016) demonstrated that a recombinant DNA vaccine containing the NDV HN gene inhibits hepatocellular carcinoma cell proliferation (HCC). Furthermore, it induces autophagy *via* the mitochondrial pathway *in vitro* and *in vivo*. This indicates that NDV-based cancer therapy is a promising candidate for liver cancer treatment.

In addition, modified NDV can significantly improve the therapeutic efficacy of NDV in the liver cancer model (Song et al., 2007; An et al., 2016). For some examples, NDV/Anh-TRAIL promotes the mouse liver cancer model to produce immune memory and protect mice from further malignant tumor challenges (Wu et al., 2017). IFN-stimulated gene (ISG)-12a mediates this process, but high basal ISG-12a may inhibit the replication and infection of NDV (Liu et al., 2014). An NDV vector with an L289A mutation inside the NDV F gene can improve NDV's oncolytic action on HCC cells *in vitro* and *in vivo*, indicating promising potential (Altomonte et al., 2010). NDV expressing the chimeric antibody (cHAb18) against tumor-associated antigen CD147 inhibits HCC cell migration and invasion, induces tumor necrosis, and prolongs the survival time of mice (Wei et al., 2015).

Combination therapy, which offers more significant advantages than single-drug therapy, is becoming an increasingly essential aspect of anticancer therapies (Nastiuk and Krolewski, 2016; Martin and Bell, 2018). The combination of NDV therapy and traditional/non-traditional therapies may become a novel choice for HCC treatment. A recent study confirms that fludarabine as an adjuvant enhances the antitumor immunity of NDV-mediated HCC treatment (Meng et al., 2019). Also, the combination treatment of NDV with 5-FU has greater antitumor efficacy than treatment with NDV or 5-FU alone (Assayaghi et al., 2019). Although OVs can strongly trigger immune activation, a negative feedback is usually upregulated in TME (Reale et al., 2019). Meng et al. (2020)indicated that dichloroacetate improved NDV-mediated viral immunotherapy for HCC by reducing the negative immunological feedback and boosting viral replication. These data suggest that more research into the clinical transformation of NDV in immunotherapy for liver cancer is essential.

Lung Cancer

Non–small cell lung cancers (NSCLCs) account for 85% of lung cancer cases and are the leading cause of cancer death (Tan et al., 2021). Increasing evidence suggests that NDV, in addition to direct oncolysis, mediates lung cancer cell proliferation by controlling the cell immune response (Ye et al., 2018; Shao et al., 2019). NDV-D90, an NDV strain isolated from natural sources, exerts an antiproliferative effect in A549 cells (human lung cancer cell lines) (Fu et al., 2011). Another NDV strain, RL-RVG, decreased tumor growth, subcutaneous tumor necrosis, tumor apoptosis, and increased clusters of differentiation (CD)3-/CD49 + NK cells in the tumor-bearing mice group (Yan et al., 2014). These findings emphasize the significance of NDV eliciting an antitumor immune response in lung cancer treatment. Furthermore, multiple studies have found that the occurrence and progression of cancer are linked to the deregulation of a range of microRNAs (miRNAs) (Hao et al., 2011; Che et al., 2020). The overexpression or suppression of miR-204 was substantially associated with NDV-induced oncolysis in A549 cells (Liang et al., 2021). Therefore, targeting some key miRNAs may provide a new direction for cancer therapy.

Autophagy is a defensive reaction to the cellular stress, such as viral infection. NDV inhibits mitophagy to increase viral replication by inhibiting intrinsic apoptosis (Meng et al., 2014). The induction of ICD determinants by NDV was significantly reduced when autophagy-related genes were knocked out in lung cancer cells (Ye et al., 2018). Moreover, the treatment of lung cancer spheroids with the autophagy inhibitor chloroquine increases NDV/FMW-induced cytotoxicity (Hu et al., 2015), indicating NDV may be a potential strategy for targeting lung cancer stem cells. These

findings imply that NDV combined with autophagy modulators helps improve NDV's cancer therapeutic activity.

Breast Cancer

According to the most recent cancer statistics, breast cancer is currently the most frequent malignancy in women and one of the significant causes of death worldwide (Sung et al., 2021). Breast cancer can be classified based on immunohistochemical markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Xupeng et al., 2021). Despite considerable advancements in breast cancer treatment, patients with triple-negative breast cancer (TNBC) have restricted treatment options due to a lack of recognizable specific markers (Kalscheuer et al., 2019). NDV represents a great potential candidate in the treatment of breast cancer. According to the Kalantari et al. (2020)study, NDV killed breast cancer cells by triggering the intrinsic apoptotic pathway, characterized by elevated Bax, caspase-9, and caspase-3. NDV-D90 induced apoptosis by differentially modulating the expression of ERα and GPER in ER-positive/negative breast cancer cells exposed to estrogen, respectively (Shan et al., 2021).

Furthermore, NDV-AF2240, as an ideal inducer of apoptosis, induces the apoptosis of breast cancer cells and is more cytotoxic to breast cancer than other NDV strains (Raihan et al., 2019). These results suggest that NDV promotes breast tumor regression *via* apoptotic-dependent pathways. In addition, the breast cancer cells infected with NDV showed a significant decrease in glycolysis activity (Al-Ziaydi et al., 2020a). NDV also plays an essential role in the combined treatment of breast cancer. The 2-DG (2-deoxyglucose), a kind of glucose analog in combination with NDV, showed more significant tumor growth inhibition than in a single treatment (Al-Shammari et al., 2019). D-Mannoheptulose, a particular hexokinase inhibitor, was employed by Ahmed et al. to prevent glycolysis and increase the antitumor activity of NDV (Al-Ziaydi et al., 2020b). The hemagglutinin–neuraminidase (HN) protein of NDV enables NDV to target breast cancer cells (Al-Ziaydi et al., 2020b) effectively. Therefore, NDV has a promising future in the treatment of breast cancer.

Other Cancers

As an oncolytic agent, NDV has been reported in other types of cancers (**Table 1**), including cervical cancer (Keshavarz et al., 2020a), prostate cancer (Wang et al., 2020), colorectal cancer (Song et al., 2019), and glioblastoma (Abdullah et al., 2014). Cancer is a dynamic disease (Dagogo-Jack and Shaw, 2018), so there are significant differences in cancer cells from different tissue sources, even if there is phenotypic and functional heterogeneity among cancer cells in the same tumor (Meacham and Morrison, 2013). In addition, NDV has other killing mechanisms in different cell lines (Ginting et al., 2019; Li et al., 2019), suggesting that we should carry out the targeted treatment when developing NDV therapy. MSCs (mesenchymal stem cells) represent a potential delivery method (Uder et al., 2018). For instance, Mohsen K et al. found that an MSC-engineered system significantly reduced tumor growth, enhancing $CD8^+$ T-cell cytolysis responses and splenic cytokine responses. This finding demonstrates that MSCs expressing oncolytic NDV may be a viable method for cancer immunotherapy (Keshavarz et al., 2020b).

CLINICAL APPLICATION OF NEWCASTLE DISEASE VIRUS

Increasing clinical evidence indicates that oncolytic NDV as a therapeutic agent (a type of immunotherapy) can eliminate glioma, metastatic cancer, and advanced solid tumor cells while stimulating patients' immune systems as well (**Table 2**).

The major NDV strains evaluated for direct human injection are PV-701 (Pecora et al., 2002), 73-T (Cassel and Garrett, 1965), MTH-68/H (Csatary et al., 1999), and ATV-NDV (Schirrmacher and Fournier, 2009), which are lytic and HUJ (Yaacov et al., 2008), which is non-lytic. In 1964, Wheelock and Dingle (1964) first reported the use of NDV in the treatment of human cancer. After a patient with acute myeloid leukemia was continuously inoculated with the NDV Hickman strain, the number of leukemia cells decreased rapidly, and the symptoms improved, which lasted for nearly 2 weeks (Wheelock and Dingle, 1964). In the following year, a study by William Cassel and his colleagues showed that patients with stage II and III melanoma resected with NDV-73T strain oncolysis were vaccinated with improved overall survival (Cassel et al., 1977; Murray et al., 1977; Cassel et al., 1983). Long-term follow-up of these patients showed a 10-year survival rate of more than 60% and a 15-year survival rate of 55% compared with historical controls (Cassel and Murray, 1992; Batliwalla et al., 1998). This is the early use of NDV-based tumor vaccines for active tumor–specific immunity. Liang et al. later confirmed using an autologous NDV–modified tumor cell vaccination to treat gastrointestinal cancers. They compared 310 patients with stage I–IV colorectal cancer who received resection and immunotherapy with 257 patients who received chemotherapy with resection alone. The median overall survival of the vaccine group was more than 7 years, while that of the resection group was 4.46 years (Liang et al., 2003; Burman et al., 2020). In non-controlled experiments, adjuvant immunization with autologous NDV–modified cancer cells was safe and advantageous.

Immune checkpoint inhibitors are one of the most promising agents in tumor therapy in recent years (Nettelbeck et al., 2021). Durvalumab is a selective, high-affinity, human IgG1 monoclonal antibody that blocks programmed death-ligand 1 (PD-L1) binding to programmed death 1 (PD-1) (Stewart et al., 2015). Recombinant NDV (MEDI5395) expressing granulocyte-macrophage colony-stimulating factor (GMCSF), based on the strain NDV-73T, is being evaluated with intravenous administration (NCT03889275) in conjunction with durvalumab in patients with various advanced malignant tumors (Burke et al., 2020). Other recombinant NDVs are at different stages of development and are expected to enter clinical practice in the next few years. Meanwhile, NDV can be armed with foreign genes *via* the reverse genetic technology to achieve more effective and diverse antitumor effects. The combination of genetic engineering NDV with computational approaches may be beneficial to enhance the efficacy of clinical cancer treatment (Lathwal et al., 2020).

TABLE 2 | NDV strains for different cancer treatments in clinical trials.

NDV strain	Reference	Cancer	Phase	Patient	Outcome
ATV-NDV-αHN-αCD28	Schirrmacher et al. (2015)	Colorectal cancer	Phase I	Fourteen patients whom all suffered from stage IV colorectal cancer (with distant metastases)	The decrease in CEA in four patients and the partial response of metastases in four patients were observed. Seven patients were still alive in 2009
ATV-NDV	Steiner et al. (2004)	Glioblastoma	Phase III	Twenty-three patients with a pathologically confirmed glioblastoma	91% of vaccinated patients survived 1 year, 39% survived 2 years, and 4% were long-term survivors
ATV-NDV	Karcher et al. (2004)	Head and neck squamous cell carcinoma	Phase III	Twenty patients with pathologically confirmed head and neck squamous cell carcinoma	Percentages of survival of vaccinated patients with stage III and stage IV tumors (n = 18) were 61% at 5 years
MTH-68/H	Csatary et al. (2004)	Glioblastoma multiforme	Phase I	Four patients with advanced high-grade glioma	All patients (n = 4) with advanced high-grade glioma were treated with MTH-68/H, resulting in survival rates of 5–9 years
NDV-73T	Batliwalla et al. (1998)	Melanoma	Phase II	Fifty-one patients with AJCC stage III melanoma	The 10-year survival of the NDV-73T group of patients was more than 60%, and the overall 15-year survival was 55%, with no adverse reactions
NDV-HUJ	Freeman et al. (2006)	Glioblastoma multiforme	Phase I/II	Eleven patients with glioblastoma multiform based on histology	Toxicity was minimal, with grade I/II constitutional fever seen in five patients. One patient achieved a complete response (1/11)

[a]*ATV-NDV: the NDV-modified autologous tumor vaccine; ATV-NDV-αHN-αCD28: the ATV-NDV strain expressing the anti-CD28 fusion protein, coupled to viral HN anchor molecules; NDV-73T: the mesogenic strain of NDV.; MTH-68/H: the live attenuated oncolytic viral strain of the NDV; NDV-HUJ: he NDV strain isolated from naturally attenuated B1 NDV vaccine strain.*

CONCLUSION

The tumor is a recalcitrant disease that poses a severe threat to human life and health. NDV acts as a potent oncolytic agent by causing apoptosis, autophagy and necrosis in tumor cells, limiting cell metabolism, and generating a series of immunological responses. At the same time, it has essentially no effect on human normal cells. NDV is also one of the few viruses that have been found to produce partial or even complete responses when treated with a single medication. The persistence of these responses suggests that the virus's therapeutic effect may depend not only on direct oncolysis but also on the virus's potential to promote long-term immunity. With the development of virotherapy, the activation of the immune responses through cancer virotherapy may eradicate tumors. NDV currently shows great promise in preclinical and clinical trials.

NDV replication occurs in the cytoplasm and does not integrate into the genome of the host, maintaining the safety of the parental virus. The oncolytic property of NDV is either lytic or non-lytic that only infect cells with a disturbed interferon system, which improves the safety of NDV as a vaccine. NDV does not need to be armed with foreign genes to have a strong antitumor effect and stable expression of foreign genes. The combination of NDV virus therapy and traditional/new tumor treatment techniques has been reported and has broad application prospects. However, many questions about NDV therapy, such as those about other OVs, remain unresolved, including the practical techniques of administration, the best genetic engineering strategies, the therapeutic sequence of immune checkpoint inhibitors, and the best combination partners. There is currently no conventional optimum method for how and when patients should use the virus. The tumor microenvironmental barrier and the cytoplasmic matrix of solid tumors may interfere with and inhibit virus invasion and replication, reducing its oncolytic action. Excessive foreign genes will affect the replication of NDV. Moreover, the preparation of NDV needs deep purification to obtain clinical-grade virus preparation.

Cancer patients are usually immunocompromised, while immunocompromised patients may benefit more from OV therapy. For example, cancer patients infected with COVID-19 have low levels of antibodies against the spike protein. An oncolytic vaccine based on the spike protein not only has a strong antitumor effect but also may be beneficial to the prevention of COVID-19. Further understanding of the immunological system may help develop more effective oncolytic NDV and the elimination of the NDV treatment barrier in solid tumors. The combination of NDV therapy and traditional/non-traditional therapies may become a novel choice for cancer treatment. Combining NDV viral therapy with existing immunotherapy, which uses NDV's effect on the immune response, may result in a higher antitumor effect. As a result, NDV is likely to be an ideal tumor therapeutic agent in the future.

AUTHOR CONTRIBUTIONS

Conceptualization, FH, YW, and GR; writing—original draft preparation, FH, CD, YZ, and YZ; writing—review and editing, FH, YW, and GR; supervision, YW and GR; project administration and funding acquisition, FH and YW.

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Glucose Deprivation Induced by Acarbose and Oncolytic Newcastle Disease Virus Promote Metabolic Oxidative Stress and Cell Death in a Breast Cancer Model

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Cancer cells are distinguished by enhanced glucose uptake and an aerobic glycolysis pathway in which its products support metabolic demands for cancer cell growth and proliferation. Inhibition of aerobic glycolysis is a smart therapeutic approach to target the progression of the cancer cell. We employed acarbose (ACA), a particular alpha-glucosidase inhibitor, to induce glucose deprivation combined with oncolytic Newcastle disease virus (NDV) to enhance antitumor activity. In this work, we used a mouse model of breast cancer with mammary adenocarcinoma tumor cells (AN3) that were treated with ACA, NDV, and a combination of both. The study included antitumor efficacy, relative body weight, glucose level, hexokinase (HK-1) level by ELISA, glycolysis product (pyruvate), total ATP, oxidative stress (ROS and reduced glutathione), and apoptosis by immunohistochemistry. The results showed significant antitumor efficacy against breast cancer after treatment with combination therapy. Antitumor efficacy was accompanied by a reduction in body weight and glucose level, HK-1 downregulation, inhibition of glycolysis products (pyruvate), total ATP, induction of oxidative stress (increase ROS and decrease reduced glutathione), and apoptotic cell death. The findings propose a novel anti–breast cancer combination involving the suppression of glycolysis, glucose deprivation, oxidative stress, and apoptosis, which can be translated clinically.

Keywords: glucose deprivation, oncolytic virotherapy, oxidative stress, apoptosis, cancer

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INTRODUCTION

Even in the presence of oxygen, breast cancer cells rely on fermentative aerobic glycolysis rather than oxidative phosphorylation, which requires huge amounts of glucose to create energy and support metabolic function (i.e., the Warburg effect) (Zheng, 2012). Cancer cells grow rapidly compared to normal cells, requiring an increase in ATP to meet their metabolic demands (Romero-Garcia et al., 2011). Cancer cells (in comparison to normal cells) exhibit signs of oxidative stress. Aerobic glycolysis benefits cancer cells by generating fewer reactive oxygen species (ROS). (Cairns et al., 2011). Thus, upregulation of glycolysis may be an adaptive response of cancer cells to increase ATP production in an oxygen-deprived environment and promote mitochondrial resistance to pro-

apoptotic permeabilization. Pro-apoptotic factors (e.g., ions, proteins, ROS) are downregulated, while antiapoptotic factors (e.g., Bcl-2, ANT2, and chaperones) and antioxidant enzymes are upregulated (Indran et al., 2011). When ROS levels are exceedingly high, p53 launches a failsafe apoptosis program, boosting ROS levels *via* the mitochondrial apoptosis pathway to assure cell death (Liu et al., 2008). Numerous cancer therapies are based on inhibiting this metabolic pathway. It is well-established that glucose deprivation has a detrimental effect on cancer glycolysis and may even result in cell death (Gatenby and Gillies, 2007). Withdrawal of glucose initiates a positive feedback loop in which NADPH oxidase and mitochondria generate reactive oxygen species (ROS), and protein tyrosine phosphatases are inhibited by oxidation and ROS-mediated cell death (Graham et al., 2012).

Acarbose is a glucoregulatory drug; it induces glucose deprivation *via* competitive alpha-amylase and alpha-glucosidase inhibitors, retards the digestion of complex dietary carbohydrates in the small intestine's brush border, and reduces the rapid rise in blood glucose following a meal (postprandial) (Chiasson et al., 2003). Acarbose is safe and well-tolerated, with a low incidence of adverse effects. Acarbose recipients' most common adverse events were gastrointestinal (abdominal pain, flatulence, and diarrhea) (Neuser et al., 2005; He et al., 2014). Besides its role as an FDA-approved medication for type II diabetes and hyperglycemia, acarbose has been explored as a calorie restriction mimetic (CRM) in longevity/healthy aging studies (Gibbs et al., 2018). CRMs are agents that mimic the benefits of caloric restriction (e.g., increased longevity and delayed onset of age-related illnesses) without restricting calorie consumption. To substantiate this, acarbose was found to prolong the lifespan of mice (Harrison et al., 2019) and is associated with a dose-dependent decline in the frequency of colon cancer in type II diabetic patients (Tseng et al., 2015).

Virotherapy is an intelligently targeted therapy since it enters and eliminates cancer cells while sparing healthy tissue. Oncolytic virotherapy using NDV was found safe even with extremely high doses in experimental animals and humans in clinical trials (Freeman et al., 2006; Schirrmacher, 2016; Ali et al., 2021; Al-Shammari et al., 2022). The viral dose injected into the tumor mass multiplies and is replicated unless eliminated by the immune system (Kirn et al., 2001; Al-Shammari et al., 2014a). Using virotherapy alone has thus far been unable to eradicate malignancies in animal and clinical trials. The most effective approach to completely eradicate the tumor is combining oncolytic virus therapies with other treatment options like gene therapy and radiation/chemotherapy (Chu et al., 2004). Oncolytic virus-based cancer virotherapy has been shown to be successful when combined with chemotherapies and radiotherapy (Harrington et al., 2010; Al-Shammari et al., 2016). Although malignant tumors are generally incurable diseases, oncolytic virotherapy research (for treatment with viruses that infect and kill cancer cells) develops quickly (Ottolino-Perry et al., 2010). Combination therapy aims to attack tumor cells *via* multiple approaches to prevent cancer cells from acquiring resistance to treatment (Kumar et al., 2008). NDV has been used to treat breast cancer by inhibiting glycolysis and downregulating GAPDH and hexokinase-2 (Al-Shammari et al., 2019; Al-Ziaydi et al., 2020a). The present study aims to investigate using acarbose, a glucosidase inhibitor, to induce glucose deprivation to increase breast cancer cell sensitivity to oncolytic NDV and understand the mechanisms of the combination therapy that enhances cell death.

MATERIALS AND METHODS

The Virus

The Iraqi attenuated NDV strain (Iraq/Najaf/ICCMGR/2013) named AMHA1 (Al-Shammari et al., 2014b) was provided by the Cell Bank Unit, Experimental Therapy Department, Iraqi Center of Cancer and Medical Genetics Research (ICCMGR), Mustansiriyah University. The Iraqi AMHA1 strain was propagated in the embryonated chicken eggs (Al-Kindi Company, Baghdad, Iraq), harvested from allantoic fluid, and then purified from debris through centrifugation (3,000 rpm, 30 min at 4°C). NDV was quantified through a hemagglutination test, aliquoted, and stored at −80°C. Viral titers were determined based on 50% tissue culture infective dose titration on Vero cells following the standard procedure (Hindi et al., 2017).

Animals

Swiss Albino female mice were housed under the ICCMGR protocols. The scientific committee of Baghdad University, College of Veterinary Medicine, authorized all experimental protocols, including ethical approval (2651/DA in 17/12/2019 from the College of Veterinary Medicine/University of Baghdad).

Experimental Design

This experiment utilizes the breast cancer model, the mammary adenocarcinoma tumor known as AN3 (Al-Shammari et al., 2008). The tumor was allotransplanted in inbreed mice, which allowed for its continuous propagation in mice. Tumors were established by injecting AN3 cells (1×10^6/100 µl per site) into the right flanks of female Swiss Albino mice aged 6–8 weeks. When the tumor nodules attained a diameter of 0.5–1 cm, the animals were separated into four groups of 10 randomly: First group: mice in this group were injected with i/p of 0.9% normal saline and received a normal diet (control group); second group: mice in this group received acarbose 1,000 ppm with diets daily (Harrison et al., 2019); third group: mice in this group were injected with NDV 70,000000 intratumorally in a single dose (Al-Shammari et al., 2019); and fourth group: mice in this group received acarbose 1,000 ppm with diets daily for 18 days plus NDV 70,000000 intratumorally in a single dose. After 18 days, the mice were anesthetized and killed with a fatal dose of chloroform. *In vivo* experiments were repeated twice.

Evaluation of Antitumor Efficacy

The tumor diameters were measured every third day, and their sizes were determined using calipers. The tumor volume was determined as the mean SD for each group using the formula

(product of 0.5 length breadth width) (Al-Shammari et al., 2011). To calculate tumor growth, the tumor volume was standardized to the volume of each tumor at time zero, corresponding to the start of therapy. During the evaluation period, tumor growth inhibition (TGI) was determined twice weekly using the following formula (Phuangsab et al., 2001):

GI% = tumor volume in the untreated group—tumor volume in the treated group/tumor volume in the untreated group x100 (1).

A tumor growth inhibition of greater than 50% was regarded as significant.

Relative Body Weight

The bodyweight of each mouse was weighed every third day using a sensitive balance. The relative body weight was calculated as RBW = (bodyweight on a measured day)/(bodyweight on day 0) × 100.

Glucose Levels

Blood glucose concentrations were quantified using a glucometer and test strips (Contour, Japan). The blood sample was obtained from the tail vein during the amputation of the tail.

Hexokinase-1 Enzyme Quantification

To determine the concentration of the hexokinase enzyme, the mouse mammary adenocarcinoma tissue sample was weighed and homogenized in PBS on ice, followed by 5-min centrifugation at 5000 g to get the supernatant. The Hexokinase enzyme concentration was measured using a quantitative ELISA kit (Elabscience, United States) following the manufacturer's instructions for a Hexokinase-1 assay.

Pyruvate Assay

The pyruvate concentration was measured using a colorimetric assay with a pyruvate assay kit (Elabscience, United States). On ice, a weighted mouse mammary adenocarcinoma tissue sample was homogenized in normal saline. To extract the supernatant, the tissue homogenate was centrifuged for 10 min at 3,500 g.

ATP Assay

ATP contents were determined using a colorimetric method using an ATP assay kit (Elabscience, United States). A fresh mouse mammary adenocarcinoma tissue sample was weighed and cut into pieces, then added to boiled distilled water and incubated in a boiling water bath for 10 min, and then mixed fully for 1 min. The samples were centrifuged for 10 min at x10000 g. The supernatant was collected for measurement (according to the manufacturer's instructions). The principle of the detection kit is that creatine kinase catalyzes creatine and adenosine triphosphate to generate creatine phosphate, then identified by phosphomolybdic acid colorimetry.

Reactive Oxygen Species Assay

ROS was measured through a fluorometric method by using the ROS assay kit (Elabscience, United States). The level of intracellular ROS was monitored in the mouse mammary adenocarcinoma tissue in the treated and control animals.

The recommended protocol was started by preparing a single-cell suspension using enzymatic digestion. This is carried out by immediately taking the mouse mammary adenocarcinoma tissue into a precooled Reagent 3 working solution and cleaning the blood and other contaminants from the tissue. The massive compositions like fiber, fat, and blood vessels were removed. The remaining tissue was minced into about 1 mm3 piece with ophthalmic scissors; then, we immersed these pieces in precooled Reagent 3 working solution to remove the cell debris. Then, we added an appropriate amount of digestion enzyme and incubated it in a 37°C water bath for 20–30 min. To stop the digestion, we added Reagent 3 working solution. After that, we filtered the mixture to remove the massive tissue component using nylon mesh (300 mesh) and collected only the cells. The cell suspension was centrifuged at 500 g for 10 min, and the supernatant was discarded; then, the cell pellet was washed with Reagent 3 working solution two times. Finally, cells were resuspended to prepare the single-cell suspension solution. The cell amount was about 10^6.

The fluorescent probe was added by adding the Reagent 1 working solution to the cells. The DCFH-DA working concentration was 20 μM. The solution is now incubated at 37°C for 30 min. After that, we collected the incubated single-cell suspension and centrifuged it at 1,000 g for 10 min to collect cells. These cells were washed with Reagent 3 working solutions two times. Later, we centrifuged the cell suspension and collected the cell precipitation and further resuspended the collected cells with Reagent 3 working solution for detection. Fluoresce intensity was determined at an excitation wavelength of 502 nm and emission wavelength of 525 nm using a fluorescence microplate reader. However, this method has some limitations (Dikalov and Harrison, 2012).

Reduced Glutathione Assay

Reduced GSH was measured in the mouse mammary adenocarcinoma tissue using a colorimetric method through a reduced glutathione assay kit (Elabscience, United States) catalog no: E-BC-K097-M. A fresh tissue sample was collected and washed with normal saline, the water on the tissue surface was absorbed, and the tissue sample was weighted, and a buffer solution with a protein precipitator was added. 10% homogenate was prepared by mechanical homogenization on an ice bath, centrifuged for 10 min at x10,000 g, and then the supernatant was collected for detection. GSSG is reduced to GSH by glutathione reductase; GSH reacted with DTNB to produce GSSG and TNB yellow color. The amount of yellow TNB was determined by the amount of reduced glutathione. The reduced glutathione was calculated by measuring the optical density value at 412 nm.

Detection of Cleaved Caspase-3

An immunohistochemistry assay was used to study the cleaved caspase-3 in tumor sections using a conventional avidin-biotin-immunoperoxidase protocol (Elabscience, United States). Tumor samples were fixed in neutral-buffered formalin 10% and processed to prepare paraffin-embedded tissue sections in a standard procedure. Before incubation with the primary

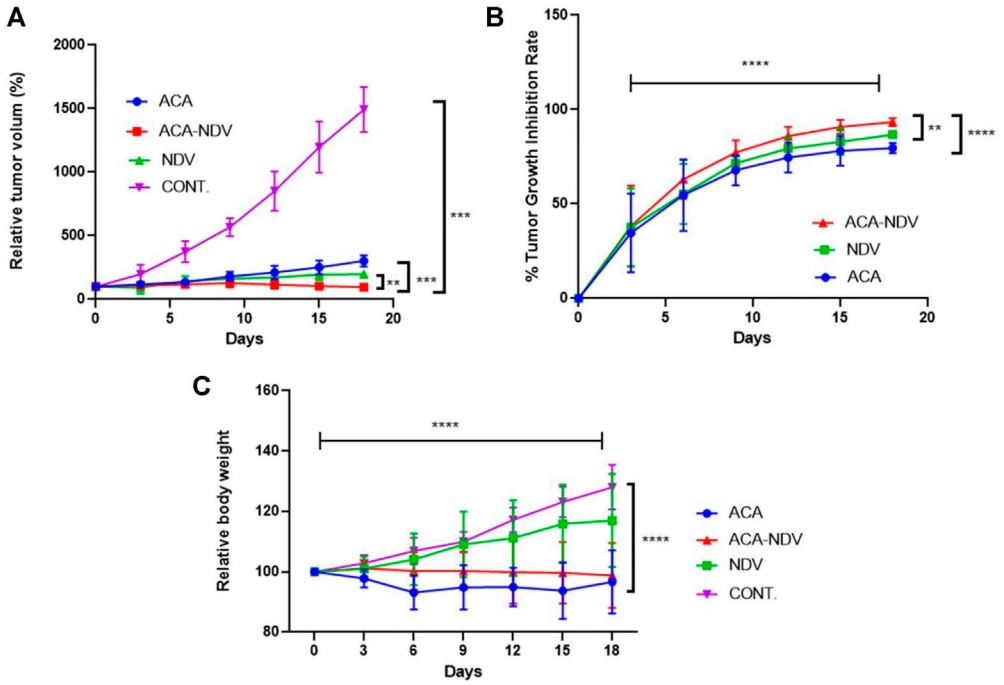

FIGURE 1 | Antitumor efficacy of acarbose (ACA), Newcastle Disease Virus (NDV), and a combination of both against a mammary adenocarcinoma AN3 *in vivo* model. **(A)** Relative tumor volumes over 18 days were plotted. Compared to the control group, ACA, NDV, or a combination of both generated a significant ($p < 0.0001$) decrease in relative tumor volume. Compared to both monotherapy groups, the combination therapy of acarbose and Newcastle Disease Virus (ACA-NDV) demonstrated significantly greater tumor size reduction. **(B)**e Growth inhibition curve demonstrated that the combined therapy group had the highest overall tumor growth inhibition, followed by the NDV group. The ACA group showed the least growth inhibition. In the control group, the tumors continued to grow during the experiment. **(C)** ACA efficiently reduced body weight in mice bearing breast cancer. Bodyweight measured after treatment with ACA, NDV, and combination every 3 days indicates that ACA induces a marked decrease in body weight. By contrast, NDV did not induce a significant effect on body weight. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$versus CONT.

antibody (1:50 dilution as supplied by the manufacturer), tissue sections were exposed to heat-induced epitope retrieval by incubation in a water bath with pH 6 and at 98°C (40 min) in a vegetable steamer, followed by cooling at room temperature and treatment with 3% hydrogen peroxide before antibody application and then treatment with rabbit polyclonal anti-cleaved caspase -3 antibody for 30 min at room temperature. Later, samples were washed with phosphate-buffered saline and incubated again with a labeled streptavidin-biotin reagent. Immunoreactive products were visualized with the DAB reaction. The sections were counterstained with hematoxylin for 2 min. The optical density (OD) of cleaved caspase-3 was determined using the FIJI image analysis tool using pictures covering all the sections. The following formula was used to determine the OD values: OD = log(maximum intensity/mean intensity), with a maximum intensity equal to 255 (Mustafa et al., 2015).

Statistical Analysis

All data analyses were performed with Graph Pad Prism version 8.01 (GraphPad software. CA, United States) and Excel version 10. Data were analyzed using one-way ANOVA analysis, which was used to perform comparison between groups. All data were presented as mean and standard deviation. The significance level was set at *p 0.05, **p 0.01, and ***p 0.001.

RESULT

Acarbose Markedly Enhances the Antitumor Efficacy of Newcastle Disease Virus

We conducted an *in vivo* experiment to examine the efficacy of the combined treatment of ACA-NDV compared to monotherapies on tumor volume of the mouse breast cancer model. Relative tumor volume was plotted over an 18-day treatment period, as shown in **Figure 1A**. All treatment modalities resulted in a statistically significant ($p < 0.0001$) decrease in tumor volume compared to the untreated control group. The ACA–NDV combined treatment significantly reduced tumor size ($p < 0.0001$) compared to the ACA and NDV mono treatment groups. Additionally, the combination therapy group achieved the highest tumor growth inhibition rate (93.34%), followed by the NDV group (86.75%). As shown in **Figure1B**, the lowest growth inhibition rate was found in the

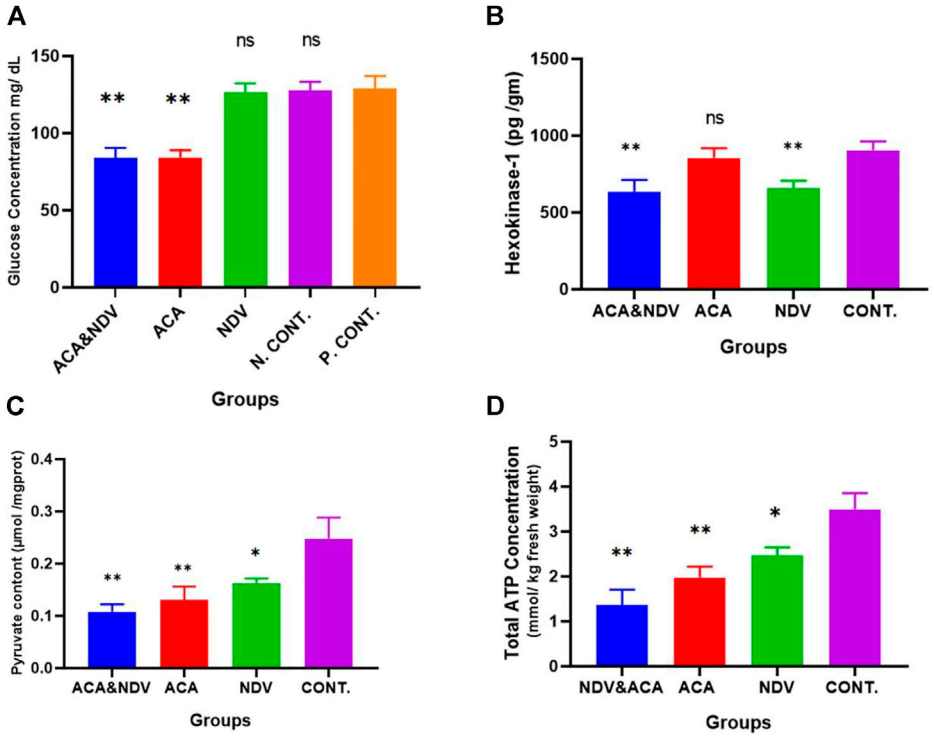

FIGURE 2 | ACA induces glucose deprivation. **(A)**, Glucose measured after Acarbose (ACA) treatment, Newcastle Disease Virus (NDV), and a combination of Acarbose and Newcastle Disease Virus (ACA-NDV), ACA exhibited a significant ** $p < 0.01$ decreased level of glucose in ACA and ACA-NDV groups compared with the positive control (P. CONT) (bearing breast cancer) and negative control N. CONT. (healthy mice) groups. **(B)** ELISA assay quantified the concentration of the Hexokinase enzyme. We observed a significant decrease in the expression of the HK-1 protein in the NDV- and ACA-NDV-treated group compared to the untreated control group. ACA alone had no significant impact on HK-1 concentration. **(C)** Measurement of pyruvate content. **(D)** Measurement of total ATP concentration. In combination with NDV, ACA efficiently inhibits glycolysis product (pyruvate) and ATP. To confirm the effect of ACA and NDV combination therapy on glycolysis products, we examined pyruvate and total ATP level in tumor tissue. We found that the ACA-NDV treatment had significantly reduced glycolysis product (pyruvate) and ATP compared to the untreated group. *** $p < 0.01$, ** $p < 0.01$, and * $p < 0.05$ versus Cont.

ACA group (79.64%). Tumors continued to grow in the untreated control group throughout the experiment.

Combined Acarbose-Newcastle Disease Virus Treatment Efficiently Maintains Mouse Body Weight Compared to Acarbose Alone

The study aimed to explore whether ACA, NDV alone, or a combination reduced body weight in mice bearing breast cancer. Combined ACA-NDV treatment efficiently maintains mouse body weight compared to ACA alone, while ACA treatment resulted in a noticeable decline in body weight (**Figure 1C**). By contrast, NDV alone did not affect body weight.

Acarbose and Combined Acarbose-Newcastle Disease Virus Treatments Induce Glucose Deprivation

We next sought to determine whether there is a relationship between ACA, NDV, and its combination with glucose deprivation. We detected glucose concentration in the blood to confirm the effect of ACA and NDV on the glucose level. As expected, compared with the control group, ACA exhibited a decreased level of glucose (**Figure 2A**). Furthermore, combined ACA-NDV treatment exhibited the same level of glucose level reduction. NDV alone does not affect glucose levels in the blood.

Newcastle Disease Virus-Acarbose Combined Treatment Efficiently Decreases Hexokinase Enzyme Level in Tumor Tissue

The present study identified and quantified HK-1 enzyme expression in the tumor tissue after treatment. The ELISA assay was used to assess the enzyme quantity according to the manufacturer's protocol. The HK enzyme level was compared between treated and untreated groups (**Figure 2B**). We detected a significant drop in the expression of the HK-1 enzyme in the (NDV and ACA–NDV) treated groups compared to the untreated control group (659.88, 636.09, and 904.22 pg/ml). The results indicated that the combination of ACA-NDV considerably decreased the HK-1 enzyme concentration. ACA alone had no noticeable effect on the concentration of HK-1. These findings imply that NDV may play a critical role in suppressing glycolysis metabolism in cancer.

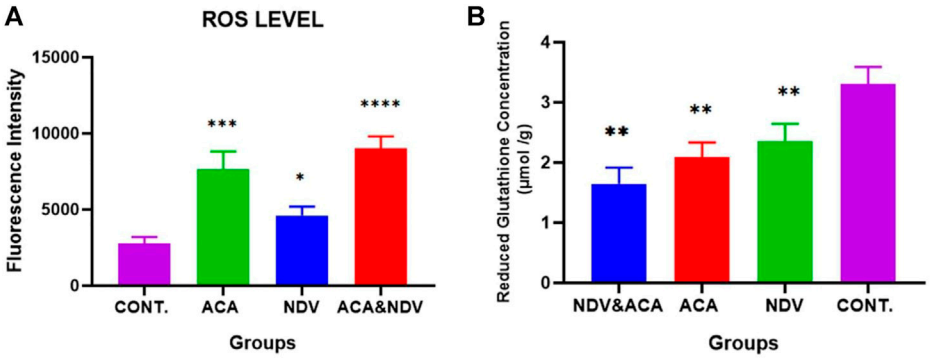

FIGURE 3 | ACA and NDV combination induces metabolic oxidative stress. We detected ROS and reduced GSH in breast cancer tissue, and we observed that ROS levels were significantly increased in treated groups (ACA, NDV, and ACA-NDV) than in the untreated groups. Reduced GSH levels were lower in ACA-NDV combination therapy than in the untreated group. **(A)** Measurement of ROS level. **(B)** Measurement of reduced glutathione concentration. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ versus CONT.

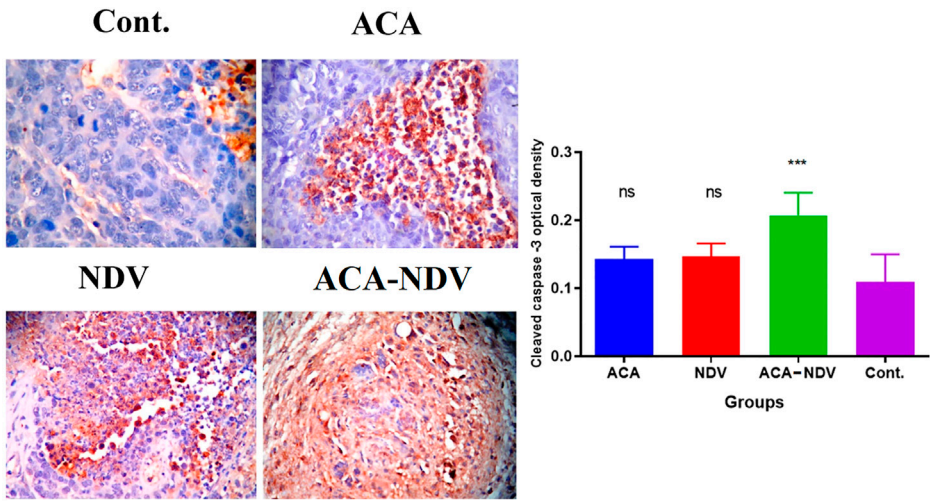

FIGURE 4 | ACA-NDV combination therapy induces apoptosis, confirmed by immunohistochemistry analysis for cleaved caspase-3. Results showed a mild effect on breast cancer cells in monotherapy (ACA and NDV alone)-treated groups, while the combination therapy of ACA-NDV has more cas-3 expression than the single therapy. *** $p < 0.001$ versus Cont.

Acarbose Suppresses Glycolysis Product (Pyruvate) and Total ATP Effectively in Breast Cancer When Combined With Newcastle Disease Virus

The impact of ACA-NDV combination therapy on glycolysis products was investigated. We examined the pyruvate and total ATP level in tumor tissue. We observed that ACA-NDV efficiently decreased glycolysis product (pyruvate **Figure 2C** and ATP **Figure 2D**) compared to the control group and better than monotherapy modalities.

Acarbose-Newcastle Disease Virus Combination Treatment Induces Oxidative Stress

To investigate whether ACA-NDV induced oxidative stress in tumor tissue, we measured ROS and reduced GSH in breast cancer tissue. We observed high ROS levels in the treated groups than in the control untreated group, but ACA-NDV combined therapy induced higher levels of ROS than monotherapies (**Figure 3A**). Reduced GSH levels were lower in ACA-NDV combination therapy than in other treated and untreated groups (**Figure 3B**).

Acarbose-Newcastle Disease Virus Combination Therapy Induces Apoptosis

The efficacy of ACA-NDV to induce apoptosis in breast cancer tissue was proven by immunohistochemistry analysis for the cleaved caspase-3 levels of expression. Combination therapy of ACA-NDV induced higher expression levels for cleaved caspase-3 than single therapy and untreated control groups. Single-therapy groups had no significant cleaved caspase-3 expression levels compared with the untreated control group (**Figure 4**).

DISCUSSION

The findings of this study demonstrate that a combination of glucose deprivation (using ACA) and virotherapy using oncolytic NDV can synergistically suppress breast cancer growth and induce breast cancer cell apoptosis *in vivo*. The combination of ACA and NDV was more effective at inhibiting the glycolysis pathway and inducing oxidative stress than each treatment administered alone. Breast cancer is associated with malignant tumors that lead to poor prognosis in women (Soerjomataram et al., 2008). Chemotherapy and radiotherapy for breast cancer have limited efficacy (Hickey et al., 2013). Cancer cells possess enhanced glycolysis and reduced oxidative phosphorylation (OXPHOS) capacity (Zheng, 2012). Aerobic glycolysis is preferred because it generates less ROS than mitochondria (Cairns et al., 2011). The increase in the uptake of glucose by cancer cells for use as a carbon source for anabolic processes, including nucleotides, proteins, and lipids, is needed to support cell proliferation (Liberti and Locasale, 2016). Cancer cells generate energy primarily by increasing the rate of glycolysis by 200 times than that of normal cells of origin (Alfarouk et al., 2014). In the presence of extracellular glucose and robust glucose transport, glycolysis drives more rapid ATP production (albeit less efficient) than ATP production *via* mitochondrial oxidative phosphorylation. Aerobic glycolysis also benefits cancer cells because it generates less ROS and allows the cells to adapt to the intermittently hypoxic conditions prevalent in a poorly vascularized tumor. The decreased glucose concentration in the cancer cell leads to pushing and activating mitochondrial oxidative phosphorylation, which causes increased ROS because of the defect in mitochondria. At the same time, it decreases ATP production because of reduced pyruvate levels due to glucose deprivation (Cairns et al., 2011). Recently it was discovered that glucose deprivation induced oxidative stress and cytotoxicity in cancer cells (Ahmad et al., 2005).

This study showed a reduction in blood glucose concentration in ACA-treated groups compared with the untreated control groups. Our results confirm the findings of an earlier study that ACA causes a reduction in glucose levels (Mustafa et al., 2015), resulting in induced glucose deprivation. Moreover, ACA (glucose deprivation inducer) reduced relative body weight compared with the control group. ACA inhibits alpha-amylase and alpha-glucosidase; therefore, delayed absorption of complex carbohydrates from the intestine leads to decreased glucose level and body weight (Zhang et al., 2020).

We measured relative tumor volume and tumor growth inhibition to determine whether free ACA or combination with NDV has an antitumor effect. We found that the ACA-NDV combination significantly reduced tumor volume compared with the untreated group. This combination had higher tumor growth inhibition than monotherapies. The Iraqi NDV AMHA1 strain recently showed anticancer properties through glycolysis pathway inhibition and apoptosis induction (Al-Ziaydi et al., 2020a). In addition to glycolysis inhibition, NDV has several antitumor mechanisms. One of these mechanisms activates the immune system by inducing cytokine secretion (IL-2 and IFN-gamma) and attracts CD56 natural killer and CD8 cytotoxic lymphocytes into infected cancer tissue (Washburn and Schirrmacher, 2002; Al-Shammari et al., 2011). Moreover, NDV is replicated within the AN3 tumor mass for many cycles after intratumoral injection, which leads to activation of caspase-3 in cancer cells (Hickey et al., 2013); therefore, this mechanism reduces tumor volume and enhances the antitumor efficacy of NDV. The current study demonstrates that ACA decreases tumor growth, and the antitumor effect may be due to ACA-induced glucose deprivation, which leads to increased ROS formation and creates oxidative stress that activates apoptosis (Graham et al., 2012).

HK1 has a key role in the glycolysis pathway at the first step *via* converting glucose to glucose-6-phosphate. Previous studies reported that the glycolysis-related gene (HKI) was overexpressed and participated in tumorigenesis; it acts as a poor prognosis biomarker in many cancers (He et al., 2016; Dai et al., 2020). Thus, we conducted HK-1 quantification by ELIZA assay. The result showed that ACA had a nonsignificant effect on HK-1 levels, which may be because ACA is an alpha-glucosidase inhibitor and acts majorly in the intestine (Kalra, 2014). Nevertheless, groups infected with NDV revealed a reduction in HK-1 levels. Our result is similar to the previous report confirming that NDV inhibits the activity of HK (Al-Ziaydi et al., 2020b). It has been reported that NDV may downregulate other glycolysis-related enzymes, such as fructose-bisphosphate aldolase C (ALDOC) and phosphoglycerate kinase (PGK). This downregulation is explained as an alteration in protein expression during the NDV infection process, which may support the control host responses to virus invasion through cell signaling pathways controlling to regulate the infection course (Deng et al., 2014).

To further confirm that this combination of ACA-NDV inhibits the glycolysis pathway in breast cancer cells, we measured products of glycolytic pathway levels (pyruvate and ATP) in breast cancer tissue. The findings revealed that the ACA-NDV combination treatment suppresses pyruvate and ATP compared with the monotreatments and the untreated group. Depending on the glucose result, ACA decreases glucose concentration, resulting in reduced pyruvate concentration in groups treated with ACA (Walton et al., 1979). Pyruvate-level reduction in ACA-NDV may be due to this combination of decreased HK activity and diminishing pyruvate concentration (Al-Ziaydi et al., 2020a). The inhibition of glycolysis causes a decline in pyruvate formation and thus a depletion of ATP (Al-Ziaydi et al., 2020b).

In support of our hypothesis, we determined intracellular ROS formation results in ACA treatment (glucose deprivation

inducer) and infection with NDV in breast cancer tissue. We found that intracellular ROS formation increased in the combination ACA-NDV group compared with the control group. ACA alone and NDV alone also increased ROS levels to lesser degrees than combined ACA-NDV treatment.

Our results suggest that glucose deprivation induced by ACA and oncolytic NDV can activate a positive response loop, including intracellular ROS generation by mitochondria and NADPH oxidase, which was described individually by others (Ahmad et al., 2005; Keshavarz et al., 2020). In addition, our result of ACA-NDV treatment induced oxidative stress, which led to reduced GSH depletion in the treated groups.

Pyruvate and NADPH have involved glucose metabolism products from glycolysis and pentose cycle; this product functions as anti-hydroperoxide. Pyruvate removed ROS through a direct reaction with hydrogen peroxide; this causes the decarboxylation of pyruvate to produce acetic acid and converts H_2O_2 to H_2O (Nath et al., 1995). In addition, NADPH was utilized as a cofactor for glutathione reductase to reduce glutathione disulfide and then detoxify ROOH and H_2O_2 by glutathione peroxidases (Sies et al., 2017). Therefore, the increased uptake of glucose by cancer cells is necessary to overcome increased intracellular ROS generated from metabolic-, genetic-, and microenvironment-associated alterations in cancer cells (Ghanbari Movahed et al., 2019). In correlation with this mechanism, we noticed that the ACA-NDV combination diminished reduced GSH. A decline in the pentose phosphate pathway accompanies glucose deprivation, dysfunction of glutathione synthesis and ROS accumulation, and a decrease in the NADPH and intracellular GSH (Zhu et al., 2020). The present study finding is consistent with previous works that reported NDV inhibition of glutathione synthesis, underexpression of glutathione peroxidase, and accumulation of ROS in tumor cells (Kan et al., 2021; Obaid et al., 2021).

Cancer cells escape the apoptotic pathway through various means, including mitochondrial pathway impairment, underexpression of pro-apoptotic proteins, and overexpression of anti-apoptotic proteins (Kluck et al., 1997; Pfeffer and Singh, 2018). Therefore, ACA (glucose deprivation inducer) and NDV synergize to overcome cancer resistance to apoptosis. In the current study, cleaved caspase-3 detection using immunohistochemistry showed that the ACA-NDV combination was the best inducer for apoptosis compared with ACA alone or NDV alone. Previous works reported that NDV induces apoptosis in caspase-dependent, caspase-independent, and endoplasmic reticulum pathways (Al-Shammari et al., 2012; Mohammed et al., 2019). A recent report postulated that NDV induces ferroptosis in tumor cells exposed to nutrient deprivation (Kan et al., 2021). Moreover, ACA-induced glucose deprivation displays cleavage of caspase and caspase substrates, which induces apoptosis (Caro-Maldonado et al., 2010; Obaid et al., 2022). Also, GD-induced stress promotes both TRAIL-RD/DR2 and receptor-mediated apoptosis (Iurlaro et al., 2017). In addition, glucose deprivation induces inhibition of glycolysis, leading to lack of proton provision and mitochondrial electron transfer chain constant proton consumption to generate energy. This deficiency in the proton is compensated by lysosomes through proton efflux, leading to an increase in lysosomal pH, resulting in necrosis or apoptosis depending on alkalinization extent (Cui et al., 2017).

In conclusion, this study's results strongly support the novel hypothesis that ACA induces glucose deprivation with virotherapy synergizing to promote metabolic oxidative stress and apoptosis. This study is the first to report that ACA-induced glucose deprivation synergizes with oncolytic NDV, featuring a very smart glycolysis pathway targeting safe and effective therapy. This novel combined therapy has a strong translational capacity in clinical therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by (2651/DA in 17/12/2019 from the College of Veterinary Medicine/University of Baghdad).

AUTHOR CONTRIBUTIONS

Conception of the work: AMA-S and KKK; collection of data: QAO and AMA-S; analysis of data: QAO, AMA-S, and KKK; writing of the manuscript: QAO, AMA-S, and KKK; final approval of the final draft: AMA-S and KKK.

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Evolving role of seneca valley virus and its biomarker TEM8/ANTXR1 in cancer therapeutics

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Oncolytic viruses have made a significant inroad in cancer drug development. Numerous clinical trials are currently investigating oncolytic viruses both as single agents or in combination with various immunomodulators. Oncolytic viruses (OV) are an integral pillar of immuno-oncology and hold potential for not only delivering durable anti-tumor responses but also converting "cold" tumors to "hot" tumors. In this review we will discuss one such promising oncolytic virus called Seneca Valley Virus (SVV-001) and its therapeutic implications. SVV development has seen seismic evolution over the past decade and now boasts of being the only OV with a practically applicable biomarker for viral tropism. We discuss relevant preclinical and clinical data involving SVV and how bio-selecting for TEM8/ANTXR1, a negative tumor prognosticator can lead to first of its kind biomarker driven oncolytic viral cancer therapy.

KEYWORDS

seneca valley virus, oncolytic virus, drug development, TEM8/ANTXR1, neuroendocrine tumors, neuroendocrine carcinomas, solid tumors

Introduction: The promise of oncolytic viruses in cancer therapeutics

Immunotherapy has revolutionized the cancer treatment landscape. The development of immune checkpoint inhibitors targeting PD-1/PD-L1 or CTLA4 has improved patient outcomes in a variety of solid tumors (Kraehenbuehl et al., 2022). Chimeric antigen receptor T cells (CAR-T) and bispecific antibodies (bsAbs)/bispecific T-cell engagers (BiTEs) well developed in hematologic malignancies, are now being advanced in solid tumors (Edeline et al., 2021). Ongoing studies are evaluating cancer vaccines as well as a variety of combination therapies. Oncolytic viruses (OVs) represent an exciting and rapidly evolving field within cancer immunotherapies. Interest in using viruses in cancer treatment has been present for many years based on observations that many hematological malignancies temporarily improved with concurrent viral infections (Kelly and Russell, 2007). Recently, interest in OVs and OV combination therapies

TABLE 1 Summary of key preclinical studies of SVV-001 in cancer cell lines and murine models.

References	Study/model	Outcomes
Reddy et al. (2007)	• Cytotoxicity evaluation in multiple tumor cell lines with neuroendocrine properties • Toxicity evaluation in immunocompetent mice • Efficacy evaluation in athymic female tumor bearing mice with tumors derived from SCLC and retinoblastoma cell lines	• Most cell lines with neuroendocrine markers were sensitive to SVV-001 mediated killing, normal human cells were resistant to SVV-001 mediated killing • Toxicity evaluation in immunocompetent mice without dose limiting toxicity. Neutralizing antibodies were noted • Efficacy analysis in athymic mice with promising anti-tumor killing efficacy in a model of tumors derived from SCLC and retinoblastoma cell lines
Wadhwa et al. (2007)	• Cytotoxicity evaluation in multiple tumor cell lines with neuroendocrine properties, including retinoblastoma, glioblastoma, and human embryonic kidney • Efficacy evaluation in murine xenograft model of metastatic retinoblastoma created with injection of human retinoblastoma tumor cells into vitreous	• Cytotoxicity was noted with SVV-001 treatment in retinoblastoma cell lines but not glioblastoma or embryonic kidney cell lines • In the murine xenograft model of metastatic retinoblastoma intravenous administration of SVV-001 decreased extraocular tumor burden and decreased extraocular extension of tumor as compared to controls
Morton et al. (2010)	• Cytotoxicity of SVV-001 evaluated in 23 cancer cell lines • Efficacy evaluation in 36 solid tumor xenograft severe combined immunodeficiency (SCID) murine models	• Cytotoxicity noted with SVV-001 treatment in cell lines from a subset of neuroblastoma, Ewing sarcoma, and rhabdomyosarcoma panels • In solid tumor xenograft murine models of rhabdomyosarcoma and neuroblastoma complete responses were observed with intravenous SVV-001 treatment, responses were also noted in rhabdoid tumor, Wilms tumor, and glioblastoma models
Yu et al. (2011)	• Efficacy evaluation of intravenous SVV-001 in a medulloblastoma orthotopic xenograft Rag2 SCID murine model	• Intravenous SVV-001 injection was associated with anti-tumor activity in medulloblastoma xenograft murine models and prolonged survival • Intravenous SVV-001 injection was associated with killing of cancer stem cells • SVV-001 treatment was associated with autophagy activation • SVV-001 was shown to cross the blood brain barrier *in vivo*
Poirier et al. (2013)	• Efficacy evaluation of intravenous SVV-001 in several classic and variant SCLC heterotransplant models immunosuppressed mice • Analysis of gene expression profiles in SVV-001 permissive tumors as compared to SVV-001 non-permissive tumors	• Efficacy was noted with tumor inhibition in variant SCLC heterotransplant models • SVV-001 permissive tumors were associated with a specific gene profile characterized by elevated NEUROD1 to ASCL1 ratio
Miles et al. (2017)	• Genome wide loss-of-function screens performed to determine factors necessary for SVV-001 infection and replication	• ANTXR1/TEM8 was necessary for SVV-001 infection in neuroendocrine cancer cell lines • In neuroendocrine cancer cell lines, genetic knock out of ANTXR1/TEM8 was shown to drive loss of SVV-001 permissivity • Defective innate immune response was associated with SVV-001 replication
Hallenbeck and Chada (2021)	• Evaluation of efficacy of SVV-001 intratumoral injection combined with anti-PD-1 and anti- CTLA4 checkpoint blockade in an immunocompetent syngeneic pancreatic cancer murine model	• Combination treatment with intratumoral SVV-001 injection with anti-PD-1 and anti- CTLA4 checkpoint blockade led to both significant tumor shrinkage and improved survival

has surged, both with new insights into immunology and with rapid improvements in techniques for genetic engineering of viruses. Talimogene laherparepvec (T-VEC or trade name IMLYGIC™) is an OV based on a modified herpes simplex virus (HSV) type 1 with the addition of a gene encoding human granulocyte macrophage colony-stimulating factor (GM-CSF). The FDA approval of intratumoral injection of T-VEC in advanced melanoma in 2015 was the first in class approval of an oncolytic viral agent and has generated interest in additional trials evaluating OVs and novel OV combinations (Andtbacka et al., 2015; Zhang and Rabkin, 2021).

OV immunotherapy employs viruses that target cancer cells, either due to inherent characteristics of the virus or engineering for tumor selectivity. The primary mechanism of action includes

TABLE 2 Human clinical trials of SVV-001.

References	Study description	Outcomes
Rudin et al. (2011)	Phase 1 dose escalation trial of systemic SVV-001 in adults with advanced cancers with neuroendocrine differentiation ($N = 30$). Primary objectives were toxicity assessment and determination of recommended dose. Secondary objectives included assessment of viral titers and neutralizing antibody titers	SVV-001 was well tolerated with no dose limiting toxicities up to 10^{11} vp/kg. Intratumoral viral replication was detected as well as evidence of disease response in a patient with SCLC. All patients developed neutralizing antibodies
Burke et al. (2015)	Phase 1 dose escalation trial of systemic SVV-001 in children with advanced cancers with neuroendocrine differentiation ($N = 22$). Primary objectives were determination of maximum tolerated dose for SVV-001 as a single infusion (cohort A) or as two consecutive infusions in combination with cyclophosphamide (Cohort B). Secondary objectives included assessment of viral titers and neutralizing antibody titers	SVV-001 was well tolerated, one patient experienced a dose limiting toxicity in Cohort A (pain successfully treated with analgesics). No objective responses were observed. Neutralizing antibodies developed in both cohorts
Schenk et al. (2020)	Phase 2 double blind, placebo controlled trial of systemic SVV-001 in adults with extensive stage SCLC after first line chemotherapy. Primary endpoint was progression free survival (PFS). Secondary objectives include overall survival, response, and presence of neutralizing antibodies and viral clearance	Systemic SVV-001 was not associated with significant change in median PFS. In the SVV-001 group patients who had persistent detection of SVV-001 in peripheral blood 7 or 14 days after treatment had shorter PFS.

two potential pathways 1) selective, replication in, and direct lytic destruction of tumor cells *in situ* and 2) induction of systemic anti-tumor immunity (Kaufman et al., 2015). The specific mechanism of action varies depending upon the viral vector, specific cancer cell type, addition of immune stimulatory agents, and modulation of the tumor microenvironment. Greater than 30 viruses have been evaluated in this setting including herpesvirus, adenovirus, poxvirus, picornavirus, reovirus among others (Cook and Chauhan, 2020). Recombinant engineering allowing enhancement of viral selectivity and response and/or removal of virulence genes has led to the creation of targeted and safe OVs (Boagni et al., 2021). Although direct destruction of tumor cells is key to the mechanism of OVs, recent studies suggest that the immune induction likely plays a more important role in their efficacy (Ramelyte et al., 2021). As OVs target and induce lysis of tumor cells, antiviral signals are triggered in the cells leading to endoplasmic reticulum stress and generation of antiviral cytokines and type I interferons (IFNs) which activate immune cells including antigen presenting cells and cytotoxic $CD8^+$ T cells (Workenhe and Mossman, 2014). As the tumor is destroyed danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) are released further prompting an adaptive immune response by activation of toll like receptors (TLRs) (Malogolovkin et al., 2021). Tumor-associated antigens and neoantigens released by the dying cells cultivate tumor antigen-specific $CD4^+$ and $CD8^+$ T cell responses (Workenhe and Mossman, 2014; Kaufman et al., 2015). However, while OVs may stimulate an anti-tumor immune response this mechanism may also lead to an immune response against the OV including the production of neutralizing antibodies. The balance of anti-tumor and antiviral effects represent an important mediator of the efficacy of OVs (Grillo et al., 2018; Zhang and Rabkin, 2021). Delivery of OV by intratumoral injection in many cases seems to thwart neutralizing antibody inactivation. However, efficient, multiple intravenous administration is still an important goal in bringing this technology to more patients with varying solid cancers and in creating more tolerable therapies.

Seneca valley virus targets neuroendocrine cancers

Seneca valley virus (SVV-001) is a naturally occurring oncolytic picornavirus first discovered in 2002 in a cell culture presumably contaminated with SVV-001 containing porcine trypsin or bovine serum. Soon after discovery, it was found to have selectivity for tumor cells with neuroendocrine properties (Reddy et al., 2007). SVV-001 is a single positive stranded, non-recombinant RNA virus (27 nm) that causes cell death *via* intracellular viral replication, cell lysis, and autophagy, with a replication cycle less than 12 h (Rudin et al., 2011; Burke, 2016). Complete genome sequencing revealed SVV-001 is a picornavirus, within a separate genus now called Senecavirus, closely related to cardioviruses (Venkataraman et al., 2008a; Venkataraman et al., 2008b; Hales et al., 2008). Of note, as an RNA virus there is no chance of insertion into the host genome and no risk of mutagenesis and SVV-001 was recognized soon after discovery as a promising candidate for OV therapy.

Most humans do not have antibodies to SVV-001 and normal, healthy human cells are not infected by SVV-001 (Molecular Theraphy, 2005). In contrast to other oncolytic viral agents under investigation, SVV-001 is not inhibited by normal human blood components (Reddy et al., 2007). The family of Seneca viruses has since been renamed Seneca virus A (SVA). SVA strains have been classified into 3 distinct clades. SVV-001, the original isolate from 2002 is in clade 1 of the Senecavirus genus. This agent, particularly when produced

on the human cell line PER.C6 appears to be non-pathogenic in humans and swine and likely most or all animals (Fernandes et al., 2018). SVA in clades 2 and 3 are causative agents for vesicular disease in pigs (Jayawardena et al., 2019). SVV-001 has several unique features that make it attractive as an OV including: 1) potential targeting of solid tumors with intravenous dosing, 2) RNA virus without insertional mutagenesis, 3) *in vivo* self-replication.

When first identified SVV-001 was found to infect and replicate in cells with neuroendocrine markers, including gastrin releasing peptide receptors, synaptophysin, neuron specific enolase, and CD56 (Reddy et al., 2007; Bolton et al., 2020). However, in the last decade our understanding of the mechanism of specificity of SVV-001 for neuroendocrine cells has rapidly expanded. The tropism of SVV-001 for specific neuroendocrine tumors was explored in a study of SVV-001 in non-permissive small cell lung cancer (SCLC) cell lines. The authors identified a subpopulation of cells infected with SVV-001 in a model of SCLC previously thought to be resistant to infection (Poirier et al., 2012). This is likely due to targeting of cancer stem cells, which in a medulloblastoma orthotopic xenograft mouse model were found to be preferentially targeted by SVV-001 (Yu et al., 2011). Further work seeking to identify markers of infectivity to SVV-001 was done using a mouse model of SCLC. In this study 2 out of 6 mice exposed to a SVV-001 had durable, complete responses to therapy. Gene profiling was done of responders and compared to non-responders. Response to SVV-001 was correlated with a high expression of the transcriptomic regulator neurogenic differentiation factor 1 (*NEUROD1*) and low expression of achaete-scute homologue 1 (*ASCL1*) (Poirier et al., 2013). Of historical interest the tropism of SVV-001 for SCLC cells with low *ASCL1* to *NEUROD1* ratio was one of the initial observations that prompted further investigation into novel subtypes of SCLC, classified by expression of master transcriptomic regulators that are emerging as an important area of investigation and biomarkers of response to treatment. In the classification described by Rudin et al. (2019), SCLC with a low *ASCL1* to *NEUROD1* ratio is labeled as SCLC-N (Gay et al., 2021) (Table 1).

Although SVV-001 was found to target SCLC-N, the details of this interaction are more complex. The specific receptor of SVV-001 was recently discovered when Miles et al. (2017) performed genome wide loss of function screens and identified anthrax toxin receptor 1 (ANTXR1), also known as tumor endothelial marker 8 (TEM8), as the receptor for SVV-001 on tumor cells. The authors also established that TEM8/ANTXR1 expression alone was not sufficient for infective permissibility, and that decreased expression of antiviral IFN genes must also be present. Again, this group confirmed the association with SCLC-N, when they evaluated neurogenic transcription factors in responders and non-responders and also found that the elevated *NEUROD1* and low *ASCL1*, markers of SCLC-N, were associated with downregulation of antiviral IFN gene signaling (Miles et al., 2017). The same group also established that glycosylation of the TEM8/ANTXR1 receptor was necessary for SVV-001 binding, cell entry, and infection (Jayawardena et al., 2021). Although this association was identified in SCLC, it is likely, given TEM8/ANTXR1 is the receptor for SVV-001, that SVV-001 permissive subtypes of other neuroendocrine cancers share similar features to SCLC-N, including elevated TEM8/ANTXR1 and low expression of IFN genes.

TEM8/ANTXR1: A marker of hypoxia, vasculogenic mimicry, and mediator of metastasis

TEM8/ANTXR1 is an integrin-like, transmembrane glycoprotein upregulated in a variety of cancer types, tumor associated stromal cells, and tumor-associated blood vessels (Yang et al., 2011; Evans et al., 2018) (Figure 1). TEM8/ANTXR1 is upregulated in the presence of hypoxia (Opoku-Darko et al., 2007). TEM8/ANTXR1 is unique in its association with tumor vessels but not normal blood vessels (Chaudhary et al., 2012). TEM8/ANTXR1 has been described as a marker for pathological, tumor-associated angiogenesis, which promotes tumor growth and may mediate resistance to therapies targeting angiogenesis (Xu et al., 2021).

Studies have demonstrated that TEM8/ANTXR1 is enriched in triple negative breast cancer (Xu et al., 2021), prostate cancer (Li et al., 2021a), gastric cancer (Li et al., 2021b; Sun et al., 2021), pancreatic cancer (Alcalá et al., 2019), angiosarcoma (Kusaba et al., 2021), colon cancer (Ł et al., 2021), and non-small cell lung cancer (NSCLC) (Gong et al., 2021). In multiple tumor types, upregulation of TEM8/ANTXR1 is a negative prognostic indicator (Li et al., 2021a; Ł et al., 2021; Ding et al., 2021). In triple negative breast cancer, TEM8/ANTXR1, is marker of vasculogenic mimicry, and is associated with poor outcomes (Fernández-Cortés et al., 2019; Xu et al., 2021). Vasculogenic mimicry is a process where tumor cells organize themselves into structures mimicking endothelial cells with functional tubes that can carry red blood cells. This process is driven by hypoxia. The presence of vasculogenic mimicry is associated with poor prognosis of multiple cancer types (Fernández-Cortés et al., 2019; Wei et al., 2021). Early evidence suggests that vasculogenic mimicry is mediated by tumor associated macrophages (Barnett et al., 2016; Rong et al., 2016; He et al., 2021). In addition, overexpression of TEM8/ANTXR1 in the setting of hypoxic tumor microenvironments is associated with the presence of cancer stem cells, increased stem cell self-renewal and increased metastasis in a Wnt pathway dependent mechanism (Chen et al., 2013). The interplay

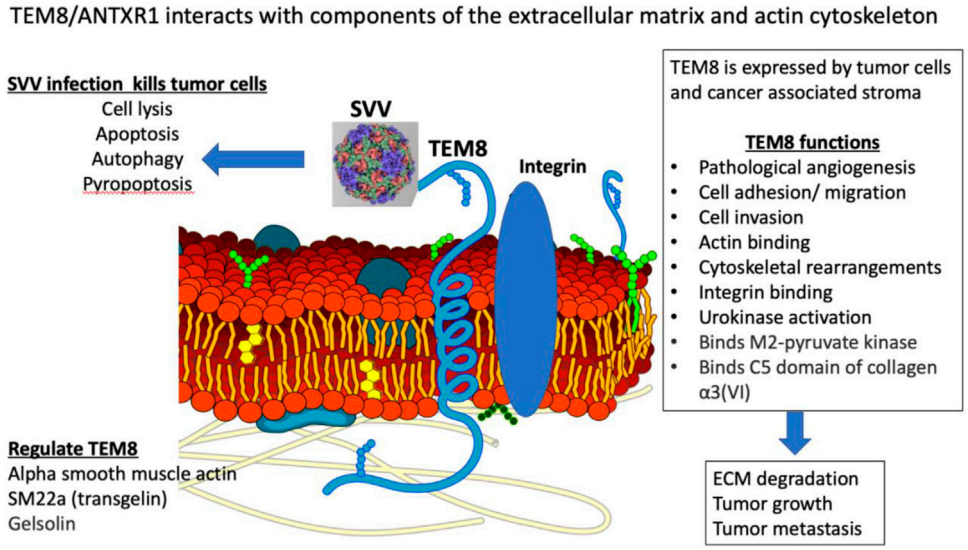

FIGURE 1
Illustration describing TEM8/ANTXR1, its function and anti-tumor effects.

between cancer stem cells, TEM8/ANTXR1, angiogenesis, and tumor associated macrophages is a potentially important area for further studies.

TEM8/ANTXR1 is an adhesion molecule and meditates cell movement by binding to components of the extracellular matrix (ECM) and interacting with the actin cytoskeleton (Hotchkiss et al., 2005; Abdel-Hamid et al., 2019). The specific interaction between TEM8/ANTXR1 and the surrounding cells that mediates increased metastatic potential is not fully understood. TEM8/ANTXR1 interacts with the alpha 3 subunit of collagen VI which has been hypothesized to mediate cell attachment to endothelial cells and influence angiogenesis (Nanda et al., 2004; Hotchkiss et al., 2005; Werner et al., 2006). Although capillary morphogenesis protein 2 (CMG2) or anthrax toxin receptor 2 (ANTRX2) is the main mediator of anthrax toxicity (Liu et al., 2013a), TEM8/ANTRX1 was first identified as another target of anthrax toxin binding, specifically a site of binding of the protective antigen (PA) component of the anthrax toxin. TEM8/ANTRX1 contains a von-Willebrand factor A (vWA) domain that is involved in binding of PA (Bann, 2012). Low density lipoprotein receptor-related protein 6 (LRP6) has also been identified as an important component of the interaction of PA with TEM8/ANTRX1 in a process that also involves the Wnt/β-catenin signaling pathway (Wei et al., 2006; Peröbner et al., 2012). Other studies have also shown a connection between TEM8/ANTXR1 and endothelial cell response to Wnt signaling in cancer, with upregulation of TEM8/ANTXR1 associated with activation of downstream targets of Wnt pathways (Verma et al., 2011). In NSCLC cell lines, TEM8/ANTXR1 promotes metastasis *via* activation of Wnt/β-catenin signaling pathway (Ding et al., 2021). In hepatocellular carcinoma (HCC) cell lines microRNA-493 suppressed tumor cell growth by targeting TEM8/ANTXR1 and R-Spondin 2 (RSPO2) and decreasing activation of the Wnt/β-catenin signaling pathway (Xu et al., 2017). In glioblastomas upregulated TEM8/ANTXR1 is also a negative prognostic factor. Specifically, in a recent preprint, upregulation of hypomethylated TEM8/ANTXR1 genes in glioblastomas is associated with increased proliferation, metastasis, and resistance to chemotherapy and radiotherapy. The authors suggest that TEM8/ANTXR1 upregulation leads to β-catenin induction in a non-Wnt ligand dependent process (Kundu et al., 2022). Additional research is needed to fully clarify the role of TEM8/ANTXR1 in activation of Wnt/β-catenin signaling pathways and the relationship between this pathway and outcomes in different cancer types.

Studies with agents directly targeting TEM8/ANTXR1 have shown promising responses. One study showed genetic disruption of TEM8/ANTXR1 in a variety of human tumor xenograft models including melanoma, breast, colon, and lung cancer led to decreased tumor growth. In addition antibodies against TEM8/ANTXR1 have demonstrated anti-tumor activity and had synergistic effects with other anti-cancer agents (Reddy et al., 2007; Chaudhary et al., 2012). TEM8/ANTXR1 has been developed as a target in CAR-T therapy in breast cancer (Byrd et al., 2018; Petrovic et al., 2019). In preclinical murine models, an antibody-drug conjugate targeting TEM8/ANTXR1 led to tumor regression and improved survival (Szot et al., 2018). Antibodies blocking the TEM8/ANTXR1 extracellular domain inhibit tumor related angiogenesis and tumor growth (Opoku-Darko et al.,

2007; Chaudhary et al., 2012; Gong et al., 2018; Szot et al., 2018). Studies are also evaluating immune-PET imaging agents to identify TEM8/ANTXR1 expression using a radiolabeled monoclonal antibody (Kuo et al., 2014). TEM8/ANTXR1 is a promising biomarker to select patients who may benefit from SVV-001 therapy, and additionally, there may be a role for combination therapy with additional agents that also target TEM8/ANTXR1 and associated pathways.

Although the receptor for SVV-001 has been identified, the role of a type 1 IFN response in SVV-001 efficacy as an OV remains to be fully clarified. Stimulator of interferon genes (STING) plays a major role in mediating type 1 interferon immune responses in viruses and cancer (Jiang et al., 2020). SCLC-N, known to be permissive to SVV-001, has decreased STING induced cytokines as compared to other SCLC subtypes, including reduced CCL5 and CXCL10 as described in the supplementary materials to the recent paper by Gay et al. (2021). In addition to host factors leading to decreased type 1 IFN signaling, SVV-001 itself seems to target local IFN host signaling response. SVV-001 inhibits type 1 IFN response when a SVV-001 associated protease, 3C protease, cleaves mitochondrial antiviral signaling (MAVS), Toll/interleukin 1 (IL-1) receptor domain-containing adaptor inducing IFN-β (TRIF), and TRAF family member-associated NF-κB activator (TANK) leading to loss of pattern recognition receptor (PRR) activation and decreased IFN production (Qian et al., 2017). In addition, SVV-001 has significant deubiquitinating activity which also contributes to the SVV-001's ability to escape innate immune responses (Xue et al., 2018). SVV-001 replication also has been shown to induce degradation of retinoic acid-inducible gene I (RIG-I) a cytoplasmic PRR involved in type 1 IFN response which likely further contributes to decreased IFN production in SVV-001 infection (Wen et al., 2019). Finally, SVV-001 was found to kill tumor cells by inducing apoptosis in a process that involves SVV-001 proteins 2C and 3C protease and activation of caspase 3. This included mechanisms of apoptosis triggering both extrinsic death receptor signaling and intrinsic mitochondrial signaling pathways (Liu et al., 2019). This is particularly important as activation of capase-3 is associated with immunogenic cell death which is a critical component of OV efficacy in the development of anti-tumor immune response (Jaime-Sanchez et al., 2020). Interestingly, in pigs the mechanism of SVV-001 induced cell death differs from humans with induction of pyroptosis, a form of necrotic regulated cell death (Tsuchiya, 2021). In pigs SVV-001 3C protease cleaves porcine gasdermin D inducing pyroptosis (Wen et al., 2021). Taken together this data suggests a process where SVV-001 exploits a cellular environment with low expression of Type 1 IFN response to infect tumors and may also act to contribute this state, however, then infects and destroys the tumor cells leading to immunogenic cell death, which, when used as an OV has the potential synergize with checkpoint blockade to destroy tumors.

Seneca valley virus studies in mice and humans

The initial preclinical and clinical studies of SVV-1 were completed in the 2000s before the current (2017) understanding of the role of TEM8/ANTXR1 as the receptor for SVV-001 had been developed. However, preclinical data for use as an OV in human tumors with neuroendocrine features was extraordinarily promising. In early mouse models of both SCLC and pediatric retinoblastoma a single dose of SVV-001 virus had remarkable efficacy with rapid killing of neuroendocrine tumor cells and minimal toxicity (Reddy et al., 2007). SVV-001 was also evaluated in a murine model of metastatic retinoblastoma and demonstrated that systemic injections of SVV-001 reduced the development of invasive disease as well as reduced central nervous system (CNS) metastatic lesions (Wadhwa et al., 2007). Another study evaluated the efficacy of SVV-001 *in vitro* in 23 cell lines including neuroblastoma, Ewing, sarcoma, and rhabdomyosarcoma panels. SVV-001 demonstrated high efficacy in both *in vivo* and *in vitro* murine models with objective responses most notably in rhabdomyosarcoma and neuroblastoma models (Morton et al., 2010). SVV-001 was evaluated in a murine model of pediatric malignant gliomas and a single injection of SVV-001 led to infection of xenografts without harming normal brain cells. This study also demonstrated efficacy and prolonged survival in permissive mouse tumor models (Liu et al., 2013b). Nonetheless, nearly all of the preclinical *in vitro* studies done with SVV-001 in murine models were somewhat limited as they were done in immunodeficient mice and the behavior of SVV-001 in immunocompetent models was not well defined.

Given the excellent preclinical data suggesting safety and efficacy in mouse models, phase 1 trials of systemic administration of SVV-001 were developed for both adults and children (Table 2). The first trial was a phase 1 dose escalation study in adults with advanced solid tumors with neuroendocrine features. Five cohorts were evaluated with a single intravenous dose of SVV-001 increasing in log increments from 10^7 to 10^{11} viral particles/kg. The primary objectives were assessment of toxicity and determination of recommended dose. Secondary endpoints included serial assessment of viral titers in body fluids and blood and of neutralizing antibody titers. Systemic infusion of SVV-001 was well tolerated, however, several patients in the lowest dose cohort developing flu like symptoms within the first week. In the SCLC patients' viral titers peaked at day 3–4 suggesting a delay in viral clearance, possibly explained by SVV-001 production within cancer cells. Response was evaluated and revealed 1 SCLC patient with rapidly progressive, extensive disease whose disease became stable after SVV-001 treatment with stability that persisted for >10 months. In five other patients with neuroendocrine tumors responses were noted; one patient with a carcinoid

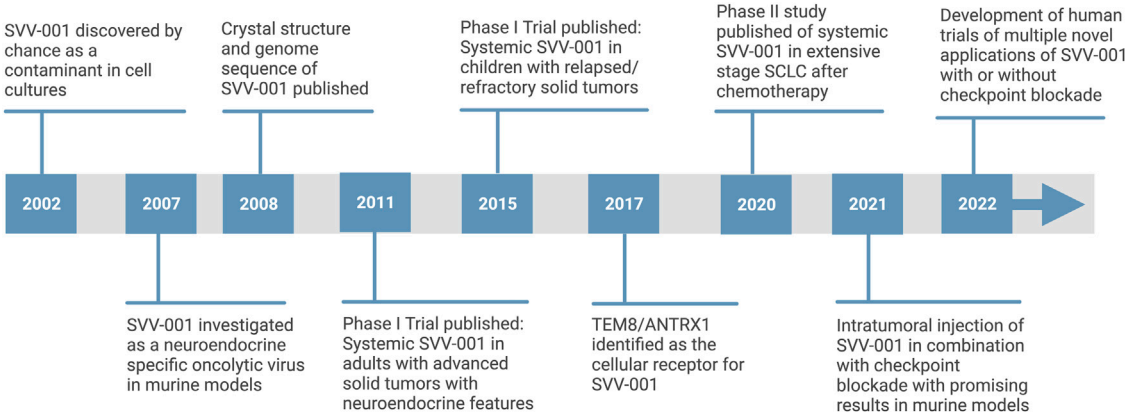

FIGURE 2
Timeline of SVV-001 oncolytic virus development [adapted from "timeline (7 segments, horizontal),"by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates].

tumor had a 50% decrease in tumor size after SVV-001 administration (Rudin et al., 2011).

A phase 1 dose escalation trial was also done of systemic injection of SVV-001 in children with advanced neuroblastoma, rhabdomyosarcoma, or tumors with neuroendocrine features. The trial had 2 cohorts, cohort A was a dose escalation group with 3 increasing dose levels. In Cohort B patients were treated with two doses of SVV-001 given at day 8 and day 29 in combination with oral cyclophosphamide to modulate immune antiviral response. In total, 22 patients were enrolled on the study. No patients had objective responses, 6 of 12 evaluable patients in part A and 4 of 6 evaluable patients on part B had stable disease. All patients in part A cleared SVV-001 from their blood within 3 weeks of treatment. In part B viral titers were cleared within 2 weeks of infusion. Neutralizing antibodies were present in all patients (Burke et al., 2015).

A phase II randomized, placebo controlled study with systemic SVV-001 versus placebo was done in adults with extensive stage SCLC with disease that was either stable or responding after at least 4 cycles of platinum based chemotherapy. The primary endpoint of this study was progression free survival. In this trial 59 patients were randomized to receive SVV-001 versus placebo. Efficacy was assessed at a prespecified interim futility analysis after 40 events. This interim analysis did not demonstrate efficacy with median progression free survival (PFS) of 1.7 months in both study and placebo arms. No significant overall survival (OS) difference was observed. Neutralizing antibodies were detected at 2 weeks in all patients tested, and viral clearance was noted in majority of patients by 14 days after treatment. There were very few patients who had persistent viral titers. Persistent viral titers were attributed to intratumoral replication of SVV-001. Exploratory analysis was performed and delayed clearance of virus was associated with decreased PFS (Schenk et al., 2020). This is now thought to be due to selective viral replication in patients with TEM8/ANTRX1 enriched tumors, which confers poor prognosis in various tumor types.

OVs can be delivered systemically or with direct intra-lesional injection into tumors (Zheng et al., 2019; Cook and Chauhan, 2020). The advantage of systemic administration include ease of administration and improved targeting of metastatic disease (Atasheva and Shayakhmetov, 2021). Prior studies in neuroendocrine cancer models with other OV therapies have demonstrated success with systemic infusions of OVs in combination with other immunomodulatory agents (Inoue et al., 2022). Disadvantages of systemic injection include development of antiviral neutralizing antibodies and cytotoxic T lymphocytes and possible off target adverse effects. The anti-viral immune response likely limits both intratumoral viral infection and anti-tumor efficacy of OVs. The only FDA approved OV, T-VEC, is delivered by intratumoral injection (Andtbacka et al., 2015). Intratumoral injection overcomes the barriers to efficacy from the development of neutralizing antibodies, but makes delivery of the OV more difficult for patients with inaccessible sites of disease.

Multiple early clinical trials showed that SVV-001 is safe with systemic administration (Figure 2). In these three studies, SVV-001 was administered in 1 or 2 IV infusions to a total of 76 patients at doses up to 10^{11} vp/kg. About 49 of these patients received highest dose with just one observed DLT. This DLT was tumor pain, which was successfully treated with analgesics. Although these studies did not show significant response with a systemic administration of SVV-001 as a monotherapy, subgroups of patients, did signal response. As stated previously, all clinical studies were done prior to the discovery that TEM8/ANTXR1 is the receptor for

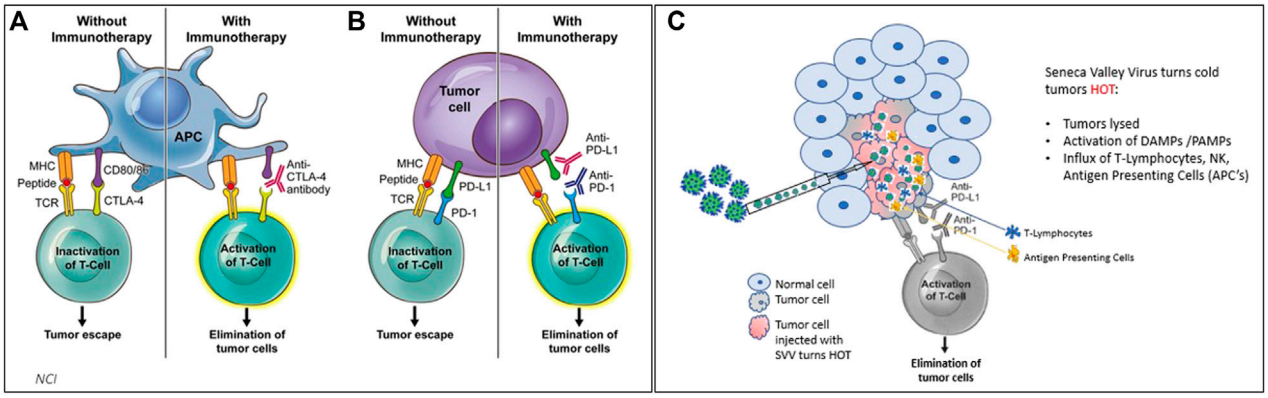

FIGURE 3
(3A,B) Treating cancer with checkpoint inhibitors (CPI's) achieves responses in solid cancers that are defined as "hot" tumors (with immune cells such as APCs and T cells) ~25% response rate on average observed. **(C)** when SVV is administered either systemically or intratumorally, SVV makes tumors HOT. SVV also replicates inside of the tumor, causing an immune response which activates DAMPs and PAMPs, and creates an influx of T-cells, NK cells, and antigen presenting cells to attack tumor cells.

SVV-001 on tumor and stromal cells and a potentially valuable biomarker for patients who would most benefit from therapy with SVV-001. In addition, rapid technological advancement in the study of OVs has shown that intratumoral injection of OVs has the potential to deliver local impact as well as distant abscopal responses and may represent a more effective means of targeting tumors than systemic administration (Melero et al., 2021). Rational combinations of intratumor administration of OVs in combination with checkpoint blockade has a great potential for synergy as OVs induce immunogenic cell death by activating both innate and adaptive immune responses can potentially enhance the efficacy of checkpoint blockade, Figure 3 (Workenhe and Mossman, 2014; Ma et al., 2020; Boagni et al., 2021; Zhou et al., 2021).

The specific proposed mechanism of combination of checkpoint blockade with OV includes modulation of an immune excluded microenvironment to enhance activity of cytotoxic T cells. Neuroendocrine cancers including well differentiated neuroendocrine tumors and SCLC-N do not respond to checkpoint blockade (Takkenkamp et al., 2020; Gay et al., 2021) which is thought to be mediated by an immune excluded tumor microenvironment. The exact mechanism of this is not clear, in SCLC-N, this may be due to evasion of natural killer surveillance (Zhu et al., 2021), however, in several types of neuroendocrine cancers tumor associated macrophages likely also play a role (Cai et al., 2019). To overcome the immune suppressive and tumor permissive environment, OV therapy with SVV-001 triggers immunogenic cell death after injection into TEM8/ANTXR1 enriched tumors cells and the associated TEM8/ANTXR1 enriched stromal cells. This leads to lysis of tumor cells and stromal cells and triggers release of DAMPs which draw innate immune cells including dendritic cells, key activators of tumor specific T cells and response to checkpoint blockade, to the microenviroment (van Vloten et al., 2018). In addition, release of tumor antigens further primes immune responses and promotes tumor infiltrating lymphocyte recruitment (Harrington et al., 2019). Lastly, RNA from both SVV and lysed cells triggers DAMPs and PAMPs to accentuate immune response. Overall, these processes enhances the efficacy of checkpoint blockade to overcome the cancer permissive and immune excluded microenvironment.

In addition OVs can be engineered to deliver cytokines to the tumor microenvironment in combination with checkpoint blockade (Nakao et al., 2020). Early studies suggest efficacy of OVs combined with CAR-T cells therapy (Rezaei et al., 2021; Rosewell Shaw et al., 2021) and bispecific antibodies (Heidbuechel and Engeland, 2021). As our understanding of the tumor microenvironment unfolds, genetically engineered OVs will allow precise manipulation of the tumor microenvironment alone or in combination with other immunotherapy agents. Given the rapid advances in immunology in the last 5 years and the discovery of a specific biomarker for SVV-001, the next generation of SVV-001 based therapies is being developed.

Studies in murine models using SVV-001 in combination with checkpoint blockade are already very promising. One study evaluated intratumoral injection of SVV-001 in combination with checkpoint blockade in two murine models of neuroblastoma and melanoma engineered with upregulated TEM8/ANTXR1 receptors. In this study both cell lines were resistant to checkpoint blockade at baseline. The combination of

checkpoint blockade plus SVV-001 increased the response rate up to 6-fold over checkpoint inhibition alone ($p < 0.01$) (Hallenbeck and Chada, 2021). Finally, a phase I/II trial is already in development exploring SVV-001 administered intratumorally in combination with ipilimumab and nivolumab compared to ipilimumab and nivolumab alone in TEM8/ANTXR1 enriched neuroendocrine tumors and neuroendocrine carcinomas (Wire, 2021a). This novel study is based on preclinical data from Seneca Therapeutics, Inc. SVV-001 was injected intratumorally in a pancreatic cancer model (Pan02) in combination with anti PD1and/or anti CTLA4 antibodies. SVV-001 not only re-sensitized tumors to immune checkpoint inhibitors but also resulted in synergistic antitumor activity as compared to immune checkpoint inhibitors alone. Over 83% of mice were noted to have compete responses with combination SVV-001 plus both immune checkpoint inhibitors. Responses were not only noted in injected lesions but also when the mice were challenged with naïve pan02 cells on the contralateral flank. Only mice from animals that had tumors regress from treatment with SVV-001 plus anti PD1 and anti CTLA4 antibodies rejected the challenge, suggesting a systemic abscopal effect. It is well known that OVs induce T-cell infiltration in injected tumors. This was also noted in SVV-001 preclinical investigations with the combination of SVV-001 and immune checkpoint demonstrating the highest T cell infiltration. Interestingly, tumors regressed with multiple injections of SVV plus CPIs despite the presence of high concentrations of SVV neutralizing antibodies, again suggesting that antibodies aren't effective in blocking SVV when injected at high concentrations inside a tumor. These data were presented at the 2022 AACR symposium (Hallenbeck and Chada, 2021).

Seneca Therapeutics has created a novel 8 gene reverse transcription polymerase chain reaction (RT-PCR) assay, performed on formalin fixed paraffin embedded patient tumor samples commonly available from most solid cancer patients. This test detects TEM8/ANTXR1 as well as seven additional genes to accurately predict if the patient's tumor is permissive to SVV infection. This test will be used to screen potential patients intended for SVV-001 therapy in clinical trials (Wire, 2021b). In addition, the development of a cancer gene delivery platform is underway allowing the incorporation of immunomodulatory transgenes into a SVV-001 delivery system allowing precise targeting of the tumor immune microenvironment of TEM8/ANTXR1 enriched tumors (Wire, 2021c).

Seneca valley virus in small cell lung cancer

SCLC is an aggressive cancer in dire need of effective treatments. The potential for SVV-001 in combination with checkpoint blockade to target SCLC has been further informed by recent advances in understanding of the pathophysiology of SCLC. Specifically, greater understanding of the SCLC molecular subgroup SCLC-N, with elevated *NEUROD1* and low *ASCL1*, targeted by SVV-001 shed light on the mechanisms of viral entry and efficacy as well as possible future targets for SVV-001-derived therapies. Rudin et al. describe four subtypes of SCLC based on expression of transcription regulators including SCLC-A, defined as *ASCL1*-high, SCLC-N, defined as *NEUROD1*-high, SCLC-Y defined as *YAP1* high, and SCLC-P defined as *POU2F3* high (Schwendenwein et al., 2021). In addition evidence from murine models suggest that *ASCL1* rather than *NEUROD1* is key to tumorigenesis of SCLC (Borromeo et al., 2016) and that over time c-MYC enriched tumor cells arise in this population and drive a switch to a *NEUROD1* high state. In mouse models MYC driven, *NEUROD1* high tumors are sensitive to Aurora kinase inhibition (Mollaoglu et al., 2017). This finding was further explored in a phase II, randomized, placebo-controlled trial of paclitaxel plus alisertib (an Aurora kinase inhibitor) as second line treatment in SCLC, with a primary endpoint of PFS. Although PFS was not significantly improved in an unselected patient population, in exploratory studies c-Myc expression by immunohistochemistry (IHC) was associated with improved PFS (4.64 months in paclitaxel/alisertib versus 2.27 months paclitaxel/placebo) (Owonikoko et al., 2020). In other models c-MYC was associated with transition from SCLC-A to SCLC-N and also regulation of Notch signaling pathways involved in epithelial-to-mesenchymal transition (Patel et al., 2021a). Whether aurora kinase inhibition could have synergy with SVV-001 is an open area of investigation.

In another recent paper by Chan et al. (2021) plasticity and immunosuppression in SCLC was explored in both primary tumors and metastases through single cell transcriptome sequencing and imaging techniques. They noted that SCLC-N was enriched in metastasis while primary tumors were more commonly SCLC-A. In addition, SCLC-N were found to express lower levels of immune-related genes as compared to SCLC-A, suggesting an immune "cold" tumor microenvironment. Consistent with this, SCLC-N was associated with T cell dysfunction including higher levels of Treg cells and CD8 + exhausted phenotype, with evidence of reduced cytotoxic $CD8^+$ effector cells. SCLC-N was also associated with increased markers of epithelial-mesenchymal transition, transforming growth factor-β (TGF-β), and other markers of pro-metastatic gene expression. Finally, SCLC-N cells were associated with a pro-fibrotic and immunosuppressive population of monocytes and macrophages.

Interestingly, somatostatin receptor 2 (SSTR2) upregulation is also associated with *NEUROD1* expression in both SCLC cell lines and primary tumors, and correlates with worse clinical outcomes (Lehman et al., 2019; Gay et al., 2021). SSTR2 is an important target in well differentiated neuroendocrine tumors which have high expression of this receptor (Caplin et al., 2014).

However, targeting of SSTR2 in high grade neuroendocrine carcinoma has not shown significant responses (Macaulay et al., 1991; Lapa et al., 2016). Given that SVV-001 targets both well differentiated and high-grade neuroendocrine tumor, and that SSTR2 may be upregulated in the same tumors, one might hypothesize that SSTR2 may play a role in a specific type of tumor microenvironment characterized by upregulated TEM8/ANTXR1, low expression of type 1 IFN associated genes, immunosuppressive myeloid infiltration, and pathological tumor associated angiogenesis. In older studies SSTR2s are upregulated in neo-angiogenesis (Curtis et al., 2000; Watson et al., 2001; Adams et al., 2005). Synergy between SSTR2 directed therapies and SVV-001 could be evaluated in future studies.

IMpower 133, a clinical trial of chemotherapy in combination with immune checkpoint blockade, was a major breakthrough in SCLC, long thought to be recalcitrant to immunotherapy based treatment regimens (Horn et al., 2018). However, this study was done in an unselected patient population and as the current understanding of SCLC pathophysiology has developed with a focus on SCLC subgroups, biomarker driven studies represent an important advancement in therapeutic trial development for SCLC. Gay et al. (2021) confirmed this paradigm in their recent exploration of SCLC treatment response in IMpower 133 classified by transcriptomic subgroups. They describe an emerging new group, SCLC-I or an inflamed SCLC subgroup, more likely to respond to checkpoint blockade. Within the population of treatment naïve patients enrolled, 17% of patients were SCLC-I and 23% of patients were found to be SCLC-N (Gay et al., 2021). The upcoming phase I/II clinical trial of intratumoral SVV-001 in combination with ipilimumab and nivolumab represents the next generation of truly biomarker-driven drug development for SCLC with selection of patients based on TEM8/ANTXR1 expression. Although SCLC-Ns are thought to be "cold" tumors poorly responsive to checkpoint blockade, with the addition of SVV-001, this trial promises to bring the advances of immunotherapy to patients with this aggressive and highly morbid disease.

Seneca valley virus in extra-pulmonary high-grade neuroendocrine carcinoma

Although SCLC is the most well-known high-grade neuroendocrine carcinoma, extra-pulmonary high-grade neuroendocrine carcinoma is also associated with significant mortality. High grade extra-pulmonary neuroendocrine carcinomas can arise throughout the body, are similar to SCLC in that they are aggressive tumors causing limited life expectancy (Dasari et al., 2018; McNamara et al., 2020). Although not as well defined as in SCLC, recent studies have also explored transcriptomic subgroups in extra-pulmonary high grade neuroendocrine carcinoma and revealed transcriptomic subgroups defined by expression of *NEUROD1* and *ASCL1* (Kawasaki et al., 2020; Li et al., 2021c; Metovic et al., 2022). However, data is limited given the rarity of these tumors, and there are no clearly defined transcriptomic subgroups as in SCLC that may predict response to checkpoint blockade in high grade extra pulmonary neuroendocrine carcinoma. Microsatellite instability and elevated tumor mutation burden (TMB) > 10 may predict response to checkpoint blockade in this setting (Sahnane et al., 2015; Girardi et al., 2017; Shao et al., 2020). First line treatment in extra pulmonary high grade neuroendocrine carcinoma is combination of platinum and etoposide (Thomas et al., 2019). The use of immunotherapy was explored in the phase II Dual Anti-CTLA-4 and Anti-PD-1 Blockade in Rare Tumors (DART) SWOG S1609 trial which reported a 26% overall response rate with ipilimumab and nivolumab in patients with high grade extra-pulmonary neuroendocrine neoplasms. In subgroup analysis of this trial there were several responders with microsatellite stable disease and TMB < 10 (Patel et al., 2021b). Further biomarkers of response to immunotherapy are needed. SVV-001 represents a promising agent in this setting, as above, with the ability to provide biomarker driven therapy. The planned phase I/II trial will include all neuroendocrine carcinomas and promises to deliver not only responses in this aggressive disease, but also an expanded understanding of these rare but aggressive cancers with help of serial tumor biopsies and exploratory correlative studies.

Seneca valley virus in well differentiated neuroendocrine tumors

Neuroendocrine tumors are distinct from neuroendocrine carcinomas in their relatively indolent disease course and characteristic morphology microscopically. Neuroendocrine tumors can originate from anywhere in the body but small intestine, lung, and pancreas constitute the most prevalent locations. The WHO classification of both pulmonary neuroendocrine neoplasms and gastroenteropancreatic (GEP) neuroendocrine neoplasms were recently updated (Assarzadegan and Montgomery, 2020; Nagtegaal et al., 2020; Nicholson et al., 2022). The incidence of well differentiated neuroendocrine tumors is increasing (Dasari et al., 2017). There are limited FDA approved therapies for oncologic treatment of neuroendocrine tumors; these include lanreotide (Caplin et al., 2014), everolimus (Yao et al., 2011; Yao et al., 2016), and Peptide Receptor Radionuclide Therapy (PRRT) (Strosberg et al., 2017). Promising studies of multi-target tyrosine inhibitors are ongoing (Chan et al., 2017; Capdevila et al., 2018; Grillo et al., 2018; Capdevila et al.,

2021). Prior studies have evaluated checkpoint blockade in well differentiated neuroendocrine tumors with limited overall response rates (Mehnert et al., 2020; Strosberg et al., 2020; Yao et al., 2021).

Well differentiated neuroendocrine tumors were known to be permissive to SVV-001. Although there is no published data about TEM8/ANTXR1 upregulation in well differentiated neuroendocrine tumors, given the permissivity towards SVV-001, it is likely that upregulated TEM8/ANTXR1 is present in a subset of these tumors. It is clear that well differentiated neuroendocrine tumors express high levels of SSTR2 (Wolin, 2012), but a connection between SSTR2 and pathologic angiogenesis, possibly associated with TEM8/ANTXR1 is only speculation at present.

The same transcriptomic subgroups explored in SCLC and more recently in extra-pulmonary high-grade neuroendocrine carcinoma have not been defined in well differentiated neuroendocrine tumors. Past data suggests that subsets of well differentiated gastroenteropancreatic neuroendocrine tumors do express elevated *NEUROD1* (Shida et al., 2008). One study examining small intestinal neuroendocrine tumors using transcriptomic expression profiling identified three clusters of small intestinal neuroendocrine tumors with different patient survival patterns. In 2 of the 3 clusters identified, *NEUROD1*, was found to be an upstream transcriptomic regulator (Andersson et al., 2016). In addition, well differentiated neuroendocrine tumors are known to be highly vascular, which is the basis of the "neuroendocrine paradox" where in contrast to adenocarcinomas, lower grade, more indolent tumors often have increased dense vascular networks as compared to higher grade more aggressive tumors (Scoazec, 2013; Carrasco et al., 2017). Well differentiated pancreatic neuroendocrine tumors are associated with hypoxia driven, abnormal angiogenesis, and vascular mimicry (Chu et al., 2013). Pancreatic neuroendocrine tumors are also associated with C-MYC overexpression which also promotes vascular endothelial growth factor C (VEGFC) expression the development of lymphatic endothelial cells (Chang et al., 2021). In addition, well differentiated neuroendocrine of the midgut are associated with an immunosuppressive (Busse et al., 2020) and intensely fibrotic tumor microenvironment with crosstalk between tumor cells and stromal cells, and upregulation of integrin signaling pathways (Laskaratos et al., 2021). All of these characteristics suggest a type of hypoxia-driven highly vascular tumor microenvironment similar to the environment that in other tumor types are enriched for TEM8/ANTXR1. However, the pathophysiology of the development of this type of environment is likely different in well differentiated neuroendocrine tumors as compared to high grade neuroendocrine carcinomas. Recent evidence suggests plasticity in SCLC, where tumors starts as SCLC-A and transition to SCLC-N over time with environmental pressure (Ireland et al., 2020; Chan et al., 2021). Well differentiated neuroendocrine tumors develop from neuroendocrine cells, which are physiologically involved in complex hormonal paracrine and autocrine processes and closely interact with the local tissue environment and vasculature. It is likely that intrinsic processes, related to neuroendocrine cell function drive the local tumor microenvironment as these cells transform to neuroendocrine tumors.

SVV-001 is a potentially transformational agent for well differentiated neuroendocrine tumors. SVV-001 intratumoral injection in combination with checkpoint blockade may lead to significant responses in patients with TEM8/ANTXR1 upregulation. Current FDA approved agents used in well differentiated neuroendocrine tumors are often cytostatic. SVV-001 and immune checkpoint combination holds the potential for significant cytoreduction based on impressive pre-clinical data. This is especially needed for patients with large, bulky symptomatic disease.

Discussion: Seneca valley virus beyond neuroendocrine neoplasms

SVV-001 is an important potential therapeutic agent in many cancer types. However, understanding SVV-001 and the unique tumor microenvironment, represented by upregulation of TEM8/ANTXR1, that it targets, has the potential to provide additional clues about mechanisms of resistance to immunotherapy and chemotherapy in neuroendocrine neoplasms and other cancers. TEM8/ANTXR1 upregulation has been described in a variety of solid tumors types including triple negative breast cancer (Xu et al., 2021), prostate cancer (Li et al., 2021a), gastric cancer (Li et al., 2021b; Sun et al., 2021), pancreatic cancer (Alcalá et al., 2019), angiosarcoma (Kusaba et al., 2021), colon cancer (Ł et al., 2021), and NSCLC (Gong et al., 2021). Lineage plasticity with a transformation from adenocarcinomas to carcinomas with neuroendocrine differentiation has been described in a variety of solid tumors, including lung and prostate primary tumors (Farrell et al., 2017; Rubin et al., 2020; Ito et al., 2021). This transformation is often associated with the development of therapy resistance and portends poor outcomes for patients. The tropism of SVV-001 for neuroendocrine cancer, mediated by upregulated TEM8/ANTXR1 can inform this paradigm and opens the door for novel uses of SVV-001 to target these tumor types. The model of lineage plasticity described in SCLC, with SCLC-A transforming to SCLC-N mediated by MYC activation (Ireland et al., 2020), has similarities to the transformation of prostate cancer (Li et al., 2021a) and pancreatic cancer (Farrell et al., 2017). The identification of TEM8/ANTXR1 as a potential mediator of neuroendocrine transformation was most clearly shown in prostate cancer, where N-MYC was found to promote dysregulated angiogenesis and tumor progression *via* TEM8/

ANTXR1 (Li et al., 2021a). The specific association between upregulated TEM8/ANTXR1, vasculogenic mimicry, and cancer stem cells, suggests the presence of a hypoxic tumor microenvironment with disordered angiogenesis, which promotes the survival and spread of cancer cells. It remains to be clarified if this same pathway of lineage plasticity is also present in other tumor types expressing TEM8/ANTXR1, however it is possible the same paradigm mediates metastasis and therapy resistance in subsets of triple negative breast cancer, gastric cancer, colon cancer, and NSCLC. Further research is needed to validate if these hypotheses prove true and if they represent additional targets for cancer therapy.

SVV-001 was first identified as neuroendocrine specific OV, with extraordinary potential to transform the landscape of neuroendocrine neoplasm treatments by inducing a significant response in a tumor type long thought to be resistant to immunotherapy. However, early studies were limited by lack of a biomarker to select SVV permissive patients. The identification of TEM8/ANTXR1 as the receptor for SVV-001, where SVV-001 can be administered *via* intratumoral injections, in a biomarker enriched patient population and in combination with dual checkpoint blockade to optimize responses has paved the way for the next generation of rationally designed clinical trials using SVV-001. Although this treatment paradigm was developed to target neuroendocrine neoplasms, recent advances in the understanding of lineage plasticity of neuroendocrine transformation in a variety of solid tumor types along with studies identifying widespread TEM8/ANTXR1 upregulation, suggest that SVV-001 has the potential to target many other tumor types that are particularly therapy-resistant and deadly. Further understanding of the precise immune tumor microenvironment associated with TEM8/ANTXR1 upregulation in high grade neuroendocrine carcinoma, well differentiated neuroendocrine tumors, and other associated tumor types is key to not only using SVV-001 to target these diseases, but also to developing other novel agents that could be used in combination with SVV-001.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

PH was employed by Seneca Therapeutics Inc. VC formerly owned equity in Pfizer, BristolMyers Squibb, Seagen, and Viatris.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3-Dimensional coculture of breast cancer cell lines with adipose tissue–Derived stem cells reveals the efficiency of oncolytic Newcastle disease virus infection *via* labeling technology

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Oncolytic virotherapy is one of the emerging biological therapeutics that needs a more efficient *in vitro* tumor model to overcome the two-dimensional (2D) monolayer tumor cell culture model's inability to maintain tissue-specific structure. This is to offer significant prognostic preclinical assessment findings. One of the best models that can mimic the *in vivo* model *in vitro* are the three-dimensional (3D) tumor–normal cell coculture systems, which can be employed in preclinical oncolytic virus therapeutics. Thus, we developed our 3D coculture system *in vitro* using two types of breast cancer cell lines showing different receptor statuses cocultured with adipose tissue–derived mesenchymal stem cells. The cells were cultured in a floater tissue culture plate to allow spheroids formation, and then the spheroids were collected and transferred to a scaffold spheroids dish. These 3D culture systems were used to evaluate oncolytic Newcastle disease virus AMHA1 strain infectivity and antitumor activity using a tracking system of the Newcastle disease virus (NDV) labeled with fluorescent PKH67 linker to follow the virus entry into target cells. This provides evidence that the NDV AMHA1 strain is an efficient oncolytic agent. The fluorescently detected virus particles showed high intensity in both coculture spheres. Strategies for chemically introducing fluorescent dyes into NDV particles extract quantitative information from the infected cancer models. In conclusion, the results indicate that the NDV AMHA1 strain efficiently replicates and induces an antitumor effect in cancer–normal 3D coculture systems, indicating efficient clinical outcomes.

KEYWORDS

labeled viruses, virotherapy, breast cancer, adipose tissue–derived adult stem cell, 3D cancer model, coculture

Introduction

Oncolytic virotherapy is an emerging biological therapeutic that needs an efficient *in vitro* tumor model to maintain the tissue-specific structure that two-dimensional (2D) culture models cannot maintain (Kloker et al., 2018; Duval et al., 2017). One of the best models that can mimic the *in vivo* model *in vitro* are the three-dimensional (3D) tumor–normal cell coculture systems (Chaicharoenaudomrung et al., 2019), which can be employed in preclinical oncolytic virus therapeutics. Oncolytic virotherapy is a kind of promising breast cancer therapy (Al-Ziaydi et al., 2020b). Breast cancer (BC) is the most invasive malignancy and the main cause of death in females (Cao et al., 2020). One of the most significant extrinsic variables in the development of BC is the tumor microenvironment (TME), which is composed of extracellular matrix (ECM) proteins, stromal cells such as adipocytes and associated cells, and the physical characteristics of neighboring cells or the ECM, all making up the TME structure (Place et al., 2011). These variables may have a cumulative effect against the development and progression of the tumor by influencing BC cell behavior *via* biophysical or biochemical interactions (Pallegar et al., 2019). Therefore, the 3D breast cancer cell systems can be used as a model that holds an inordinate promise for the discovery of drugs and cancer-targeted therapy. The NDV has been suggested as a biological agent with the potential to break therapy resistance, as it can replicate in non-proliferating tumor cells that are resistant to chemotherapy and radiotherapy (Schirrmacher, 2015). The NDV can also interfere with cancer angiogenesis (Al-Shammari et al., 2020a). The induction of apoptosis and immunogenic cell death is involved in NDV-mediated cancer cell killing (Al-Shammari et al., 2020c). Several methods have been developed to study virus adsorption and internalization, such as fluorescent *in situ* hybridization (Brabec-Zaruba et al., 2009), signal molecule tracking (Seisenberger et al., 2001), and radioactive labeling (Gotoh et al., 2006). Fluorescent lipid molecule tracing using amphipathic carbocyanine probe techniques has also been reported for labeling and visualizing enveloped RNA virus–cell interactions (Balogh et al., 2011). Here, we aim to demonstrate the efficiency of the NDV AMHA1 strain labeled with the PKH67 linker in replicating the 3D coculture spheroid cells augmented with scaffold developed to mimic the *in vivo* interaction between the BC cells and human adipose tissue–derived mesenchymal stem cells (hATMSCs).

Material and method

The study was approved by the Scientific Committee of the Department of Biotechnology, College of Science, Baghdad University. The experiments were performed at the Experimental Therapy Department, Iraqi Center of Cancer and Medical Genetics Research (ICCMGR), Mustansiriyah University, Baghdad, Iraq.

Newcastle disease virus propagation

An attenuated NDV AMHA1 strain (Al-Ziaydi et al., 2020a) was propagated in a 9-day chicken egg embryo (Al-Kindi, Baghdad, Iraq). The NDV was collected from the allantoic fluid of chicken eggs, purified from debris by using centrifugation at 3,000 rpm for 30 min, and stored at −80°C after being tested for hemagglutination (HA test). The viral titers were determined on Vero–SLAM cells (kindly provided by Dr. S.J. Russell, Mayo Clinic, United States) using a 50% tissue culture infective dose titration assay (TCID 50) per the standard procedure.

Newcastle disease virus purification

The virus was purified from the allantoic fluids through the sucrose gradient method. First, the debris-free NDV was concentrated by ultracentrifugation at 120,000 *g* in an SW 32 Ti rotor (Beckman, United States) for 3.5 h at 4°C to make viral pellets suspended in 1 ml phosphate-buffered saline (PBS) of each tube and collected and stored at −80°C. The sucrose gradient was prepared as 60%, 50%, 40%, and 25% w/v sucrose in Milli-Q water, 0.22 mM filter sterilized, in 13.2 ml ultracentrifuge tubes at 4°C. The gradient was prepared by adding 1 ml of 60% w/v sucrose to the bottom of the tube. Later, it was carefully layered with 2 ml, 2 ml, and 2.5 ml of 50%, 40%, and 25% w/v sucrose, respectively. Finally, a layer of 4 ml of concentrated NDV was added to the top. The gradients were ultracentrifuged at 120,000 *g* in an SW 41 Ti rotor for 3.5 h at 4°C. The virus typically bands between the 40% and 50% sucrose layers. All tubes had to be clamped to retort stands to collect the virus, then wiped with 70% ethanol from the outside. A wide-mouthed container was placed below the ultracentrifuge tube to collect the liquid waste that was poured out after removing the syringe. An 18-G needle was attached to a 3 cc syringe positioned to the side of the tube at around 5 mm below the virus-containing band and the virus (2 ml) was slowly withdrawn (Santry et al., 2018).

Cancer and normal cells

The human breast cancer cell line AMJ13 derived from Iraqi patients (estrogen and progesterone receptors negative) (Al-Shammari et al., 2015), the MCF7 human BC cell line (estrogen, progesterone receptors positive), and normal human adipose tissue–derived mesenchymal stem cells (hATMSCs) were supplied as a cell line established by Dr. Ahmed Majeed Al-Shammari, Experimental Therapy

department, the Iraqi Center of Cancer and Medical Genetics Research (ICCMGR), Mustansiriyah University, Baghdad, Iraq. The original hATMSCs sample isolation was described by Hammadi and Alhimyari (2019). AMJ13 was cultured in an RPMI-1640 medium. The MCF7 cell and hATMSCs were cultured in MEM (US Biological, United States) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 IU penicillin and 100 µg streptomycin (Capricorn-Scientific, Germany). The cells were checked regularly for contamination.

Three-dimensional multicellular spheroids coculture system

We modified the 3D-culture protocol initially developed by Wong et al. (2012) and Rolver et al. (2019). Briefly, Both AMJ13 cancer cells and hATMSCs normal cells, MCF-7, and hATMSCs, were trypsinized from a monolayer to a single-cell suspension and seeded as coculture at 50,000 cells each (ratio: 1:1) in a 24-well cell floater plate (SPL3D™, SPL Life Sciences, South Korea) and allowed for spheroids formation for 3 days at 37°C. Later, we collected spheroids and transferred them to a scaffold spheroids dish for culturing for an additional 72 h, as the meshes inside the well facilitate identifying and counting the spheroids (mesh thickness: 137 µm, pore size: 200 µm, cat. No. 110350, SPL Life Sciences, South Korea).

Newcastle disease virus particles' labeling with PKH67 linker

The PKH67 Fluorescent Cell Linker Kits (Sigma-Aldrich, United States) were used to label the oncolytic NDV AMHA1. A total of 1.5×10^8 particles in PBS were labeled as follows: 1 µl of PKH67 dye (Sigma, St. Louis, MO) was dissolved in 2 ml of Diluent C before labeling as per the manufacturer's recommendations (Sigma, St. Louis, MO). Two volumes of diluted PKH67 were mixed with one volume of NDV suspension by pipetting. After 30 s, the labeling reaction was stopped by adding three volumes of the full medium and pipetting the suspension 5–6 times. The labeled virus was ready for exposure (Balogh et al., 2011).

Coculture spheroids infection

Coculture spheroids were treated with labeled NDV at a multiplicity of infection (MOI) of 10 for 24 h before ending the experiment. Another group of spheroids was treated with NDV AMHA1 without adding PKH67 Fluorescent Linker to act as the non-labeled control. The PKH67–Diluent C mixture was incubated with ×1 PBS as described for virus labeling (without adding NDV) to prepare the mock-infected control. The reaction was halted by adding the full medium, and the mixture was then used to treat the cells. After 24 h, we aspirated the medium from the spheres and rinsed them twice with ice-cold PBS. We then fixed the culture with 4% paraformaldehyde at room temperature for 10 min and stopped the fixation process by aspirating 4% paraformaldehyde and injecting at least two medium-volume PBS for 10 min. The samples were incubated overnight at 4°C before removing paraformaldehyde to stain the cell nuclei with propidium iodide dye (PI) (10 µl PI + 1 ml PBS) (Balogh et al., 2011).

Two-dimensional coculture protocol

In an 8-well chamber slide (SPL Life Sciences, South Korea), we seeded 2,500 cells of AMJ13 or MCF7 breast cancer cells with 25,000 normal hATMSCs in 400 µl of the growth media to allow cell growth overnight to create the 2D coculture system of AMJ13 with hATMSCs and MCF7 with hATMSCs. By day 2, the cells were treated with an NDV-PKH67 to detect virus infectivity and selectivity in the 2D coculture system.

Image quantitative analysis

Images were analyzed using the ImageJ software to measure the green fluorescent dye and the sphere's area. Moreover, the fields were quantified for sphere numbers. The growth inhibition of the spheres was assessed using the following formula:

$$\text{Growth } \textit{inhibition}\,\% = \left(\text{spheres area}_{\text{control}} - \text{spheres area}_{\text{treated}}\right) / \text{spheres area}_{\text{control}} \times 100 \quad (1)$$

where the spheres $\text{area}_{\text{control}}$ is the mean sphere area of untreated wells, and spheres $\text{area}_{\text{treated}}$ is the sphere area of treated wells.

Investigation of Newcastle disease virus AMHA1 hemagglutinin–neuraminidase protein expression in cancer and normal spheroids to confirm virus replication in three-dimensional systems

The cancer spheroids (AMJ13 and MCF7) and normal hATMSCs spheroids were grown separately and infected with NDV at an MOI of 10 for 24 h to confirm infectivity and replication according to cell identity. These spheroids were washed with PBS, then fixed using 4% paraformaldehyde for 30 min at RT. Permeabilization was done using 0.5% Triton-X for 30 min, RT. They were blocked by using 10% goat serum for 60 min. The spheroids were stained with 1 µg/100 µl of the

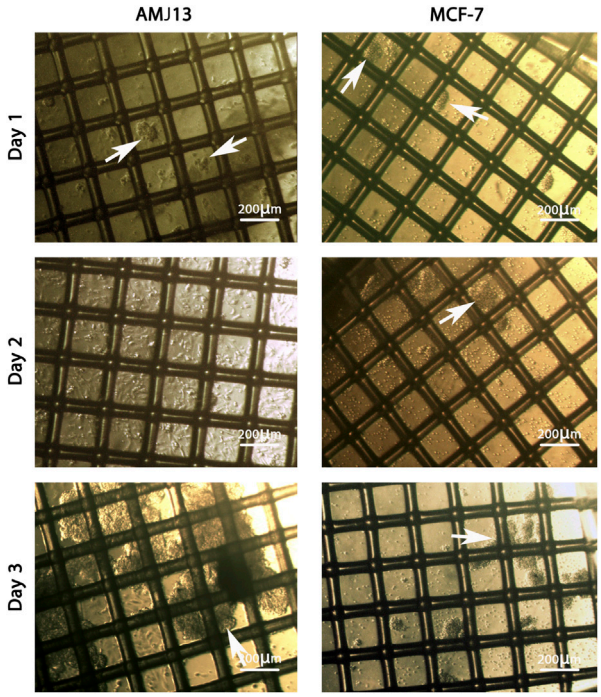

FIGURE 1
The growth of the spheroids coculture system of AMJ13-hATMSCs and MCF7-hATMSCs on a scaffold dish under an inverted microscope. On the first day, the spheres were minor in size, and the tiny aggregates of spheres (white arrows) were organized as a small network. At 48 h, the spheres contained a small number of cells individually, while another aggregate had many cells aggregated (white arrows). At 72 h, the spheres of the coculture extend in size to connect with the scaffold fiber and form massive spheroids (white arrows).

mouse monoclonal antibody raised against hemagglutinin–neuraminidase (HN) of the Newcastle disease virus [NDV-HN (11F12):sc-52112: dilution 1:30, Santa Cruz Biotechnology, CA, United States], diluted in blocking buffer, and incubated overnight at 4°C. Then, the spheroids were treated with 1 μg/100 μl of the secondary antibody Alexa Fluor 568–conjugated goat anti-mouse IgG for 2 h at RT. The spheroids were examined using a Micros fluorescent microscope and photographed using a Micros 5-megapixel camera (Micros, Austria). The fluorescence intensities were measured using the ImageJ software.

Statistical analysis

Statistical significance was determined by unpaired two-tailed Student's *t*-test or two-way analysis of variance with multiple comparisons using GraphPad Prism 6 (GraphPad Software, San Diego, CA). The statistical significance was set at $p < 0.05$.

Result

Spheres formation

The 3D coculture spheres showed steady expansion and growth in the mesh scaffold. In general, during the first 24 hours, the aggregates of spheres were very tiny in size and less in number, which floated in the culture media (Figure 1). During the second day, the spheres had developed in size and increased in numbers. However, such spheres contained a small number of cells individually, while another aggregate had many cells aggregated. Nonetheless, during the third day, we observed hyperactive growth of spheres with a layer of huge aggregate on the surface of the suspended media (Figure 1).

PKH67-labeled Newcastle disease virus AMHA1 lyses breast cancer–hATMSCs spheroid cocultures

We first assessed the efficiency of PKH67-labeled NDV (PKH67-NDV) AMHA1 to revoke the 3D breast cancer–hATMSCs growth. Three-dimensional cocultures of AMJ13 and MCF7 cell lines were exposed to the labeled NDV AMHA1 at an MOI of 10 or mock-infected (control) and checked for spheroid size and numbers at 24 h post-infection. PKH67-NDV infected AMJ13, and MCF7 spheres were lysed or reduced in size when compared to mock-infected cells for the first 3 days before infection (Figures 2A,B). Likewise, PKH67-NDV infection significantly decreased the sphere numbers (Figure 2C). Moreover, the growth inhibition of the spheres show that more than 80% for each cell line has been tested (Figure 2D), indicating that PKH67-NDV can efficiently prevent breast cancer spheroids' growth.

Efficient infectivity of PKH67-labeled Newcastle disease virus in AMJ13 and MCF7 breast cancer three-dimensional spheroids

To evaluate the efficacy of the oncolytic NDV AMHA1 strain, we employed PKH67 dye to label the virus. Through green fluorescence intensity analysis, we determined both AMJ13-hATMSCs and MCF7-hATMSCs 3D cancer cell spheroids susceptibility to PKH67-NDV (Figures 3, 4). The PKH67-NDV replicated within 24 h, infecting most of the spheroids, which when infected with an MOI of 10, dyed all cancer spheres with clear green fluorescence. Unlike untreated cells that fluorescent red-orange only for the propidium iodide that stains nuclei of live cells, prove the specificity of our labeling dye as a tracking system for the labeled virus. By examining

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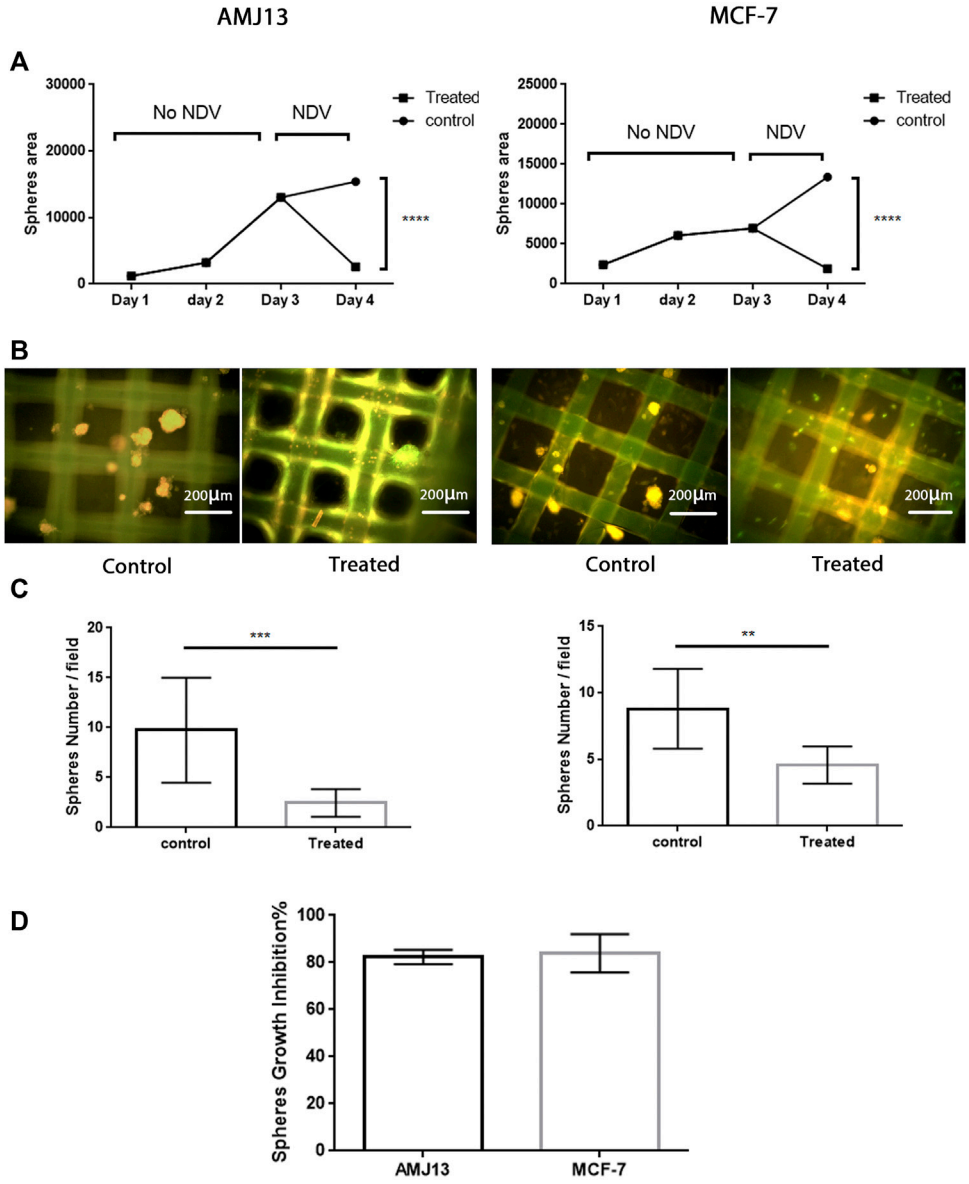

FIGURE 2
Antitumor assessment of the PKH67-NDV AMHA1 against the 3D breast cancer–hATMSCs spheres. Three-dimensional cocultures of AMJ13 and MCF7 cell lines and normal stem cells were exposed to labeled NDV AMHA1 at an MOI of 10 for 24 h post-infection. **(A)** Growth curve showing the steady growth of the spheres in size as measured by area, through 3 days until exposed to PKH67-NDV, where the spheres size drops due to oncolysis effect when compared to that of mock-infected cells. **(B)** Immunofluorescence image showing mostly green fluorescent PKH67-NDV infection decreased the number of spheres when compared to the control noninfected spheres. The control spheres show orange propidium iodide that stains the cell nuclei red. **(C)** Significant decreases seen in spheres number. **(D)** Sphere's growth inhibition showed more than 80% for each cell line tested, indicating that PKH67-NDV can efficiently prevent breast cancer spheroids' growth.

under a fluorescent microscope (Macros, Austria), the infected 3D spheres revealed lytic cytopathic effects and robust green fluorescent signaling (Figures 3, 4). The PKH67-NDV tracking system results show that both AMJ13 and MCF7 cancer spheroids are greatly sensitive to PKH67-NDV infectivity, confirming the broad-spectrum antitumor nature of the oncolytic NDV. The efficient infectivity subsequently causes cancer cell death and virus progeny release. Moreover, the images of PKH67-NDV–infected spheres show a few spheres that are fluorescent red-orange only with no green fluorescence, which can be explained as a sphere of normal hATMSCs only spared by the NDV, as they do not replicate in normal cells.

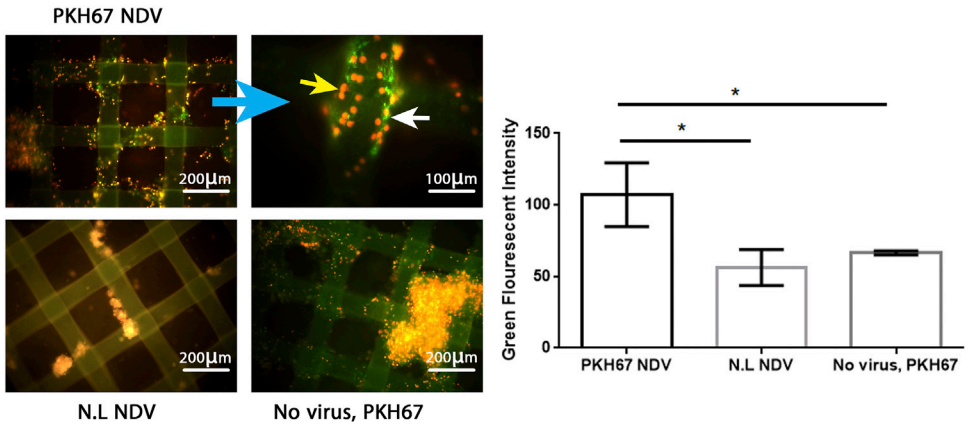

FIGURE 3
Infectivity analysis of PKH67-NDV in AMJ13-hATMSCs 3D spheroids. In representative fluorescent images of the 3D coculture of AMJ13-hATMSCs in the (3D) scaffold system, spheroids were infected at an MOI of 10 in the image labeled with PKH67-NDV. Cells were infected with labeled NDV (virus-carrying PKH67 linker). At 24 h post-infection, coculture spheroids were fixed and propidium iodide (PI)–stained for nuclear staining. PI staining is seen as red/orange, while the cytoplasm is seen as clear green fluorescence, ensuring the entry of the labeled NDV. The top left figure shows images of AMJ13 coculture (×100), and the top right figure shows the magnified PKH67-NDV–infected spheroids (×400). While the figure on the bottom left shows an image of non-labeled NDV infecting the 3D coculture system, showing lysed and small spheroids stained only with red-orange propidium iodide dye. The picture at the bottom right presents coculture spheroids of the AMJ13 spheroid cells treated with PKH67 linker only without any virus (mock-infected) as the second control. While the histogram clarifies the intensity of PKH67 green fluorescence, revealing that PKH67-NDV–infected spheres are the ones showing the most intense green fluorescence.

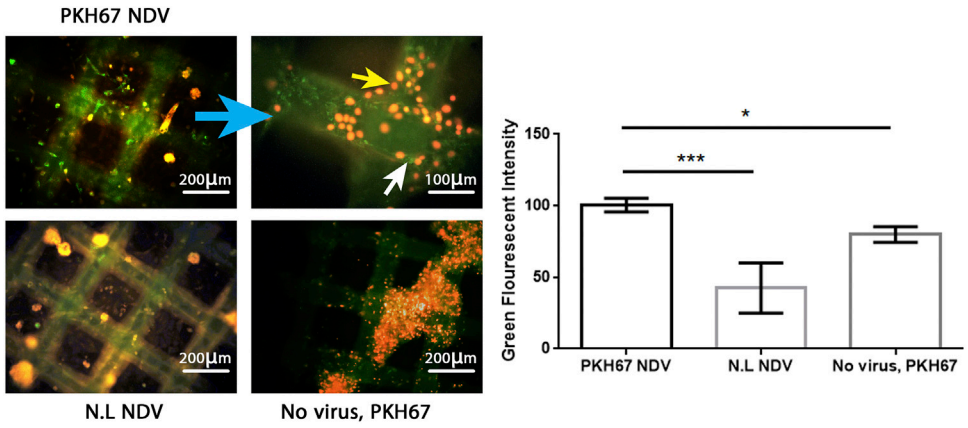

FIGURE 4
Infectivity analysis of PKH67-NDV in MCF7-hATMSCs 3D spheroids, coculture images of MCF7 with hATMSCs in the (3D) scaffold system; spheroids are infected at an MOI of 10 as seen under a fluorescence microscope. Cells were infected with labeled NDV (virus carrying PKH67 linker). At 24 h post-infection, coculture spheroids cells were fixed and stained with propidium iodide (PI) for nuclear staining. PI staining is shown in red/orange, while the cytoplasm is shown in green fluorescence for infectivity of the PKH67-NDV inside cancer cells' cytoplasm, which confirms the entry of labeled NDV. Also seen are few fluorescent red-orange spheres with no green fluorescence, which can be explained as a sphere of normal hATMSCs only spared by the NDV as it is not replicated in the normal cells. The top left figure shows images of MCF7 coculture x100, and the top right figure is of a higher magnification of PKH67-NDV–infected spheres, which appear destroyed, and shows the presence of cells with green cytoplasm (white arrow) and red-orange nuclei (yellow arrow) (×400). While the bottom left figure (non-labeled NDV) shows MCF7-hATMSCs coculture spheres treated with non-labeled NDV, having a nucleus stained red-orange with propidium iodide dye. The bottom right figure presents cocultured spheroids of MCF7 spheroids cells, treated with PKH67 linker only without any virus (mock-infected), treated as the second control. The histogram clarifies the intensity of PKH67 green fluorescence, demonstrating that PKH67-NDV–infected spheres have the highest intensity while showing high infection of the labeled virus.

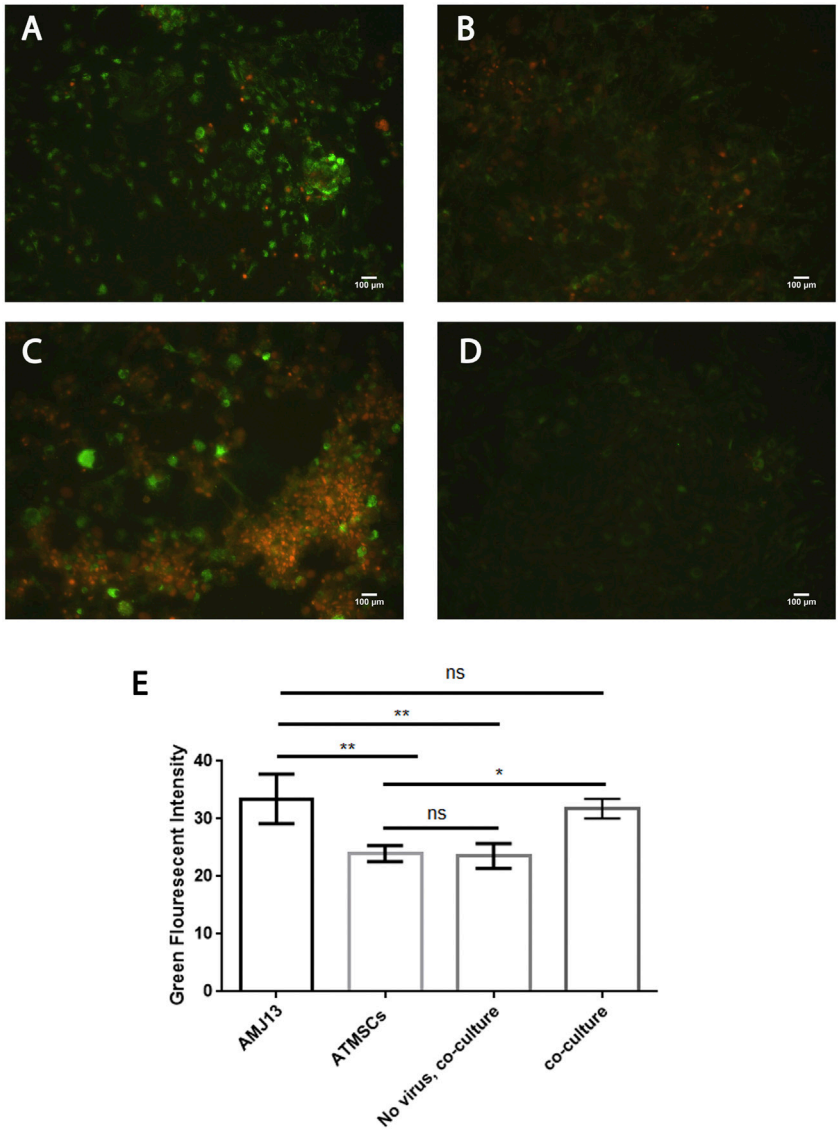

FIGURE 5
Newcastle disease virus infects and replicates in AMJ13 breast cancer cells while infecting but not replicating in normal hATMSCs. **(A)** AMJ13 breast cancer cells infected with labeled NDV. **(B)** Human normal stem cells infected with labeled NDV. **(C)** Coculture of AMJ13 cancer cells with human normal stem cells infected with labeled NDV. **(D)** Coculture model stained with PHK67 dye showing hardly detectable green fluorescence. **(E)** The quantitative image analysis for green fluorescence by ImageJ confirmed significant green fluorescence intensity of infected cancer cells when compared to normal infected cells and the noninfected coculture cell model. There was no significant difference in green fluorescence intensity seen between the infected cancer cells and infected cocultured cells. Propidium iodide was used as the counterstain.

Newcastle disease virus infects and replicates in cancer cells while infecting but not replicating in normal hATMSCs in a two-dimensional system

We tested the hypothesis of NDV infection and replication in cancer cells while infecting but not replicating it in normal stem cells in a 2D cell culture model of cancer, hATMSCs, and coculture of both. We simulated the 3D experiments in a 2D model by culturing AMJ13 and MCF7 cancer cells with human normal stem cells and infecting them with labeled NDV, while adding the labeling dye to the cells as control. The results showed that both cancer cell lines (AMJ13 and MCF7) showed intense green fluorescent staining (Figures 5A, 6A), which reflects efficient NDV infection and replication in cancer cells as compared to normal cells that have very low green fluorescent intensity which is hardly detectable (Figures 5B, 6B). The 2D

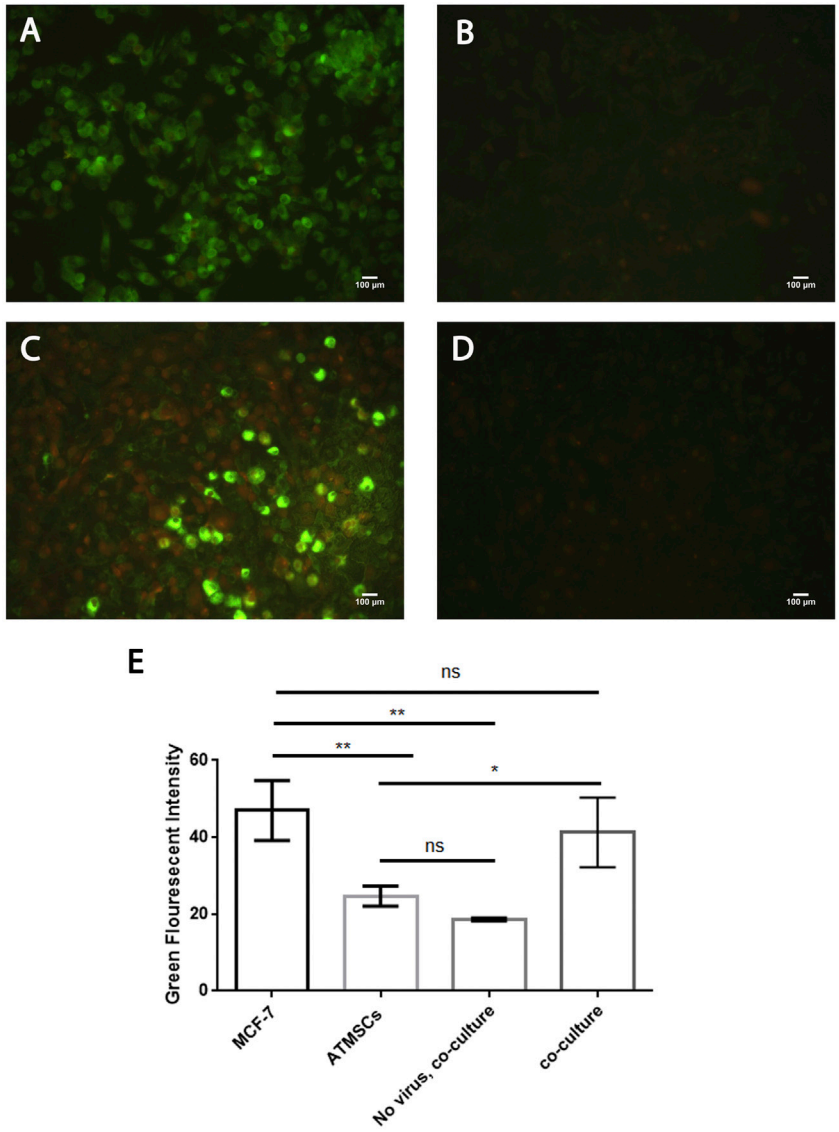

FIGURE 6
Newcastle disease virus infects and replicates in MCF7 breast cancer cells while infecting but not replicating in normal hATMSCs. **(A)** MCF7 breast cancer cells infected with labeled NDV. **(B)** Human normal stem cells infected with labeled NDV. **(C)** Coculture of MCF7 cancer cells with normal human stem cells infected with labeled NDV. **(D)** Coculture model stained with PHK67 dye showing hardly detectable green fluorescence. **(E)** The quantitative image analysis for green fluorescence by ImageJ confirmed significant green fluorescence intensity of the infected cancer cells as compared to normal infected cells and the noninfected coculture cell model. There was no significant difference in the green fluorescence intensity seen between the infected cancer cells and the infected cocultured cells. Propidium iodide was used as the counterstain.

coculture model shows intense staining of some of the cells while others show only counterstaining (Figures 5C, 6C) after infection with PHK67-labeled NDV. Adding the PHK67 dye to the coculture model results in hardly detectable green fluorescence, which is even less than that seen in infected hATMSCs (Figures 5D, 6D). The quantitative image analysis for green fluorescence by ImageJ confirmed significant green fluorescence intensity of the infected cancer cells when compared to normal infected cells and the noninfected coculture cell model (Figure 5E, 6E). There was no significant difference in green fluorescence intensity between the infected cancer cells and infected cocultured cells. These data suggest that labeled viruses attached or entered normal cells and transferred their PHK67 to the cells without replicating in them while showing efficient infection and presence inside cancer cells.

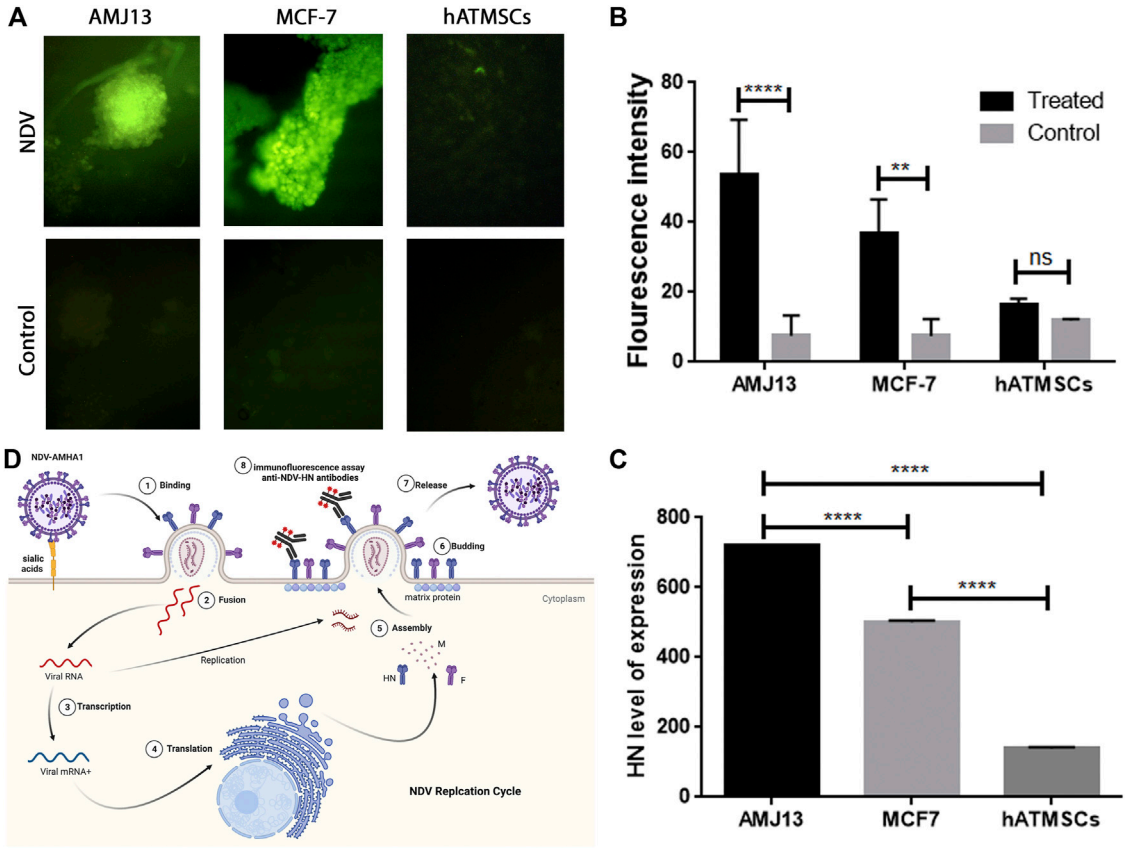

FIGURE 7
Immunofluorescence (IF) assay for NDV-HN protein detection confirms NDV AMHA1 infectivity and replication inside the spheroids through staining with anti-HN NDV mAbs. **(A)** Comparison between infected cells and the controls of all three spheroid types. Infected cancer spheroids expressing HN protein heavily, whereas there is no expression seen in the control or noninfected spheroids, while normal hATMSCs spheroids show low signal levels. **(B)** The fluorescence intensity analysis for the expression of HN-NDV protein in NDV-infected cancer and normal spheroids and comparison of the levels of expression showing significant HN protein expression levels in infected cancer spheroids when compared to noninfected cancer spheroids. Also, there were no significant HN protein levels in the infected normal cell spheroids when compared to noninfected normal spheroids. **(C)** Our findings show significant expression levels of HN-NDV in cancer cell spheroids that are NDV treated when compared to the untreated control cancer spheroids. Moreover, there is a significantly higher expression of HN-NDV in cancer spheroids (AMJ13 and MCF7) when compared to normal cells (hATMSCs). These results have confirmed that NDV AMHA1 could infect and replicate efficiently in cancer spheroids only with no significant replication in normal spheroids. **(D)** NDV AMHA1 replication cycle shows that HN protein is an important NDV protein inserted in the infected cell surface during the NDV replication cycle inside the cells. HN-NDV presence on the cell surface proves virus replication and infectivity.

Expression of Newcastle disease virus AMHA1 hemagglutinin–neuraminidase protein in cancer spheroids but not in normal spheroids to confirm virus replication in three-dimensional systems according to cell identity

Immunofluorescence (IF) assay for NDV-HN protein detection was performed to prove NDV infectivity and replication inside the spheroids by staining them with anti-HN NDV mAbs. HN is one of the main and important NDV proteins inserted in the infected cell surface during the NDV replication cycle inside the cells. Moreover, its presence in the cell is a great proof of virus replication and infectivity. Cancer and normal cell spheroids were grown separately to prove infectivity and replication according to cell identity. Figure 7A compares the infected cells and controls for all three spheroid types. Infected cancer spheroids express HN protein heavily, while there is no expression on the noninfected control spheroids and the normal hATMSCs spheroids show low signal levels. The fluorescence intensity analysis for the expression of HN-NDV protein in NDV-infected cancer and normal spheroids and comparison of the levels of expression showing significant HN protein expression levels

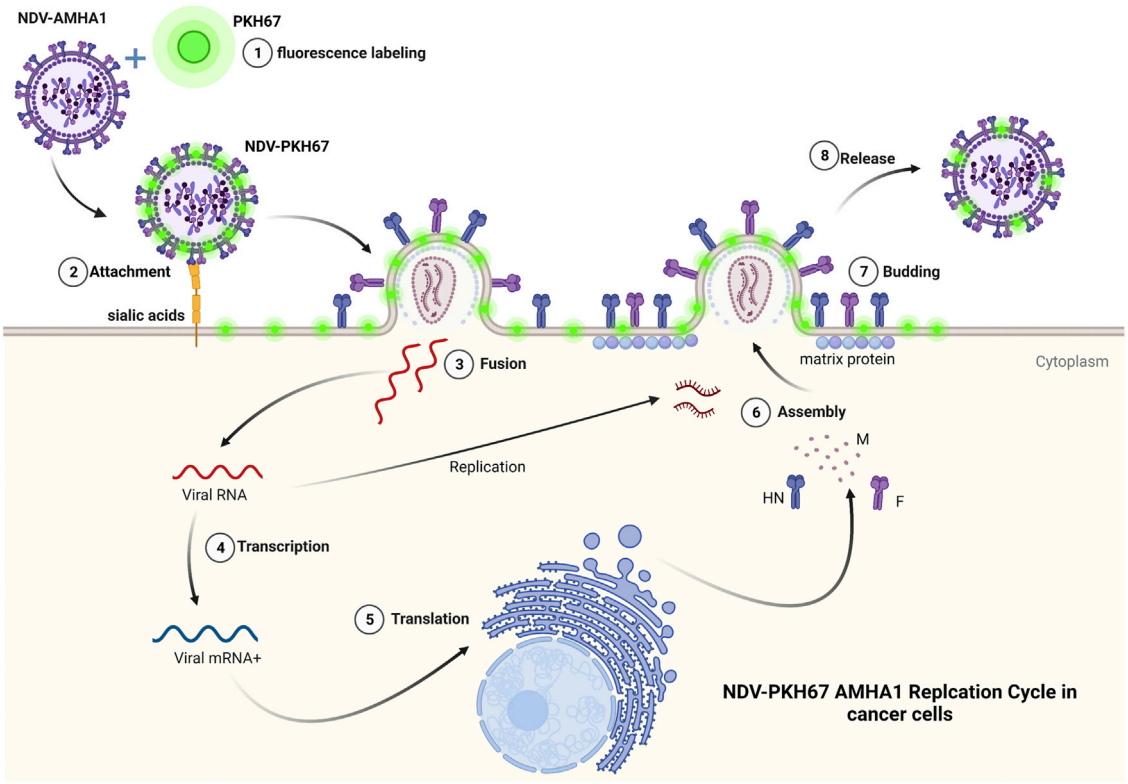

FIGURE 8
PKH67 fluorescent dye used to label the phospholipid membrane of NDV particles and used them as a tracking system to evaluate NDV oncolytic activity in cancer cells.

in infected cancer spheroids compared to noninfected cancer spheroids. Also, there were no significant HN protein levels in the infected normal cell spheroids when compared to noninfected normal spheroids (Figure 7B).

We analyzed the expression of HN-NDV in NDV-infected cancer and normal spheroids and compared the levels of expression to prove NDV replication according to cell identity by replicating in cancer spheroids and sparing normal spheroids. Our finding was that there were significant expression levels of HN-NDV in infected AMJ13 cancer cell spheroids as compared to MCF7 cancer spheroids. Moreover, there were significantly higher expression levels for HN-NDV in cancer spheroids (AMJ13 and MCF7) when compared to normal infected cells (hATMSCs) (Figure 7C). These results confirm that new NDV AMHA1 could infect and replicate efficiently in cancer spheroids only, with no significant replication in normal spheroids. Figure 7D summarizes the NDV replication cycle inside the infected cancer cells, where the NDV inserts its HN protein with fusion protein in the cell surface to help for a new virion assembly, and the budding and staining with HN-specific mAbs after 24 h of infection.

Discussion

To the best of our knowledge, this is the first study testing the oncolytic efficiency of NDV virotherapy in a 3D model of breast cancer and normal mesenchymal stem cells. In cocultures with normal stem cells, we used the following breast cancer cell lines: AMJ13, estrogen, progesterone receptor–negative, and MCF7, progesterone receptor–positive breast cancer cell lines. We used two different breast cancer cell lines to cover both important types of breast cancer: hormone dependent and hormone independent.

We used PKH67 fluorescent dye to label the phospholipid membrane of NDV particles and used them as a tracking system to evaluate NDV oncolytic activity in breast cancer–normal stem cell spheroids (Figure 8). This model represents a good model for oncolytic virotherapy to simulate *in vivo* experiments as Mandel et al. (2013) had described breast cancer cells cocultured with stem cells seemed to facilitate tumor cell expansion through interaction that promoted extracellular matrix structures of a tumor microenvironment, which protects breast cancer cells from chemotherapy treatment. One of the major obstacles to cancer virotherapy is the stromal and extracellular barriers those

prevent viruses from dissemination inside the tumor mass (Howells et al., 2017). In the current study, we have reported the initial characterization of the 3D coculture sphere morphology and growth patterns in the scaffold panel, which successfully generated big spheroids of different sizes and shapes. It has been shown that 3D spheres are more like *in vivo* circumstances as nutrient, growth factors, and drug exposure are also heterogeneous, with cells on the outside edge of the spheroid being more exposed than cells in the inner core, thus resembling real tumors *in vivo*. Furthermore, malignant cells are grown on their microenvironment with normal cell signals for survival, growth as spheres aggregated to clusters, and metastasis (Aggarwal et al., 2012).

The promising finding of our work was that a simple technique of PKH67 fluorescent dye was used to label our NDV AMHA1 strain successfully to track virus entry, infectivity, and antitumor activity in our novel 3D coculture model. Another group used NDV expressing green fluorescent protein rFMW/GFP as a valuable method to track the efficiency of their strain NDV/FMW in killing cancer cells and determine the oncolytic mechanisms of NDV in inducing cell death (Jiang et al., 2018).

One possible way to investigate virus penetration of sphere cells is to label viruses with florescent dye and track the event of entry and vesicular trafficking to examine the virus's penetrance as compared to controls. Since viruses are believed to bind to multiple receptor molecules, single-particle detection may also be used to study the effects of multiple receptor molecules (Ewers and Schelhaas, 2012). The Fluorescent Cell Linker PKH67 uses proprietary membrane labeling technology to stably incorporate a green fluorescent dye with long aliphatic tails (PKH67) into lipid regions of the cell membrane (Wallace et al., 2008). PKH67 is well suited for cytotoxicity assays that use propidium iodide or 7-aminoactinomycin D as viability probes (Zaritskaya et al., 2009; Awan et al., 2010; Tario et al., 2011). The PKH-based adsorption assay, besides its simplicity, is more rapid; after fusing NDV with the cell membrane, either receptor-mediated endocytosis of entire virus particles or constitutive pinocytosis of viral envelope sections might cause this. In the host cells, lateral migration of the dye molecules is expected to occur when the viral membranes fuse with the cell membrane (Balogh et al., 2011). PKH67 is often used for proliferation monitoring based on dye dilution (Barth et al., 2010; Rong et al., 2015), which includes the estimation of antigen-specific precursor frequencies (Schwaab et al., 2007), as well as for *in vivo* cell trafficking studies (Ledgerwood et al., 2008).

The labeled virus, oncolytic PKH67-NDV, replicated efficiently in breast cancer–hATMSCs spheroids. Furthermore, the PKH67-NDV AMHA1 strain showed significant oncolytic activity in breast cancer–hATMSCs 3D cultures as it inhibited sphere growth, as we can see a reduction in sphere size and number. PKH67-NDV inhibited the 3D growth potential of AMJ13 and MCF7 cells through lysing the spheres and other death mechanisms. Previously, we indicated that NDV AMHA1 induces both intrinsic and extrinsic apoptosis cell death involving caspase-dependent and -independent pathways (Mohammed et al., 2019). In the current study, PKH67-NDV induced lysis of breast cancer coculture 3D spheres as observed inside the cancer cell cytoplasm, which is fluorescent green, indicating virus presence that induces cancer cell death in response to PKH67-NDV infection. Our studies found that the NDV AMHA1 strain inhibits the glycolysis pathway in breast cancer cells by interfering with glycolysis enzymes such as hexokinase and GAPDH (Al-Shammari et al., 2019; Al-Ziaydi et al., 2020a). It has been reported that NDV can replicate in lung cancer spheroids and induce cell death in lung cancer stem cells (Hu et al., 2015).

We conducted a 2D culture and coculture assay to confirm the selectivity of NDV to infect cancer cells more than normal stem cells. The results show a significant increase in the high intensity of green fluorescence in cancer cells alone and in cocultures of cancer and normal cells treated with PKH-67 linker–labeled NDV as compared to the control coculture (PKH-67 linker without NDV) and labeled NDV–infected normal hATMSCs. The normal cells showed mild staining, reflecting some viral entry, but there was a huge difference when compared to that of cancer cells regarding the staining intensity, reflecting higher infectivity in cancer cells.

Moreover, the coculture system showed two populations of cells, first with a high-intensity green fluorescence and second, with a low intensity, in a very clear pattern, in both cancer cell lines tested: AMJ13 and MCF7. The high-intensity cells are the cancer cells, and the low-intensity ones are the normal cells. It has been found that MSCs can be used to carry oncolytic NDV to cancer cells such as glioma (Kazimirsky et al., 2016).

We established a 3D sphere system for cancer and normal cells separately to analyze the HN-NDV protein expression level by IF assay to confirm viral infectivity and replication inside cancer cells but not inside normal cells. The HN-NDV protein showed significant expression levels in the cancer spheroids for both cell lines, AMJ13 and MCF7, while there were no significant expression levels in the normal cells. These results prove that HN protein is expressed in infected cancer spheroids and has no expression in normal spheroids, confirming NDV selectivity in replication. These results confirmed the 2D experiment for halted virus replication in normal cells but efficient replication inside cancer cells.

The NDV genome encodes many proteins, such as NDV HN (hemagglutinin–neuraminidase); NDV HN is a glycosylated component of the external envelope responsible for NDV binding to host cells. Moreover, NDV HN binds to a receptor on target cells, causing an increase in the production of death receptors (DRs) and other proteins in the host cell (Chen et al., 2001).

After the virus and cell surface membranes have been bound, the NDV F protein regulates how they merge. The HN and F proteins promote infecting neighboring cells, contributing to NDV pathogenicity and virulence (Brown et al., 2021). HN protein contributes significantly to the replication and pathogenicity characteristics of NDV (Jin et al., 2016). NDV glycoprotein HN is synthesized by the rough endoplasmic reticulum and transported *via* the smooth intracellular membranes to the cell surface plasma membrane and into new NDV progeny (Nagai et al., 1976). Therefore, HN detection in the infected cell only confirms virus replication in cancer cells, as our immunofluorescence assay results have shown.

Our previous work revealed that classical chemotherapeutics, as well as different types of phytotherapeutics, enhance NDV AMHA1 oncolytic activity against breast cancer cells both *in vitro* and *in vivo*, as well as against many other cancer cell types (Al-Shammari, et al., 2016; Al-Shammari et al., 2020b). These data suggest promising strategies of NDV AMHA1 combination with chemotherapy or novel antitumor treatments that can increase oncolytic activity in breast cancer coculture 3D spheres to overcome the expected resistance of different types of cancer. NDV oncolytic activity involves the induction of a specific immune response against the infected cancer cells by inserting its antigens in the cell plasma membrane, as documented by our study in detecting HN protein on the infected cancer cell surface. This antigenic modification to the tumor cell surface can increase its recognition by the immune system leading to a specific immune response that helps in tumor elimination.

Conclusion

We introduced the first use of breast cancer–normal stem cells 3D cocultures *in vitro* model to evaluate NDV antitumor activity. The 3D coculture model better resembles the *in vivo* environment *in vitro* regarding the barriers preventing oncolytic virus spread inside the tumor mass. The results showed that oncolytic NDV could destroy breast cancer 3D spheres while sparing the normal cells. Such results are encouraging for the clinical trials of oncolytic NDV AMHA1 as breast cancer therapy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving animals were reviewed and approved by the Scientific Committee of the Department of Biotechnology, College of Science, Baghdad University.

Author contributions

AA-S, and ME. designed the experiments. ME, AA-S, and MS conducted experiments. ME, AA-S, and MS. wrote the manuscript. All authors approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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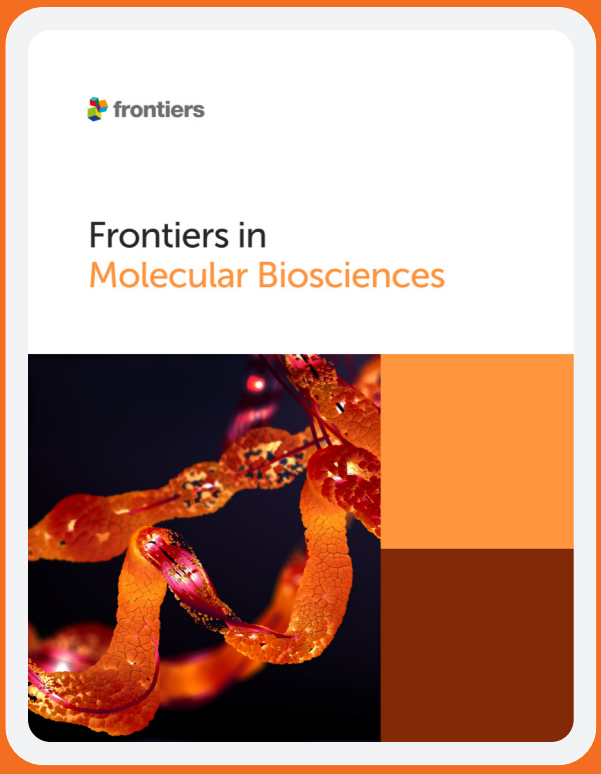

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