ONCOLYTIC VIRUSES—GENETICALLY ENGINEERING THE FUTURE OF CANCER THERAPY

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ONCOLYTIC VIRUSES—GENETICALLY ENGINEERING THE FUTURE OF CANCER THERAPY

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The ability to genetically engineer oncolytic viruses in order to minimize side effects and improve the selective targeting of tumor cells has opened up novel opportunities for treating cancer. Understanding the mechanisms involved and the complex interaction between the viruses and the immune system will undoubtedly help guide the development of new strategies. Theranostic biomarkers to monitor these therapies in clinical trials serve an important need in this innovative field and demand further research.

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EDITORIAL
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Editorial: Oncolytic Viruses—Genetically Engineering the Future of Cancer Therapy

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Editorial on the Research Topic

Oncolytic Viruses—Genetically Engineering the Future of Cancer Therapy

Since the 1960s, oncolytic viruses (OVs) have been a target of research as a therapeutic modality for cancer. The mechanism of these viruses involves both direct tumor cell lysis and the induction of immunogenic cell death. Clinical trials have explored a wide variety of viruses including naturally occurring viruses and genetically engineered viruses. Indications have spanned the gamut from hepatocellular carcinoma to soft tissue sarcoma to glioblastoma multiforme to multiple myeloma. Recently, the U.S. Food and Drug Administration announced the first FDA-approved OV therapy, for the treatment of melanoma lesions in the skin and lymph nodes. OVs have been used as single agent therapy or in combination with conventional cancer therapies. Current challenges including both scientific and regulatory do not diminish the significant potential for the future of this modality. Questions about the advantages of one virus over another, the synergistic potential of multiple viruses used in combination, dosage, and optimal route of administration remain unresolved and demand further research. Furthermore, the possibility that a particular OV might be more suitable for a specific cancer type than other OVs depending on the mechanism of the virus and the nature of the cancer raises additional research challenges. The precise role of adjuvant therapies such as dendritic cells in combination with OVs is yet another unresolved area in this innovative field.

While some of the research in this volume focused on specific viruses, others have confined their investigations to specific cancers, the role of the immune system in oncolytic virotherapy, or various strategies in developing recombinant viruses. Ocathail et al. looked at what might be the most familiar and widely understood OV, namely, adenovirus; however, they did not look at the virus in isolation. Rather, they investigated the interaction between the virus and radiation and found that the virus can actually sensitize the tumor to radiation therapy. Yin et al. chose to concentrate on another well-known specific OV, namely, the herpes simplex virus (HSV). They described the particular characteristics that enable HSV to evade T cells and highlighted various strategies in modifying the virus to increase its efficacy along with approaches to combinatorial therapy. Similarly, Eissa et al. also directed their research toward HSV. Their study focused on HF-10, a HSV, which has shown the ability to reduce tumor growth and prolong survival rates. They surveyed the various preclinical and clinical trials with HF-10 in monotherapy and combination therapy. They found that HF-10 has high tumor selectivity and a potent effect against tumors. Shifting to other specific viruses, Kleinlutzum et al. narrowed their investigation to comparing a recombinant measles virus MV-CD133 to a recombinant vesicular stomatitis virus, VSV-CD133. They found that VSV-CD133 infected a much wider area of the tumor than CD133 over the same amount of time. In addition, Angelova et al. focused on a specific virus, namely, the H1 Parvovirus. They reviewed the use of H1 Parvovirus in pancreatic carcinoma and in glioblastoma. They then surveyed the preclinical use of the virus in hematological

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malignancies specifically. They found that H-1PV can infect and kill cancer cells efficiently in these malignancies. Haddad looked specifically at the vaccinia virus (VACV). She reviewed preclinical studies with genetically engineered VACV strains, analyzing the advantages of this virus as an OV such as its large genomic capacity. Various strategies employed in the newer generations of the engineered viruses were discussed including transgene delivery for treatment, imaging, and combination therapy.

In terms of research on specific cancers Pease and Kratzke honed in on mesothelioma. Since mesothelioma tends to grow locally and in a location that allows for direct injection of the virus into the tumor, we would expect it to be an ideal candidate for oncolytic virotherapy. They summarized the preclinical studies using various viruses for mesothelioma including: adenovirus, HSV-1, vaccinia, measles, and others. Overall, they see much potential for the future for treating mesothelioma with combination approaches including OVs.

The role of the immune system in oncolytic virotherapy is the subject of intense research. Generally, it is assumed that viruses trigger the immune system and that the immune system attacks the tumor cells. Surprisingly, Filley and Dey explain why that is an oversimplification. In fact, the role of the immune system is sometimes actually the opposite. More specifically, there are immunological barriers to oncolytic virotherapy including: neutralizing antibodies, complement proteins, and type I interferon signaling, among others. The vector and timing of the viral infection along with the specific malignancy involved can all play a role in whether the immune system serves as an ally or not in the fight against cancer. Guo et al. also reviewed the nature of the relationship between the immune system and OVs. They highlighted various hurdles preventing OVs from broader use. These included the following: limited range of OVs, premature clearing of viruses by the immune system, and toxicities. Similarly, Holay et al. looked at studies of viruses that have incorporated specific tumor antigens to improve the response of the immune system to the tumors. They suggested that improvements in sequencing, computational techniques, and peptide isolation have enabled better tumor antigen discovery. Jhawar et al. looked at both naturally occurring and engineered viruses and their immune and non-immune pathways. More specifically, they summarized approaches involving improved antigen presentation, heat shock protein, and serotype switching. Meyers et al. focused on three main strategies for developing recombinant viruses. They included: improving host immune response by inserting transgenes such as granulocyte-macrophage colony-stimulating factor, combining OVs with drugs that modulate the immune system such as immune checkpoint inhibitors, and the prime boost strategy. In the prime boost approach, tumor-specific antigens can be built into the one viral platform to prime the immune system before being exposed to a second viral platform carrying the same antigens that upregulate the antitumor immune response. Shifting gears, another extremely innovative strategy in developing recombinant viruses, Bofill et al. investigated the insertion of miRNA response elements recognizing miRNAs expressed in specific tissues, but downregulated in tumors, into viral genes. They explained the complex nature of the interaction between viruses and host cells and how it can be maximized using miRNA. Howells et al. reviewed both gene insertion and gene deletion strategies for generating recombinant OVs. They also reflected on a third strategy involving the control of gene promoters both in tumor cells and in the viral genes.

Irwin et al. analyzed various pathways in the production of deoxynucleotide triphosphate (dNTP). The production of dNTP is often dysregulated in cancer cells. This difference between cancer cells and normal cells can be leveraged to selectively target the cancer cells. They found that the supply of dNTP can affect viral replication and the immune response. Further studies are necessary to explore how viruses can be engineered to capitalize on these findings to improve therapy.

Finally, with the recent success of clinical trials for oncolytic virotherapy, the need to improve methods for monitoring this treatment and to better understand the mechanism of action is great. Ansel et al. reviewed four primary strategies for monitoring oncolytic virotherapy *via* gene expression and highlighted advantages and disadvantages of each one. They concluded that combined gene expression studies looking at both the tumor expression and the viral expression could potentially provide much more information about the efficacy of the treatment modality and its pathway.

AUTHOR CONTRIBUTIONS

BG contributed substantially to the conception of the work and revised it critically. JR contributed substantially to the conception, design, and analysis of the work and also drafted the work.

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REVIEW
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Combining Oncolytic Adenovirus with Radiation—A Paradigm for the Future of Radiosensitization

Sean M. O'Cathail[1†], Tzveta D. Pokrovska[2†], Timothy S. Maughan[1], Kerry D. Fisher[2], Leonard W. Seymour[2] and Maria A. Hawkins[1]*

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Oncolytic viruses and radiotherapy represent two diverse areas of cancer therapy, utilizing quite different treatment modalities and with non-overlapping cytotoxicity profiles. It is, therefore, an intriguing possibility to consider that oncolytic ("cancer-killing") viruses may act as cancer-selective radiosensitizers, enhancing the therapeutic consequences of radiation treatment on tumors while exerting minimal effects on normal tissue. There is a solid mechanistic basis for this potential synergy, with many viruses having developed strategies to inhibit cellular DNA repair pathways in order to protect themselves, during genome replication, from unwanted interference by cell processes that are normally triggered by DNA damage. Exploiting these abilities to inhibit cellular DNA repair following damage by therapeutic irradiation may well augment the anticancer potency of the approach. In this review, we focus on oncolytic adenovirus, the most widely developed and best understood oncolytic virus, and explore its various mechanisms for modulating cellular DNA repair pathways. The most obvious effects of the various adenovirus serotypes are to interfere with activity of the MRE11-Rad50-Nbs1 complex, temporally one of the first sensors of double-stranded DNA damage, and inhibition of DNA ligase IV, a central repair enzyme for healing double-stranded breaks by non-homologous end joining (NHEJ). There have been several preclinical and clinical studies of this approach and we assess the current state of progress. In addition, oncolytic viruses provide the option to promote a localized proinflammatory response, both by mediating immunogenic death of cancer cells by oncosis and also by encoding and expressing proinflammatory biologics within the tumor microenvironment. Both of these approaches provide exciting potential to augment the known immunological consequences of radiotherapy, aiming to develop systems capable of creating a systemic anticancer immune response following localized tumor treatment.

Keywords: oncolytic virus, radiation, radiosensitizer, adenovirus, immunotherapy

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INTRODUCTION

Radiation therapy is responsible for an estimated 40% of all the cured cancers worldwide (1). Modern radiotherapy techniques allow for reduced toxicity due to improved accuracy and modulation of radiation delivery. The therapeutic window between efficacy and toxicity is often optimized by the

addition of a radiosensitizer. However, the development of novel radiosensitizers is challenging, with cytotoxic chemotherapy remaining the mainstay of radiosensitization in most solid organ tumors. There is an unmet need for rational development of radiation–drug combinations, as a relatively modest change in therapeutic index could have significant implications regarding improving outcomes. Apart from the addition of cetuximab to radical head and neck radiotherapy (2), no other targeted agent has been approved for radiosensitization in the last decade. Better understanding of the biological effects of radiation at a molecular level and the expanding availability of drugs that act on the specific pathways of radiobiological damage offers new opportunities. The unique nature of radiation injury and its cellular damage means that it is ideally suited for combination with oncolytic viruses. Here, we will discuss the nature of both radiation-induced DNA damage and the interaction between oncolytic adenoviral proteins and the DNA damage response, the behavior of oncolytic viruses in a cancer context and the proposed mechanism of synergy gained from their combination. We will also summarize the preclinical and clinical data to date, including toxicity therein, the role of the immune system in optimizing the effectiveness of combination therapy and, finally, the ability to arm oncolytic viruses to maximally contribute to effective synergy.

RADIATION-INDUCED DNA DAMAGE AND REPAIR

The therapeutic effect of radiation on cell kill is mediated through DNA damage, specifically double-strand DNA damage, resulting in irretrievable cell death. Here following is a basic overview of the two main types of DNA damage produced by ionizing radiation (see **Figure 1**). It is important to understand the role of both non-homologous end joining (NHEJ) and homologous recombination repair (HRR) in order to understand how the combination with oncolytic virus may prove useful in the future. NHEJ is classically described as occurring in the G1 phase of cell division when there is no sister chromatid to act as a template. HRR is described mainly in G2/S phase when the presence of an undamaged chromatid acts as a template for repair of the affected DNA strand. Although the processes are not mutually exclusive, a cursory separation allows for an informed

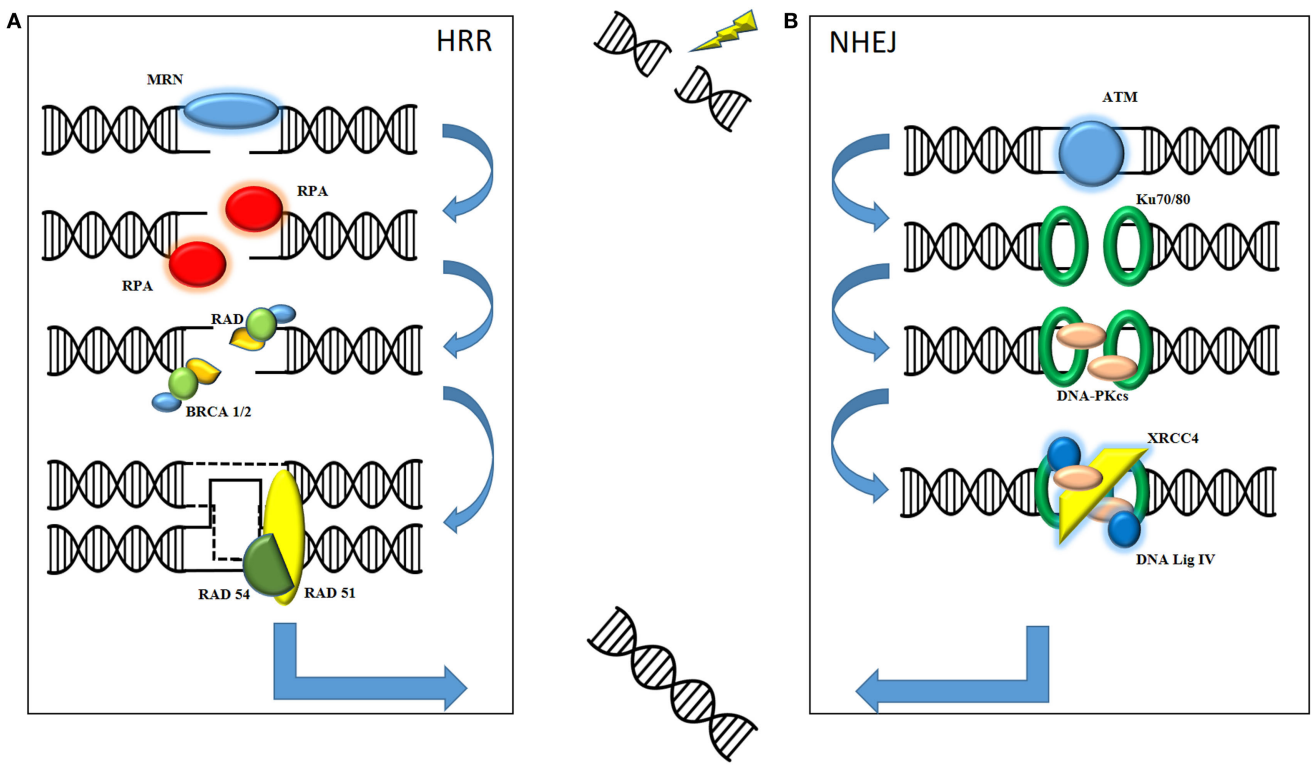

FIGURE 1 | Ionizing radiation causes fatal double-strand breaks. DNA damage repair is mediated by two main pathways: homologous recombination repair (HRR) and non-homologous end joining (NHEJ). **(A)** In HRR, damage is sensed by the MRE11–Rad50–Nbs1 (MRN) complex, consisting of MRE11, Rad50, and Nbs1, which facilitates recruitment of downstream mediators to the site of damage. These include replication protein A (RPA), the Rad family of proteins and BRCA1 and BRCA2. Final sequence homology for the damaged DNA is provided by invading, and requires the presence of, the sister chromatid. **(B)** NHEJ is initiated by the recruitment of phosphatidylinositol-3-kinase-related kinase (PIKK) family such as ataxia-telangiectasia mutated (ATM). These facilitate the recognition of damaged strands by Ku70/Ku80, subsequent processing by DNA-PKcs and final repair and processing of strand ends by XRCC4 and DNA Ligase IV. The final product of both pathways is a repaired, complete strand of DNA.

mechanistic explanation. The role of the MRE11–Rad50–Nbs1 (MRN) complex appears to be central to both forms of repair as a sensor for double-strand breaks (3).

Non-Homologous End Joining

Protein kinases belonging to the phosphatidylinositol-3-kinase-related kinase (PIKK) family, such as ataxia-telangiectasia mutated (ATM) and ATR, are recruited to the site of DNA damage. Recognition of the damaged strands is facilitated by Ku70/Ku80. Subsequent recruitment of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which autophosphorylates to allow release from the DNA, means that end processing and ligation of the strands can commence. This is primarily mediated by DNA ligase IV and X-ray repair cross-complementing group (XRCC4). Consequently, these often form the basis for translational work, with their presence indicating significant DNA damage in need of repair.

Homologous Recombination Repair

In contrast to NHEJ, HRR is a high fidelity repair mechanism for mammalian DNA. The double-strand break is sensed by the MRN complex, which processes the DNA into 3′ DNA single strands. The MRN complex allows for recruitment and activation of ATM, a key regulator of HRR. Autophosphorylation of ATM allows for downstream recruitment of repair proteins, such as BRCA1 and BRCA2, replication protein A (RPA), and other mediators such as the Rad family of proteins (4). Rad51 is a key protein as it mediates the invasion of the sister chromatid of the homologous strand to allow for accurate replication of DNA. The 3′ end of the ssDNA invades the homologous strand of the sister chromatid to form a "displacement" loop and the sequence is then extended by synthesizing new DNA to form a Holliday junction. Gap filling occurs by DNA synthesis beyond the original break site before Rad54 facilitates release of the newly synthesized end (5). The DNA strand is then annealed with the other end of the ssDNA to complete the repair process.

Although these two pathways are key mediators of the DNA damage response pathway, it has become clear recently that post translational modifications have a key role to play in coordinating the cell's repair (6). This can include modification of the proteins themselves, phosphorylation of cyclin dependent kinases to control cell phase, ubiquitylation and sumoylation, and the regulation of checkpoints.

ONCOLYTIC ADENOVIRUSES AND THE DNA DAMAGE RESPONSE

The Concept of Virotherapy

Oncolytic virotherapy uses lytic viruses that replicate selectively within cancer cells. This approach combines targeted cytotoxicity with amplification of the therapeutic agent actually within tumor cells. With the first Federal Drugs Administration (FDA) approval of an oncolytic virus, Talimogene laherparepvec (T-vec) in 2015 (7), interest in oncolytic virotherapy is rapidly expanding. This is, however, a field that has been developing for many years (8) and a wealth of information is now available on the ways in which these viruses can be incorporated in the current anticancer therapeutic paradigms (9).

A range of oncolytic viruses are currently undergoing clinical trials, including adenovirus, vaccinia virus, herpes simplex virus (HSV), reovirus, poxvirus, coxsackievirus, Newcastle disease virus, measles virus, parvovirus, Seneca Valley Virus, poliovirus, and vesicular stomatitis virus (10). The mechanism through which these viruses lead to tumor cell death varies between types, however it is the ability of certain oncolytic viruses to interact with and inhibit the DNA damage response which is of particular interest with regard to combining treatment with radiotherapy (11). Nuclear-living DNA viruses might be particularly sensitive to cellular DNA repair mechanisms and, therefore, could have developed strategies to interact with these cellular factors to protect viral DNA from unwanted repair (12).

There is considerable evidence available on the ability of adenoviruses, in particular, to inhibit the DNA damage response as part of the normal virus life cycle (see **Figure 2**). It is hypothesized that this is to prevent the linear double-stranded DNA viral genome from being recognized by the cell as a double-stranded DNA break, potentially leading to concatemerization of virus genomes (13, 14). This was first observed using a range of mutant adenovirus type 5 and type 2 on KB cells, a human epidermoid cell line. The mutations were all located in the early region 4 (E4) region of the genome and, when both E4orf3 and E4orf6 regions were mutated, concatemerization of the viral genome was noted (15).

The Effect of Virus Proteins on the MRN Complex

The key cellular factor involved in sensing double-stranded DNA damage, particularly in the HRR pathway, is the MRN complex. The majority of the evidence relating to the interaction between adenoviral proteins and the DNA damage response is consistent with an effect on this complex. This was initially demonstrated by Stracker et al (16). Adenoviral genome concatemers formed in cells infected with adenovirus serotype 5 (Ad5) lacking the E4 region, but not with wild-type virus. However, no concatemers formed following infection with this mutant virus in cell lines containing any of mutant DNA ligase IV, DNA-PKcs, Nbs1, or MRE11, suggesting that these cellular proteins are all required for recognition and concatemerization of the adenoviral genome. In addition, in cells infected with the E4 mutant virus, the MRN complex could be seen to form foci surrounding viral replication centers. This paper was the first to provide evidence for MRN degradation mediated by the Ad5 E4orf6–E1b55K complex and MRN mislocalization mediated by Ad5 E4orf3 (16). These two mechanisms of MRN inhibition will be briefly explored below.

Degradation of the MRN Complex

Stracker et al. initially demonstrated that intracellular levels of MRE11, Rad50, and Nbs1 decreased following infection with wild-type adenovirus serotype 5 (Ad5). This was due to enhanced protein turnover and was not seen following infection with E4-deficient Ad5. Infection with Ad5 mutants lacking different E4 genes demonstrated that there was no degradation of MRE11

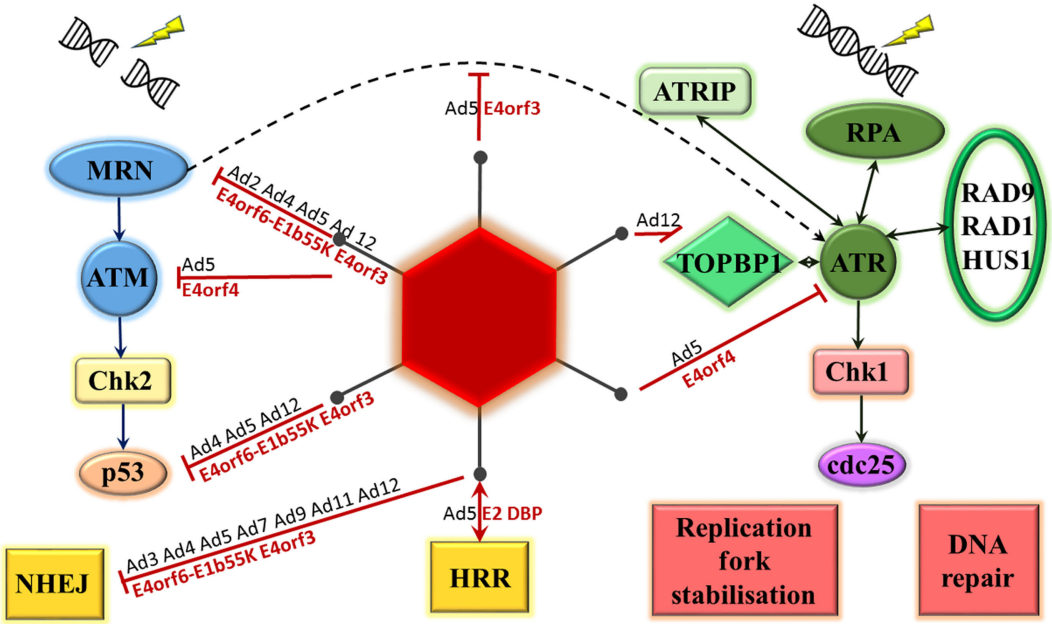

FIGURE 2 | Adenovirus proteins interact with the DNA damage response. Double-strand breaks result in MRE11–Rad50–Nbs1 (MRN) complex activation of ataxia-telangiectasia mutated (ATM). This leads to phosphorylation of checkpoint kinase 2 (Chk2), activation of p53 and DNA damage repair through either non-homologous end joining (NHEJ) or homologous recombination repair (HRR). Single-stranded DNA is bound by replication protein A (RPA), which recruits ATM and RAD3-related (ATR) kinase, ATR-interacting protein (ATRIP), RAD9-RAD1-HUS1 and topoisomerase-IIβ-binding protein 1 (TOPBP1) to site. ATR phosphorylates checkpoint kinase 1 (Chk1) resulting in phosphorylation of the cell division cycle 25 (cdc25) phosphatases and a number of cellular changes, including DNA repair, effects on cell cycle, and stabilization of replication forks. Adenoviral proteins interact with a number of these steps, the most studied is adenovirus 5. Serotype of interacting adenovirus (Ad) denoted in black, adenovirus 5 protein identified as mediating interaction denoted in red.

and Rad50 when cells were infected with Ad5 specifically lacking E4orf6–E1b55K. Furthermore, transfection of 293 cells with an E4orf6 expression vector resulted in MRE11 and Rad50 degradation, but not transfection with an expression vector containing mutant E4orf6 unable to form a complex with E1b55K. These, therefore, demonstrated that MRN degradation following infection with Ad5 is mediated by the E4orf6–E1b55K complex (16). Karen et al. further showed that this MRN degradation occurs prior to viral DNA accumulation (14).

The majority of evidence available is on the function of Ad5 proteins. Stracker et al. compared effects on MRN following infection with Ad5, Ad4, and Ad12. Infection of HeLa cells with all three serotypes led to decreased levels of MRE11 and Rad50. Likewise, transfection of 293 cells, which stably express Ad5 E1b55K, with plasmids encoding E4orf6 from these serotypes led to degradation of all MRN components as well as p53. These results suggest that the ability of the E1b55K–E4orf6 complex to cause MRN degradation is conserved between these serotypes (17). Forrester et al. have also demonstrated MRE11 degradation following infection with Ad4, Ad5, and Ad12, but not following infection with Ad3, Ad7, Ad9, and Ad11 (18). Therefore, although MRN degradation does appear to be targeted by a range of adenovirus serotypes, this is not conserved across all serotypes.

Relocalization of the MRN Complex

Stracker et al. demonstrated that early after infection by adenovirus serotype 5 there appears to be relocalization of the MRN complex to areas of nuclear speckles partially overlapping with promyelocytic leukemia protein (PML) (16). In uninfected cells PML is found in oncogenic domains (PODs/ND10), however Ad5 E4orf3 causes relocalization of PML into nuclear tracks (16, 19, 20). This function appears to be conserved across serotypes and has been demonstrated for Ad2, Ad4, and Ad12 E4orf3 (17, 20).

Stracker et al. further showed that this MRN relocalization does not occur following infection with Ad5 lacking E4orf1 to E4orf3. Transfection of 293 cells with different expression vectors showed that expression of E4orf3 is sufficient for relocalization of the MRN complex to occur. The relocalization of MRN can, therefore, be linked directly to expression of Ad5 E4orf3 (16).

It has since been demonstrated that at later time points of infection Ad5 E4orf3 causes redistribution of the MRN complex to large single juxtanuclear cytoplasmic accumulations suggestive of aggresomes. These contain both E4orf3 and E1b55K, but seem to be able to form in the absence of the latter (21, 22). Aggresomes are areas of ubiquitin-rich cytoplasmic inclusions containing aggregated misfolded proteins and surrounded by a cage of the intermediate filament protein vimentin. They are proposed to be a cellular response to undegraded, aggregated protein (23) and their formation has been linked to a number of degenerative diseases (24). Infection of A549 cells with an E4orf6/E4orf3 mutant Ad5 has been shown to greatly delay both aggresome formation and MRE11 localization to these areas, but not infection with Ad5 lacking only one of these viral proteins. Therefore, both Ad5

E4orf3 and E4orf6 can be seen to be key proteins involved in MRE11 relocalization during adenovirus infection, and appear to be functionally redundant (22). This relocalization to the cytoplasm accelerates degradation, thereby acting to protect the viral genome from recognition by the MRN complex and subsequent concatemerization (22).

Evidence provided by Liu et al. suggests that E1b55K also plays an important role in the formation of MRN-containing cytoplasmic aggresomes. They utilized leptomycin B to inhibit an exportin interacting with an E1b55K nuclear export signal. In cells infected with an Ad5 E4orf6 deletion mutant, which allows visualization of MRN without its degradation, leptomycin B caused inhibition of MRE11 relocalization from the nucleus to juxtanuclear aggresomes. Furthermore, addition of leptomycin B to wild-type Ad5-infected cells resulted in a decreased rate of MRE11 depletion. Finally, there was minimal exportation of MRE11 out of the nucleus following infection of cells with either an E1b55K deletion mutant Ad5 or Ad5 containing mutant E1b55K unable to bind MRN. These results suggest that E1b55K plays an important role in the relocalization and degradation of the MRN complex following infection with Ad5 (22).

Interestingly, when comparing the effects of Ad5 E4orf3 with that of Ad4 and Ad12, Stracker et al. found that, though Nbs1 appeared to be relocated away from viral replication centers following infection with Ad5, Ad4 and Ad12 did not have the same effect. These results would suggest that there are some differing effects on the relocalization of MRN components by different viral serotypes (17). Results published by Forrester et al., on the other hand, demonstrated that Ad5, Ad9, and Ad4 infection caused MRE11 relocalization to PML tracks. Infection with Ad3, Ad7, Ad11, and Ad12 resulted in relocalization of MRE11 to viral replication centers, as demonstrated by areas of RPA32 staining (18). These results are consistent with the view that different virus serotypes have varying effects on the MRN complex, and demonstrate some interesting differences in the results pertaining to Ad4 effects on members of the MRN complex.

The Effects of Virus Proteins on Phosphatidylinositol-3-Kinase-Related Kinase (PIKK) Family

Ataxia-telangiectasia mutated and ATR play pivotal roles in the cellular response to DNA damage. Brestovitsky et al. have recently presented some data on the role of Ad5 E4orf4 in inhibiting the DNA damage response through effects on these proteins and their substrates. Comparison of the phosphorylation status between cells infected with an Ad5 E4 deletion mutant and an Ad5 E4 deletion mutant expressing only E4orf4 was carried out. Addition of E4orf4 significantly decreased the levels of phosphorylation of all ATM and ATR pathway proteins tested. This was shown to be dependent on E4orf4 interaction with the cellular protein phosphatase 2A (PP2A), which is known to dephosphorylate a number of proteins involved in the DDR including ATR, ATM, DNA-PK, and a range of their substrates. Both the cytopathic effect of E4orf4 and viral replication were significantly enhanced in cells lacking wild-type ATM and treated with an ATR inhibitor. Lack of wild-type ATM appeared to be beneficial during the early part of the virus life cycle and ATR inhibition during the late part of the virus life cycle. Moreover, expression of E4orf4 alone in cells was sufficient to inhibit DNA damage repair and to sensitize cells to the effects of genotoxic drugs even out with the context of infection (25). These results suggest inhibition of ATM and ATR is beneficial for the Ad5 viral life cycle and is mediated via an interaction between E4orf4 and PP2A.

The Effects of Virus Proteins on ATM

It has been suggested that, in the context of infection with Ad5 mutant viruses lacking E4 and unable to interfere with the MRN complex, ATM plays an important role in viral inhibition (26). Given this and the results presented by Brestovitsky et al. (25), it is perhaps somewhat surprising that Forrester et al. demonstrated that infection of HeLa cells with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11, and Ad12 all resulted in an increase in KAP1 phosphorylation, a marker of ATM activation. These results suggest that all viral serotypes investigated caused activation of ATM, however the kinetics of this varied between different serotypes (18).

This discrepancy can be explained by allowing for two different mechanisms of ATM activation secondary to Ad5 infection. The first is proposed to be localized MRN-mediated ATM activation associated with blocking of viral genome replication at the earliest stages of the viral life cycle, prior to MRN inhibition by viral proteins. The second is an MRN-independent, global ATM activation that occurs subsequent to viral genome replication and does not impact on viral replication (27). Thus, the increase in KAP1 phosphorylation representative of increased ATM activity would not be necessarily associated with a direct impact on viral replication.

The Effects of Virus Proteins on ATR

ATR is traditionally associated with recognition of single-stranded DNA (28). It would initially seem counter-intuitive for DNA viruses to have developed mechanisms of ATR interaction, nonetheless there is evidence that this is the case with adenovirus (25, 29, 30). It is frequently seen that infection with Ad5 leads to widespread histone 2AX phosphorylation (γH2AX) (31, 32). γH2AX is usually a marker for double-strand DNA breaks; however following infection, it can be seen throughout the nucleus. Nichols et al. demonstrated that, in adenovirus-infected cells, the greatest decrease in phosphorylation of γH2AX following inhibition of ATM, ATR, or DNA-PK was seen following ATR inhibition, suggesting that ATR is the primary mediator of adenovirus-induced H2AX phosphorylation. Large amounts of γH2AX phosphorylation were seen after the onset of viral genome replication and some γH2AX of a differing pattern was seen following input of viral DNA at high multiplicities (31). This suggests that there is a cell response to the presence of viral DNA and potentially a mechanism by which there is ATR stimulation in response to viral replication. As ATR is known to respond to a wide range of cellular stresses (33), it may be that viral replication is a sufficient cell stressor to cause this. Alternatively, it has been demonstrated that a significant number of single-stranded DNA sequences are generated during viral replication (34), the virus may, therefore, have developed a mechanism to interact with ATR to prevent this stage of the viral replication cycle from causing

cell cycle arrest. There are a number of interactions between adenoviral proteins and the cellular ATR pathway that have been demonstrated.

E1b-AP5 (Ad E1b55K-associated protein 5) is a cellular protein that binds E1b55K in both Ad5-infected and Ad5-transformed cells (35). Blackford et al. have demonstrated that, in non-infected cells, this protein is localized to the nucleus but excluded from nucleoli. In the context of infection with adenovirus serotype 5 and 12, however, E1b-AP5 levels increase and it is redistributed to viral replication centers where is colocalizes with RPA32. Moreover, infection with these virus serotypes also seemed to lead to relocalization of ATR-interacting protein (ATRIP) to viral replication centers and colocalization with RPA. The two different viral serotypes appeared to have different effects on ATR kinase substrates. Whereas Ad12 infection was associated with marked E1b-AP5 and ATR-dependent hyperphosphorylation of RPA32 as well as hyperphosphorylation of Rad9, Ad5 infection did not have this effect, adding evidence that different virus serotypes have varying effects on the cell's DNA damage response pathway (36). Forrester et al. have provided further evidence in support of this. The effect on checkpoint kinase 1 (Chk1) phosphorylation, an ATR substrate, in response to infection with different virus serotypes was investigated. The authors demonstrated that the overall effect on phosphorylation levels, as well as the timeline over which changes took place, differed markedly between serotypes (18). In addition, the ATR-activator protein topoisomerase-IIβ-binding protein 1 (TOPBP1) was degraded following infection with Ad12, but not with other viral serotypes investigated (18, 30).

Carson et al. provide evidence that the inhibition of ATR function by Ad5 is through effects on MRN. Cell lines with hypomorphic mutations in either Nbs1 or MRE11 were infected with either wild-type or E4-deleted Ad5. Results were compared when these cell lines were transduced with Nbs1 or MRE11 wild-type cDNA. There was decreased phosphorylation of the ATR substrates Chk1 and RPA32, suggesting decreased ATR function, when cells infected with E4-deleted Ad5 did not express wild-type Nbs1 or MRE11. Furthermore, infection of HeLa cells with an E4-deleted Ad5 mutant virus lead to phosphorylation of Chk1 and RPA32. This was markedly decreased in cells infected with Ad5 expressing E4orf3, but not in cells infected with Ad5 expressing E4orf6 or E1b55K. The authors did not find this effect on ATM signaling. Interestingly, when HeLa cells were infected with Ad5 mutants lacking E4, transfection with an Ad5 E4orf3 expression vector was sufficient to lead to a decrease in phosphorylation of Chk1 and RPA32. This was not, however, the case following transfection with an Ad12 E4orf3 expression vector or with an Ad5 E4orf3 expression vector mutated to abrogate the protein's ability to mislocalize MRN. Ultimately, the authors provided evidence that MRN is key in the hyperphosphorylation of ATR substrates following infection with Ad5 lacking E4, and that this can be abrogated through expression of Ad5 E4orf3 and subsequent mislocalization of the MRN complex (29).

The Effects of Virus Proteins on p53

The full range of p53's effects within the cell is still being elucidated; however, it is clear that p53 has an inherent role in the cell's response to stressors and DNA damage (37, 38). It is, therefore, perhaps no surprise that its function is targeted by adenoviruses, though the exact effects of this targeting appear to vary between serotypes. Ad5 E4orf3 and E4orf6 are known to have an effect on p53 through interaction with the E1b55K protein (39, 40). Liu et al. have demonstrated that Ad5 E1b55K co-localizes with p53 to aggresomes. Following transfection of 293 cells with E4orf6, the majority of cells demonstrated p53 depletion, and in those cells where p53 was visible, it was associated with E4orf6 in aggresomes (22). Harada et al. used a proteomics-based approach to demonstrate that the E1b55K–E4orf6 complex interacts with a number of cellular proteins to lead to the polyubiquitination of p53 *in vitro* (41). It is thought that E1b55K–E4orf6, therefore, leads to p53 depletion through ubiquitin-dependent proteasome-mediated destruction.

The degradation of p53 does not appear to be conserved across all virus serotypes tested. Forrester et al. have demonstrated that, though infection with Ad4, Ad5, and Ad12 leads to p53 degradation, this does not occur following infection with Ad3, Ad7, Ad9, and Ad11. Interestingly, there appeared to be, in fact, a marked increase in p53 levels in cells infected with Ad3, Ad7, and Ad11. The authors present evidence that this is, however, not transcriptionally active. It was shown that MDM2 levels were decreased following infection with all virus serotypes investigated, including those associated with increasing levels of p53. Furthermore, following Ad3 and Ad7 infection of cells containing a p53 plasmid and a luciferase reporter construct, though there was a significant increase in p53 levels, there was only a minor increase in reporter transcription. In addition, cells infected with these virus serotypes demonstrated decreased protein levels of p21, a p53-regulated gene, secondary to decreased levels of p21 mRNA (18).

It remains to be seen how this variable interaction between p53 and the different adenovirus serotypes will impact on a potential synergistic effect with radiotherapy. The role of p53 within the cell is a complex and far-reaching one, hence its moniker "the guardian of the genome" (42). In particular, p53 inactivation is key in oncogenesis and it is mutated in approximately 50% of all cancers (43). Its function as a key modulator of apoptosis is, therefore, in balance with that of a DNA damage responder. Wild-type p53 is associated with enhanced chemosensitivity and radiosensitivity, but the exact p53-mediated cellular response to these stressors appears to be reliant on a range of cellular factors (44). As such, given the complex interactions between adenovirus infection and the cellular stress response, it is difficult to predict which p53 transcriptional pathway will ultimately be most affected by combination therapy.

Effects of Virus on DNA Repair Proteins

Effects of Virus Proteins on NHEJ

A physical interaction between an oncolytic viral protein and a protein heavily involved in the DNA damage response was first demonstrated by Boyer et al (45). Glioma cells with or without DNA PK were infected with either wild-type Ad5 or a mutant lacking E4. Concatemers formed in cells expressing DNA PK when these were infected by mutant virus, but not wild-type. Interestingly, no concatemerization was seen in cells lacking

DNA PK, and following plasmid transfection of 293 cells with plasmids expressing DNA PK and either E4orf6 or E4orf3, there appeared to be co-immunoprecipitation of DNA PK and these viral proteins.

Since this time, the major effect of multiple virus serotypes on NHEJ has been demonstrated to be through degradation of DNA ligase IV. This was initially shown in Ad5 by Baker et al., who demonstrated that degradation was dependent on expression of the E1b55K–E4orf6 complex and was likely mediated by ubiquitination and subsequent targeting by the proteasome (46).

Degradation of DNA ligase IV has also been observed post infection with adenovirus serotypes 3, 4, 5, 7, 9, 11, and 12 (18, 30). Crucially, it was the only cellular protein degraded by all adenoviral serotypes tested by Forrester et al., suggesting that it plays a key role in the inhibition of the DNA damage response pathway by human adenoviruses (18).

Effects of Virus Proteins on HR

There is currently limited evidence on the effect of oncolytic viruses on HR. Tookman et al. investigated the impact of HR status in ovarian cancer on adenovirus infection. Two cell lines were utilized, both from the same ovarian cancer patient. The first was obtained during a platinum-sensitive relapse, contained a deleterious BRCA2 mutation and was, therefore, HR defective, the second was obtained during a subsequent platinum-resistant relapse following development of a secondary BRCA2 mutation which restored the open reading frame and had, therefore, regained HR function. Cells were infected with wild-type Ad5, Ad11 or Ad35, or one of two Ad5 mutants, both containing an E3b region deletion, one of these also containing a deletion in the region of E1A CR2. Interestingly, the authors found that the cell line with functional HR demonstrated a significant decrease in cell survival following infection by all three Ad5 viruses when compared to the cell line with defective HR. Confocal microscopy showed colocalization between viral replication centres (VRC) and BRCA2. Surprisingly, the authors also noted colocalization of VRC and Rad51 foci in both the presence and absence of BRCA2, and this was confirmed in a number of other cell lines. This is the first demonstration of recruitment of HR proteins to the adenovirus VRC. Co-immunoprecipitation was consistent with an interaction between Rad51 and Ad5 E2 DNA binding protein. Furthermore, depletion of Rad51 resulted in reduced cytotoxicity and viral replication following infection with the above-mentioned Ad5 mutants, BRCA2 depletion likewise leads to reduced cytotoxicity (47). These data suggest these HR proteins are utilized by Ad5 to improve viral replication and cytotoxicity.

ADENOVIRUS AS A RADIOSENSITIZER

Adenoviruses have developed a range of interactions with cellular DNA damage repair proteins to allow successful viral replication. This has implications for the initiation of a number of DNA repair pathways activated in response to radiation-induced damage, in particular all adenoviral serotypes tested appear to target NHEJ repair (18). The hypothesis that oncolytic adenovirus infection would work synergistically with radiotherapy has been tested by a number of groups. The combination of CG7870 with radiation resulted in a synergistic increase in cell killing, both *in vitro* and *in vivo* in the LINPAC xenograft model, than either agent alone (48). Toth et al. studied 3 Ad5-based vectors and combined them with radiation in A549 lung cancer cells (49). Again they found that *in vivo* and *in vitro* tumor cell kill was increased with the combination approach than was seen with either agent alone. Similar findings have been noted with a variety of different adenoviral vectors in other cell types, including ovarian cancer cell lines (50) and glioma xenografts (51). Importantly, however, the effect of radiosensitization does not appear to extend to normal tissues. The combination of Ad5/CMV/p53 radiosensitized two non-small cell lung cancer cell lines (A549 and H322) *in vitro* and in xenograft models, in a synergistic fashion, but did not show an increased radiosensitization effect on normal lung fibroblasts (52). These observations provide a framework to consider the clinical rationale for adenovirus and radiation combinations, as discussed in section "Clinical Efficacy and Toxicity."

Although radiation damage to the viral genome could render the particle inactive, the small size of the genome is, statistically, unlikely to be affected by standard X-ray photons. X-rays are sparsely ionizing meaning that primary ionization events are well separated, at least microscopically. In comparison to the human genome (3×10^9 bp), the relative size of the genome for most therapeutically employed viruses (adenovirus is about 3.5×10^3 bp) means the ionization events are unlikely to trouble the majority of particles. The ability to influence ATR and by extension single-strand break repair, which is a far more common type of cellular response to radiation, is also beneficial. Single-strand breaks are easily repaired avoiding cell death. The arrest of single-strand break repair increases the likelihood of future catastrophic damage and apoptotic death.

RADIOTHERAPY AND THE IMMUNE RESPONSE

Immune Inhibition versus Immune Stimulation

The interplay between immunostimulatory and immunoinhibitory pathways in response to radiation is a complex and intricate one (53–55). Radiotherapy has long been thought to elicit an immunosuppressive effect (56, 57). A number of cell types have been implicated in this. Regulatory T cells have been shown to play an important role in the inhibition of an antitumor immune response following radiotherapy (58). There is also evidence for immunoinhibitory roles played by, and potential increased influx resulting from radiotherapy, of both tumor-associated macrophages (59, 60) and myeloid-derived suppressor cells (60, 61). Furthermore, radiation-induced effects on dendritic cells have been demonstrated *in vitro* to shift cytokine release away from activation and toward tolerization (62). Effects on different cell types and the tumor microenvironment have recently been elegantly reviewed elsewhere and an in-depth discussion is outwith the scope of this article (53, 55, 60).

On the other hand, the body of literature presents us with multiple preclinical and clinical examples of ways in which the host antitumor immune response can actually be augmented by

radiotherapy (63–71). A number of mechanisms have been proposed as mediating this. These include preferential radiotherapy-mediated killing of radiosensitive suppressor T cells (63, 64), increased immune cell infiltration of tumors (65), activation of dendritic cells (66, 67), improved antigen presentation both in draining lymph nodes (65) and within the tumor itself (68), induction/upregulation of cell surface markers that interact with cytotoxic T lymphocytes (69, 72), release of immunostimulatory molecules subsequent to radiation-induced immunogenic cell death (70, 71, 73), and the production of pro-inflammatory cytokines and chemokines (73).

We are still unable to predict which way the fine balance will tip in response to tumor irradiation. Interestingly, it has been proposed that ablative radiotherapy (typically greater than 6 Gy per fraction) favors a T-cell-dependent immunostimulatory anti-tumor response when compared to more traditional fractionation schedules (1.8–2 Gy per fraction), which may favor an immunosuppressive response (66, 68, 74, 75), though Dewan et al. have presented data that would appear to contradict this (76). The ideal fractionation schedule favoring immune stimulation has yet to be determined and is likely to be influenced by a large number of host and tumor-specific variables (72). Currently, focus is shifting toward combining ionizing radiation with immunotherapy to tip the scales in favor of promoting an antitumor immune response (54). In particular, a number of studies are currently planned or ongoing focusing on combining radiation with checkpoint inhibitors (53).

The Abscopal Effect

The abscopal effect, a phenomenon whereby ionizing radiation of a tumor leads to reduction of tumor growth outwith the field of radiation, was initially described by Mole (77). Demaria et al. demonstrated that it is at least partially immune mediated (78). Though a number of clinical cases describing the abscopal effect have now been documented, this phenomenon is by no means common (75, 79). With the advent of combination radiotherapy with checkpoint inhibitors, it is hoped and anticipated that cases demonstrating beneficial abscopal effect will become more common in the coming years.

ONCOLYTIC ADENOVIRUSES AND THE IMMUNE RESPONSE

Viruses utilize a number of mechanisms to evade the normal immune response. It has been proposed that the aberrant intracellular pathways in cancer cells, however, make them particularly vulnerable to targeting by oncolytic viruses for destruction via a mechanism of immunogenic cell death (10). This would provide an alternative means by which the viruses may act synergistically with radiotherapy and tip the scales in favor of immunostimulation. One potential mechanism to enhance this further is the development of armed oncolytic viruses that express immunostimulatory molecules (produced locally by virus-infected tumor cells and released into the tumor microenvironment), providing further signals that can enhance an antitumor immune response.

Oncolytic Adenovirus Mechanisms of Immune Stimulation

The different mechanisms of immunogenic cell death have recently been elegantly reviewed (80). With regard to cellular responses to invasion of pathogens, microorganism-associated molecular patterns (MAMPs)/pathogen-associated molecular patterns (PAMPs) interact with intra- and extracellular pattern recognition receptors, such as toll-like receptors and nucleotide-binding oligomerization domain-like receptors. In response to viral infection, a subsequent danger response is elicited and is associated with formation of inflammasome complexes and secretion of type I interferons. Pathogens, including viruses, demonstrate a range of mechanisms developed to avoid this immune detection and stimulation (80, 81).

In addition to PAMPs/MAMPs, virus-mediated cytotoxicity pathways may also be intrinsically immunogenic via damage-associated molecular patterns. For example, Dyer et al. have recently published data on the ability of Enadenotucirev (EnAd), a chimeric group B adenovirus currently undergoing clinical trials, to induce proinflammatory cell death (82). Mode of cytotoxicity displayed features consistent with oncosis, and infection with EnAd led to release of the pro-inflammatory markers heat shock protein 70 (HSP70) and high-mobility group box-1 (HMGB1) from cells. The pro-phagocytic marker calreticulin was also increased on tumor cells infected with EnAd. Moreover, infection of tumor cells lead to a significantly higher level of dendritic cell-mediated T cell activation than wild-type Ad5. These data suggest that induction of immunogenic cell death may be an additional mechanism by which oncolytic viruses could work synergistically with radiotherapy.

Arming Oncolytic Adenoviruses to Enhance Immune Stimulation

A number of different mechanisms have been utilized to "arm" oncolytic adenovirus and enhance the immune response. For example, Li et al. carried out a phase I dose escalation study investigating the effect of H103, a recombinant Ad2 virus over-expressing HSP70 (83). 27 patients with advanced solid tumors received intratumoral treatment. Clinical benefit rate (partial response, minor response, or stable disease) was 48.1%, with 11.1% experiencing partial response and, interestingly, three patients demonstrating transient regression of some distant non-injected areas of metastasis, though RECIST criteria for response were not met. The most frequently experienced side effects were local injection-site reaction and fever, hematological toxicities were observed in five patients.

A phase I study on the intravesical use of a GM-CSF expressing adenovirus, CG0070, in non-muscle invasive bladder cancer was carried out by Burke et al. (84). Of 35 patients treated, 17 had complete response (CR, 48.6%), with a median CR duration of 10.4 months. No clinically significant treatment-related toxicities were reported.

CGTG-602 (Ad5/3-E2F-Δ24-GMCSF) was another oncolytic adenovirus engineered to express GM-CSF (85). *In vivo* this virus demonstrated selective replication in tumor cells and appeared to induce an immune-mediated antitumor response. Intratumoral

administration was carried out in 13 patients with advanced metastatic tumors. 6 were able to be assessed with PET CT, of these 83% demonstrated radiological disease control and PET response rate was 50%, including one patient who demonstrated complete metabolic response in a non-injected site. Tumor marker assessment indicated potential benefit in 6 out of 10 patients who had elevated markers at baseline.

Sova et al. present data on the use of a TNF-related apoptosis-inducing ligand (TRAIL) expressing adenovirus vector (86). This vector was able to induce tumor-specific apoptosis both *in vitro* and *in vivo*. In a mouse model for colorectal liver metastases, when compared to untreated controls, intravenous administration of the vector resulted in an approximately 10-fold tumor burden reduction versus approximately 1.5 to 3 fold in response to non-TRAIL expressing vectors, and complete eradication of metastases in three out of five mice. There was transient elevation of a liver enzyme following vector infusion but no histological changes of normal liver tissue.

Finally, Hirvinen et al. generated Ad5/3-D24-hTNFa, an oncolytic adenovirus expressing human TNFa and selective to retinoblastoma protein defective cells (87). Cell death caused by this virus *in vitro* was associated with a significant increase in ATP release compared to control virus, and increased, but not significant, levels of calreticulin exposure and HMGB1 release, thereby displaying some features of immunogenic cell death. *In vivo*, intratumoral injection resulted in significant tumor growth delay and prolonged survival compared to control virus. There was likewise significant reduction in tumor growth compared to control virus in a syngeneic mouse melanoma model. Interestingly, the authors investigated the combination of this virus with radiotherapy treatment *in vivo* and *in vitro*. Combination treatment had no impact on cell viability *in vitro. In vivo*, treatment with Ad5/3-D24-hTNFa combined with radiotherapy in a prostate cancer xenograft model led to a significant reduction in tumor growth when compared to treatment with mock or control virus and radiotherapy, but there was no difference between control virus and Ad5/3-D24-hTNFa in the immunocompetent mouse melanoma model, which the authors postulated may have been related to the inherent radioresistant nature of melanoma.

CLINICAL EFFICACY AND TOXICITY

To date, clinical experience with virus/radiation combinations has been limited to local (most commonly intratumoral) administration. This mode of delivery facilitates direct infection, ensuring correct dosing and avoiding the rapid hepatic uptake seen with systemic delivery (88). The downside is only tumor types that can be easily accessed with a needle, such as skin, head, and neck cancers or prostate cancers, are considered suitable for clinical trials. Nevertheless, the results of these studies provide us with useful mechanistic indicators as well as guiding assessment of toxicity. While the authors acknowledge that there are other oncolytic viruses in clinical practice, we will focus on the clinical experience with adenoviral agents.

A study of intraprostatic injection of an oncolytic Ad5 PSE/PBN E1A-AR (Ad5, adenovirus serotype 5; prostate-specific enhancer; PBN, rat probasin promoter; E1A, early region 1A; androgen receptor), combined with either low or high dose rate radiation therapy, showed remarkably few side effects (89). Although DNA damage, as assessed by γH2AX foci, viral replication and viral induced cell death all favored the high-dose radiation arm, the side effect profile was similar in both arms. This indicates that the therapeutic efficacy is separate from the toxicity, in contrast to traditional radiosensitizers where a higher dose often increases both efficacy and toxicity. These findings support the large body of preclinical data that there is little additive toxicity to that seen with either agent alone (90).

A phase I trial of intraprostatic injection of a replication-competent adenovirus in combination with radical dose (74 Gy delivered in daily 2 Gy fractions) of intensity modulated radiotherapy (IMRT) showed no significant differences in gastro-intestinal or genitourinary toxicity in comparison to the toxicity seen when administering the adenovirus as a single agent (91). The investigational agent had already proven safe and efficacious as a single agent (92). These results were confirmed in a follow on randomized phase II trial (93). There was a non-significant 42% reduction in biopsy positivity in the investigational arm, suggesting improved efficacy and synergy with radiation. Clinical outcomes at 2 years show no difference, likely reflecting the excellent prognosis of both groups. A phase II/III (ReCAP) open label adaptive trial of 280 men, randomized to combination treatment or radiation efficacy, with biochemical failure free survival as the primary endpoint (94). Other groups have shown that administering a different type of adenovirus is safe, both concurrently and after radiation to the prostate (95, 96), when all cells should be at maximal damage and repair rates. Again, the viral compound was administered intratumorally.

There is also evidence from early phase clinical trials that a combination approach of radical dose (76 Gy delivered in 2.17 Gy daily fractions) with Ad5 replication defective adenoviral vector stimulates a systemic response (97). IMRT was commenced 48 h after the second of three doses of the viral agent, therefore, patients were effectively loaded and then treated concurrently. Again, drug was administered intraprostatically. Both HLA DR+ CD8+ and CD4+ T cells were increased in the combination arm compared to the radiation alone arm, suggesting the potential development of a Th1 response.

In a mixed solid tumor cohort, an adenovirus vector under the control of EGF-1 promoter was combined with radiation in 36 patients (98). 70% of subjects showed evidence of a partial response, with the main side effects relating to intratumoral administration of the agent. Using the same agent in combination with chemoradiation for squamous cell carcinomas of the head and neck a phase I dose escalation trial was performed (99), again with intratumoral administration. The main dose limiting toxicity (DLT) seen was thrombosis, with no increase in acute radiation side effect incidence or intensity, underlining the safety of the combination approach. Locoregional response was 83.3%. Preclinical studies with this agent have shown impressive ability to suppress regional metastatic node formation highlighting its ability to influence intrinsic tumor biology (100). Incrementally increasing doses of the same agent were also combined with radiation in soft tissue extremity sarcoma (101). No DLT was

noted and the combination was well tolerated. 91% of patients undergoing surgery showed a pathological CR to treatment highlighting significant potential synergy between both agents. The same adenovirus composite has been successfully combined with radical chemoradiotherapy (50.4 Gy delivered in 1.8 Gy daily fractions concurrently with fluoropyrimidines) for locally advanced pancreatic cancer in a non-randomized phase I/II setting (102). The main DLTs were pancreatitis and cholangitis but no specific increases in observed acute radiotherapy or chemotherapy side effects, respectively, were seen. The adenovirus was administered intratumorally.

Combination of yet another adenovirus, designed to transfer p53 to malignant cells, in a radically treated non-small cell lung cancer population has shown impressive response data (103). This prospective phase II trial of 60 Gy in combination showed no evidence of pathologically viable tumor in 63% of patients (12 out of 19) evaluable. The most common adverse events were virus related; fevers (79%), and chills (53%).

Ongoing studies in brain malignancies, such as glioblastoma multiforme (GBM), are also encouraging. Intratumoral injection at the time of surgery of an adenoviral vector expressing HSV thymidine kinase gene, combined with radical chemoradiation post operatively (104), has been tested prospectively. 12 of 13 patients completed therapy, at varying dose levels in this phase Ib trial, with no DLTs or significant toxicity. A phase II trial is ongoing (NCT00589875). Further evidence of safety in GBM patients is provided by the small phase I study that used a conditionally replicating HSV, G207 (105). Following two prior safety studies with single-agent use, they showed, in nine patients, that intratumoral injection followed by 5 Gy of radiation 24 h later had no increased risk of toxicity. Preclinical data with G207 also points to efficacy in other tumor sites, such as head and neck SCC and lung cancer (106, 107) (see above).

Taken together, these clinical data support the safety of a combination approach of radiation with a range of adenovirus constructs. The most commonly reported adverse events are related to the local administration and investigational agent itself rather than any increase in expected normal tissue toxicity mediated by excessive radiosensitization. Although clinical trials have not yet progressed to the point of assessing efficacy as an endpoint, several are in conduct. The optimum timing and sequencing of the two modalities has yet to be decided. So far concurrent delivery has been most used, appears safe and effective with no negative effects on viral biology. Further mechanistic work on sequencing is required, however. The need to access the tumor directly has limited the scope of clinical investigation, both in terms of tumor type and tumor stage. The future of oncolytic viral therapy really lies in newer agents with the ability to be delivered systemically (9, 108). This approach would not only allow the treatment of many more tumor sites but also, potentially, target micrometastatic disease such as nodal spread. The ability to successfully access tumors remotely will offer novel opportunities to further understand the reciprocal biology and elucidate the optimal approach to combination therapy.

FUTURE DIRECTIONS

Oncolytic adenoviruses have developed several mechanisms to inhibit cellular DNA damage repair pathways. This creates a logical case for additive and potentially synergistic efficacy when oncolytic viruses are combined with radiotherapy, although the outcome may also depend on the radiotherapy protocol employed. Not only do these viruses promise activity as cancer-selective radiosensitizers, the observation that some oncolytic adenoviruses naturally mediate an immunogenic cell death mechanism provides the possibility of provoking an anticancer immune response. Coupled with the option to express therapeutic proteins selectively at the tumor site, this creates an appealing and potentially unique targeted immunostimulatory strategy that builds on the strengths of both component approaches. Accordingly, we anticipate the development of systemically administered viruses designed as cancer-targeted radiosensitizers, capable of stimulating potent anticancer immune responses to stimulate activity against tumor deposits both inside and outside the field of irradiation. Such an approach should be perfectly complementary to the current generation of checkpoint inhibitors, and it is feasible to anticipate a further increase in utility if all three approaches are combined in future treatment strategies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: LS and KF own equity in Psioxus Therapeutics Ltd., which is developing an oncolytic adenovirus for treatment of cancer. All other authors declare no conflict of interest.

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MINI REVIEW
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Modulation of the Intratumoral Immune Landscape by Oncolytic Herpes Simplex Virus Virotherapy

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Vaccines and immunotherapeutic approaches to cancers with the advent of immune checkpoint inhibitors and chimeric antigen receptor-modified T cells have recently demonstrated preclinical success and entered clinical trials. Despite advances in these approaches and combinatorial therapeutic regimens, depending on the nature of the cancer and the immune and metabolic landscape within the tumor microenvironment, current immunotherapeutic modalities remain inadequate. Recent clinical trials have demonstrated clear evidence of significant, and sometimes dramatic, antitumor activity, and long-term survival effects of a variety of oncolytic viruses (OVs), particularly oncolytic herpes simplex virus (oHSV). Acting as a multifaceted gene therapy vector and potential adjuvant-like regimens, oHSV can carry genes encoding immunostimulatory molecules in its genome. The oncolytic effect of oHSV and the inflammatory response that the virus stimulates provide a one-two punch at attacking tumors. However, mechanisms underlying oHSV-induced restoration of intratumoral immunosuppression demand extensive research in order to further improve its therapeutic efficacy. In this review, we discuss the current OV-based therapy, with a focus on the unique aspects of oHSV-initiated antiviral and antitumor immune responses, arising from virus-mediated immunological cell death to intratumoral innate and adaptive immunity.

Keywords: oncolytic virotherapy, herpes simplex virus, tumor microenvironment, immune crosstalk, innate immunity, adaptive immunity, metabolic programming, immunogenic cell death

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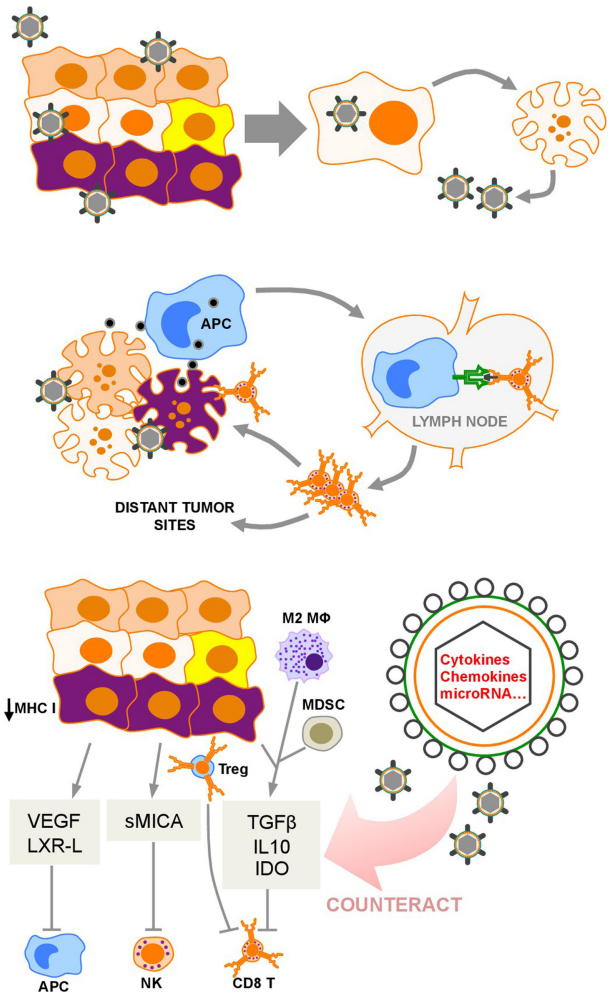
INTRODUCTION

The various cellular subsets within the tumor, including cancer cells, stromal cells, and infiltrating immune cells, interplay and contribute to a highly immunosuppressive microenvironment. Cancer cells undergoing stochastic genetic and epigenetic changes generate the critical modifications necessary to circumvent both innate and adaptive immunological defenses. Tumors evade immunity by downregulating antigen presentation, upregulating inhibitors of apoptosis, or expressing inhibitory surface molecules (e.g., programmed death-ligand 1) (1). In addition, tumor cells secrete factors [e.g., transforming growth factor beta (TGF-β), indoleamine 2,3-dioxygenase (IDO)] that directly inhibit effector immune cell functions or recruit regulatory cells, tumor-associated macrophages, and myeloid-derived suppressor cells (MDSC) to intensify an immunosuppressive microenvironment (**Figure 1**) (1). The specific intratumoral immune landscape within a certain type of cancer further contributes to tumor progression by selecting more aggressive tumor variants. In light of



Viral infection, replication and lytic cell death	
Objective	**Key factors**
• Selective infection and replication in tumor cells • Sufficient viral replication • Immunogenic cell death	• Specificity and efficiency of tumor cell receptor targeting • Physical barriers of tumor • Virus type, size, dose, delivery routes, natural and induced viral tropism • Viral replication and host cell antiviral response elements • Susceptibility of tumor cells to different forms of cell death (apoptosis, necrosis, pyroptosis and autophagy)

Tumor-associated antigens release, presentation and induction of local and systemic antitumor immunity	
Objective	**Key factors**
• Sufficient release of novel cancer antigens (neo-antigens) • Efficient antigen processing and induction of systemic adaptive immune response	Appropriate extent of host antiviral immune response: • Virus induced immune response promotes antigen presentation and local immunity • Antiviral immune response inhibits virus replication and spreading

Counteract tumor-mediated immune evasion	
Objective	**Key factors**
• Recover immunogenicity of tumor cells • Reverse immunosuppressive microenvironment of tumor • Sufficient immune cells recruitment and function	• Intrinsic immunogenic and immunosuppressive identities of tumor cells • Cytokines, chemokines and tumor cell secretive factors in tumor microenvironment • Distributions of immune cell and immunosuppressive cell in tumor microenvironment • Modification of virus as expression vector carrying factors with immunoregulatory functions

Normal cell
Poorly immunogenic transformed cell
Highly immunogenic transformed cell
Immunosuppressive transformed cell

FIGURE 1 | Sequential oncolytic herpes simplex virus-induced events: virus infection, cell death, and innate and adaptive immune responses within the tumor microenvironment. VEGF, vascular endothelial growth factor; LXR-L, liver X receptor ligand; sMICA, soluble MHC class I polypeptide-related sequence A; TGF-β, transforming growth factor-β; IL10, interleukin 10; IDO, indoleamine 2,3-deoxygenase; APC, antigen-presenting cell; M2 MΦ, M2 macrophages; MDSC, myeloid-derived suppressor cells; Treg, regulatory T cell; NK, natural killer.

the importance of immune regulation in tumor growth, cancer immunotherapeutic approaches, aimed to interfere with tumor immunosuppressive microenvironment and boost antitumor immune responses, have emerged as promising strategies. Among these approaches, checkpoint inhibitors [PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies] have been successfully used to treat several types of cancers (2, 3). However, only limited numbers of cancer patients show remission after treatment (2), indicating a pivotal effect of heterogeneous immune background on the outcome of immunotherapy, and suggesting that alternative or combined immunotherapeutic strategies should be considered.

Since the discovery of the oncolytic effect of virus infection a century ago, oncolytic virotherapy with a variety of viruses, including wild-type viruses, attenuated viruses and transgenic viruses, has emerged as a potential therapeutic approach to treat cancer (4). To date, OVs based on 11 DNA and RNA virus platforms are actively tested in clinical trials (5). The most successful one is the talimogene laherparepvec (T-VEC) derived from the herpes simplex virus (HSV), which has finished the Phase III clinical trial and been approved for the treatment of advanced metastatic melanoma in 2015 by US Food and Drug Administration (6).

Herpes simplex virus-1 is a double-stranded DNA virus possessing a large and well-characterized genome (152 kb), and about 30 kb is dispensable for viral infection. This unique feature makes HSV-1 suitable for genetic manipulation. In addition, although HSV-1 replicates in the nucleus, it does not cause insertional mutagenesis and is sensitive to aciclovir and ganciclovir (7). These safety features make HSV-1 an attractive candidate for oncolytic virotherapy. Besides T-VEC, we and others have developed several other oncolytic herpes simplex viruses (oHSVs) that have proceeded into clinical trials, for example, G207, an HSV-1 mutant with deletions of both copies of $\gamma_1$34.5 gene encoding the infected-cell protein 34.5 (ICP34.5) and a lacZ insertion into the UL39 neurovirulence gene (8); HSV1716, a $\gamma_1$34.5 null mutant

with an intact UL39 gene that replicates selectively in actively dividing cells; and G47Δ, which is built from G207 by the deletion of the α47 gene (9). These oHSVs have been evaluated in multiple tumor types in murine models and patients (10–12). In particular, the neurotropic feature of oHSV makes it an attractive option for brain cancer therapy (7).

oHSVs mediate antitumor activity through direct lysis of tumor cells and the subsequent induction of systemic antitumor immunity. The induction of antitumor immune reaction is pivotal for the effect of oHSV therapy (13). We have recently reviewed the oHSV-based therapy for malignant glioma (7). Here, we focus on the sequences of immune responses to such therapy (**Figure 1**) and provide insight into how we can utilize these information to improve this therapy and/or combine with other approaches to increase the oHSV antitumor efficacy.

INTRATUMORAL IMMUNE LANDSCAPE

The immune system is capable of recognizing tumors and eliminates early malignant cells. Nonetheless, cancer progression ultimately escapes immune-mediated destruction. Based on biopsies and gene profiling analysis of various types of tumor samples from individual patients, accumulating evidence shows that there are two distinct subsets of patients (14). One subset of patients shows evidence of spontaneous T-cell priming and immune infiltration into tumors. This phenotype has been characterized as the T cell-inflamed tumor microenvironment with the expression of various T-cell transcripts and chemokines that likely mediate T-cell recruitment, antigen-presenting cell (APC) activation, and a type I interferon (IFN) signature (14). Immunohistochemical analysis has confirmed the presence of $CD8^+$ T-cells, macrophages, some B-cells, and plasma cells in these tumors (15). In contrast to this spontaneous immune activation, the non-T cell-inflamed tumors lack all of these parameters and are devoid of T-cells (15). The characteristics of these two distinct phenotypes have suggested two broad categories of tumor evasion of host immunity. In T cell-inflamed tumors, immune failure appears to occur at the effector phase, and some patients with this type of tumor show good clinical responses to cancer vaccines, high-dose interleukin (IL)-2, anti-CTLA-4, and anti-PD-1 antibodies (16–18). Non-T cell-inflamed tumors suggest immune exclusion (15), and the current wave of immunotherapies being explored clinically seems unlikely to be successful in these cases. Further characterization of the immune contexture of individual tumors based on the tumor genomic landscape, extent of DNA damage, mutational load, and neoantigen presentation may direct more efficient approaches and better prediction of therapeutic responses (19).

All cells, including cancer and immune cells, need to produce ATP through oxidative metabolism and synthesize macromolecules through glycolysis and/or glutaminolysis to maintain their basic cellular functions (20, 21). Tumor cell proliferation and growth depend on glycolysis and glutaminolysis, a hallmark of cancer metabolism (20, 22). Metabolites secreted from tumors alter the microenvironment, enable tumors to adapt to hypoxia, and also regulate intratumoral immune cells. Metabolic pathways of oxidative metabolism, glycolysis, and glutaminolysis preferentially fuel the cell fate decisions and effector functions of all immune cells (21). Immune cells can rapidly shift between glycolysis and oxidative phosphorylation in response to external signals, which is important for their development, activation, and normal function (21, 23). Although the metabolic regulation of immune cells is not the focus of this review and has been extensively reviewed by others (21), it should be noted that complex metabolic interactions between stromal cells, cancer cells, and immune cells in the microenvironment can promote tumor growth and suppress immune reactions. Tumor cells with high metabolic demand may compromise the function of some immune cells by competing glucose and other nutrients, leading to T-cell dysfunction such as anergy and exhaustion and may also support the function of immunosuppressive cells by forming a metabolic symbiosis. Future immunotherapeutic approaches to reprogramming the metabolic pathways of immune cells and normalizing the intratumoral immune landscape should be considered.

INTRATUMORAL oHSV REPLICATION AND INDUCTION OF IMMUNOGENIC CELL DEATH (ICD)

OVs preferentially accumulate and replicate in tumor cells with aberrant apoptosis, proliferation, and antiviral signaling pathways. In normal healthy cells, double-stranded viral RNA and other viral elements can be recognized by protein kinase R (PKR), which is a component of intracellular antiviral machinery (24). Activated PKR phosphorylates eukaryotic initiation factor (eIF2α), leading to cell protein synthesis termination and rapid cell death. Wild-type HSV escapes antiviral response due to expression of the ICP34.5 protein which activates a phosphatase that then dephosphorylates eIF2α, restoring protein synthesis in the infected cell (25). Another important antiviral mechanism is mediated by intracellular toll-like receptors (TLRs) that recognize virus-related pathogen-associated molecular patterns (PAMPs) and subsequently induce local IFN release (26, 27). In cancer cells, abnormal IFN pathway and PKR activity promote tumor-specific replication of oHSV. Attenuated oHSVs, including G207 and HSV1716, are depleted of ICP34.5, which render oHSV unable to block PKR phosphorylation, resulting in preferential lysis of tumor cells compared to normal cells (7). oHSVs can also mediate targeted lysis of cancer stem cells (CSCs) (28). These cells are rare populations of tumor-initiating cells that are capable of self-renewal and have pluripotent capacity (29). CSCs are particularly resistant to chemotherapies and radiation therapies, making them the primary source of drug resistance, metastasis, and tumor recurrence. The efficacy and potential of oHSV in targeting CSCs have been extensively discussed previously (30). We have recently found that xenografts of pediatric medulloblastoma CSCs are highly sensitive to killing by oHSVs G207 or M002, a neuroattenuated oHSV expressing murine IL-12 (31).

Replication of OVs in tumor cells can induce different types of cell death including necrosis, apoptosis, pyroptosis, and autophagic cell death. Depending on the initiating stimulus,

cancer cell death can be immunogenic or non-immunogenic (32). ICD involves changes in the composition of the cell surface as well as the release of soluble mediators, which operate on a series of receptors expressed by dendritic cells (DC) to stimulate T-cells (33). Cancer cells undergoing ICD expose calreticulin (CRT) on the outer leaflet of their plasma membrane followed by a sequential secretion of ATP and high mobility group box 1 (HMGB1) (33). ATP, CRT, and HMGB1 bind to their respective receptors on immature DCs to facilitate the recruitment of DCs into the tumor bed, the engulfment of tumor antigens by DCs, and optimal antigen presentation to T-cells (32). ICD constitutes a prominent pathway for the activation of antitumor immunity, which involves release of danger-associated molecular patterns (DAMPs) and tumor-associated antigens (TAAs). By inducing ICD of tumor cells, OVs facilitate TAAs cross-presentation to DCs and finally induce antitumor immune responses. A recent study conducted with squamous cell carcinoma cells shows efficient ICD after oHSV infection (34). ICD is the mainstay of long-term success for anticancer therapies, and it may also hold promise for developing oHSVs as potential cancer vaccines or adjuvants for these vaccines.

INNATE IMMUNITY IN oHSV THERAPY

The generation of a robust adaptive immune response against cancer must, in principle, rely on upstream innate immune activation that leads to productive T-cell priming. In a non-T cell-inflamed tumor, restoring dysfunctional innate immunity is the key point of new therapeutic interventions. Here, we focus on the innate immune responses mediated by NK cells and DCs.

OV-induced cancer cell death releases PAMPs or DAMPs that are recognized by pattern recognition receptors, such as TLRs, located in the cytoplasm or on the cell surface. Their engagement induces expression of inflammatory cytokines (e.g., IFNs, tumor necrosis factor-α, IL-6, and IL-12), which bind to receptors on other cells, resulting in recruitment and activation of innate immune cells, such as NK, NKT, and γδ T-cells (5, 7). NK cells have been recognized as a relevant first-line defense against viruses. NK cells can sense infected cells either through direct interaction with PAMPs *via* TLRs or through recognition of viral and/or virus-induced ligands *via* activating NK cell receptors (35). Upon activation NK cells directly kill infected cells through cytotoxicity or boost immune responses *via* cytokine secretion. NK cells may exert either positive or negative effects on oHSV therapy, depending on several factors such as virus type, dose, and replication rate (36, 37). An optimal balance of NK activating and inhibiting signals may be particularly relevant for oHSV-based therapies. Alvarez-Breckenridge et al. have elegantly demonstrated that HSV-induced upregulation of the ligands for natural cytotoxic receptors triggers NK cells to mediate premature clearance of oHSV in a mouse glioblastoma model, suggesting a potential limitation in glioblastoma virotherapy (38). In contrast, studies using UV-inactivated HSV suggest that the surface components of UV-HSV directly activate NK cells and enhance NK-cell killing of leukemia cells (39).

One of the important immune cells that bridge innate and adaptive immune responses is the DC. DCs are classically divided into two major categories: plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (40). pDCs are specialized in the secretion of high levels of type I IFNs upon stimulation *via* TLRs. Within the cDC compartment, the $CD8\alpha^+$ DC subtype is most efficient at phagocytosing dead cells and in cross-presenting antigens to $CD8^+$ T-cells (40). Sufficient production of type I IFNs by APC, including DCs, in the tumor microenvironment is critical for induction of adaptive antitumor T-cell responses. Tumors absent of type I IFN signature usually respond poorly to conventional immunotherapies (41). The stimulator of interferon genes (STING) is a key cytosolic DNA sensor for the detection of intracellular pathogens, notably DNA viruses like HSV (42, 43). DNA released from dying tumor cells can be sensed by the cytosolic enzyme cyclic GMP-AMP synthase (cGAS). Cyclic dinucleotides generated by cGAS bind to STING and induce type I IFN production through phosphorylation of interferon regulatory factor 3 (40). Xia et al. have provided evidence that STING is frequently functionally suppressed in human cancers. Loss of STING prevents DNA damage-mediated type I IFN production, which renders tumor cells highly susceptible to OV infection (44), suggesting that STING activity might be a crucial indicator to stratify cancer patients for OV-based therapies.

ADAPTIVE IMMUNITY IN oHSV THERAPY

Sufficient innate immune responses lead to APC maturation and antigen presentation to naïve T-lymphocytes, which activates antigen-specific $CD4^+$ helper T (T_H)-cells and $CD8^+$ effector T-cells. Once activated, these T-cells expand and traffic to tumor sites, where they mediate antitumor immunity. Although priming adaptive immunity plays a critical role in OV-mediated antitumor activity, the natural ability of viruses to induce host antiviral immune responses may result in clearance of the virus through neutralizing antiviral antibodies and/or cytotoxic T-cell-mediated immune responses (5). The extent to which viral neutralization influences the induction of antitumor immunity is complex and can be influenced by many variables, most notably the characteristics of the virus and the tumor microenvironment. For example, HSV-1 evades $CD8^+$ T-cells by producing ICP47, which limits immune recognition of infected cells by inhibiting the transporter associated with antigen processing (TAP) (45). An engineered oHSV carrying a bovine herpesvirus homologous gene of ICP47 shows superior efficacy in treating bladder and breast cancer in murine models, which is dependent upon $CD8^+$ T-cells (46), suggesting that arming oHSVs with TAP inhibitor may enhance local and systemic antitumor responses.

Unlike innate immunity, the adaptive immune response generates immune memory, implying that any subsequent exposure to the same antigen that immune cells encounter previously will induce a stronger response. When using OV therapy, the antiviral memory response must be taken into consideration because it prevents retreatment, which is an essential component of OV-based therapy (4, 47). Humans are naturally (or artificially through vaccination) exposed to HSV and may therefore have preexisting neutralizing antibodies or cellular immunity against HSV. Strategies to limit virus neutralization include utilizing alternative virus serotypes or developing wild-type, non-human

viruses. However, OV-induced immune memory to tumor antigens due to epitope spreading is an integral immune component of OV therapy (5). This is exemplified by the finding that immunocompetent mice treated with a parvovirus OV do not develop glioma and long-term survivors fail to develop tumors when rechallenged with uninfected tumor cells (48). Antitumor memory response is also essential for the development of tumor vaccines. Therefore, understanding mechanisms for the generation of antitumor memory responses is required for designing strategies to enhance OV and oHSV therapies.

IMPROVING THERAPY: MODIFIED oHSV AND COMBINATORIAL THERAPY

OVs revive the suppressive microenvironment through a variety of mechanisms that alter the cytokine milieu and the type of immune cells within the tumor (5). Clinical efficacy can be increased by modifying the viral backbone or by developing OVs with multimodal activity. An extensive panel of transgenes, including inflammatory cytokines, antiangiogenic and antivascular proteins, monoclonal antibodies, proapoptotic genes, and enzymes that degrade extracellular matrix, have been used to modify the oHSV backbone to enhance their therapeutic efficacy in preclinical and clinical studies. The oHSV T-VEC is armed with human granulocyte-macrophage colony-stimulating factor, an inflammatory cytokine that bolsters antitumor immune responses by recruiting NK cells and inducing TAA-specific cytotoxic T-cells (49). oHSVs armed with other cytokines (e.g., IL-2, IL-12, IL-15, IL-18, and IFN-α/β), chemokines (e.g., CCL5), or costimulatory molecules (e.g., B7.1 and CD40L) can also induce antitumor immunity (50). For instance, an oHSV armed with IL-12, a potent antitumor cytokine with antiangiogenic activities, reduces neovasculature and Tregs, and induces T_H1-mediated immunity in an immunocompetent CSC model (51). We have developed a neuroattenuated oHSV expressing human IL-12, termed M032, which is currently in Phase I clinical trial on patients with recurrent gliomas.

Combinatorial therapy using drugs or distinct immunomodulatory methods with oHSV to activate the immune response and/or block the immunosuppressive tumor microenvironment also has great potential to improve the overall clinical efficacy. Combinatorial therapy regimens that circumvent intracellular and microenvironmental antiviral responses are good options. Depending on the cancer type, tumor immunogenicity, and tumor microenvironment, OVs can be combined with approved immunoregulatory approaches, including epigenetic modifiers (e.g., histone deacetylase inhibitors, DNA methylation inhibitors, and histone methyltransferase inhibitors) (52–54), adoptive T-cell transfer therapy (e.g., chimeric antigen receptor T-cell therapy) (55), immune checkpoint inhibitors (antibodies targeting CTLA-4, PD-1, lymphocyte-activation gene 3, or T-cell immunoglobulin and mucin-domain containing-3) (56–59), activation of stimulatory pathways (antibodies targeting CD137, OX-40, and inducible T-cell costimulator) (60, 61), targeting suppressive mechanisms in the microenvironment (IDO and TGF-β inhibitors) (62–64), novel multifunctional immunoregulatory targets (e.g., osteopontin) (65, 66), and chemotherapeutic drugs (e.g., gemcitabine, 5-fluorouracil, and retinoic acid) that delete immunosuppressive cells (Tregs, MDSCs, and M2 macrophages) (67–69). Promising results have been obtained when OVs are combined with an antibody that blocks T-cell checkpoint inhibitory receptors, such as CTLA-4 or PD-1 (56, 57). However, successful combinatorial therapy is context dependent, and additional studies are needed to define the optimal therapeutic conditions.

CONCLUSION

By virtue of its safety and suitability for genetic manipulation as a multifaceted gene therapy vector, oHSV-based therapy has emerged as a promising cancer immunotherapeutic approach. It may be particularly desirable for those non-T cell-inflamed tumors that are refractory to other immunotherapies. oHSV infection not only lyses the tumor but also induces cytokine production and immune cell recruitment into tumors, which reinvigorate the immunosuppressive environment and may restore the metabolic landscape within the tumors. Although promising results have been obtained using oHSV alone or combined with other approaches on several types of cancers, challenges remain regarding how to improve the therapeutic outcomes by simultaneously maximizing both oHSV replication and antitumor immune responses. Additional studies are also needed to determine if oHSV can be combined with metabolic interventions to adjust the metabolic interplay within the tumor, how to sustain the oHSV-induced responses, particularly memory responses, and how to develop it as a cancer vaccine or adjuvant for current tumor-targeted DC vaccines. A more complete understanding of the crosstalk between tumor and immune system will guide the development of optimal interventions on cancer without compromising antitumor immunity.

AUTHOR CONTRIBUTIONS

JY, JM, and JL drafted the manuscript, revised it critically, and approved this final version for publication.

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REVIEW
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Genomic Signature of the Natural Oncolytic Herpes Simplex Virus HF10 and Its Therapeutic Role in Preclinical and Clinical Trials

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Oncolytic viruses (OVs) are opening new possibilities in cancer therapy with their unique mechanism of selective replication within tumor cells and triggering of antitumor immune responses. HF10 is an oncolytic herpes simplex virus-1 with a unique genomic structure that has non-engineered deletions and insertions accompanied by frame-shift mutations, in contrast to the majority of engineered OVs. At the genetic level, HF10 naturally lacks the expression of *UL43*, *UL49.5*, *UL55*, *UL56*, and latency-associated transcripts, and overexpresses *UL53* and *UL54*. In preclinical studies, HF10 replicated efficiently within tumor cells with extensive cytolytic effects and induced increased numbers of activated $CD4^+$ and $CD8^+$ T cells and natural killer cells within the tumor, leading to a significant reduction in tumor growth and prolonged survival rates. Investigator-initiated clinical studies of HF10 have been completed in recurrent breast carcinoma, head and neck cancer, and unresectable pancreatic cancer in Japan. Phase I trials were subsequently completed in refractory superficial cancers and melanoma in the United States. HF10 has been demonstrated to have a high safety margin with low frequency of adverse effects in all treated patients. Interestingly, HF10 antigens were detected in pancreatic carcinoma over 300 days after treatment with infiltration of $CD4^+$ and $CD8^+$ T cells, which enhanced the immune response. To date, preliminary results from a Phase II trial have indicated that HF10 in combination with ipilimumab (anti-CTLA-4) is safe and well tolerated, with high antitumor efficacy. Improvement of the effect of ipilimumab was observed in patients with stage IIIb, IIIc, or IV unresectable or metastatic melanoma. This review provides a concise description of the genomic functional organization of HF10 compared with talimogene laherparepvec. Furthermore, this review focuses on HF10 in cancer treatment as monotherapy as well as in combination therapy through a concise description of all preclinical and clinical data. In addition, we will address approaches for future directions in HF10 studies as cancer therapy.

Keywords: herpes simplex oncolytic viruses, genomic structure, HF10, talimogene laherparepvec, preclinical studies, combination therapy, clinical trials, future directions

INTRODUCTION

Oncolytic viruses (OVs) are currently being used effectively with therapeutic drugs to change the landscape of cancer treatment. OVs are considered immunotherapeutic targeted agents due to their selective replication within tumor cells and enhancement of the immune response. As a consequence, recent advances in viral genomics and tumor immunology have addressed OVs as a type of cancer therapy. To date, over 30 OVs belonging to seven DNA or RNA virus families have been successfully translated from preclinical studies to clinical trials (**Table 1**) (1). The Herpesviridae family includes human alphaherpesvirus-1 [Herpes simplex virus-1 (HSV-1)]. HSV-1 is the first human herpesvirus to be discovered and the most intensively investigated virus (2). The HSV family has common features, such as double stranded DNA (dsDNA) and an icosahedral capsid (3). The HSV family has taken precedence over other families in cancer treatment. For example, dlsptk, a type of HSV-1 virus, was the first OV to be engineered by deletion of HSV thymidine kinase (4). Talimogene laherparepvec (T-Vec, Imlygic™ formerly Oncovex$^{GM\text{-}CSF}$), an HSV-1 virus encoding granulocyte macrophage colony-stimulating factor (GM-CSF), was the first OV approved by the US Food and Drug Administration for the treatment of melanoma (5).

Most OVs, including the approved T-Vec, have been engineered to increase tumor selectivity and efficacy. HF10, on the other hand, is a spontaneously mutated virus without any insertion of foreign genes. The HF10 genome consists of linear dsDNA with a natural deletion of 6,127 kb and insertions of 6,027 bp accompanied by frame-shift mutations located at different nucleotide positions within the genome. These deletions and insertions caused a loss of expression of *UL43*, *UL49.5*, *UL55*, *UL56*, and latency-associated transcript (LAT) genes and overexpression of *UL53* and *UL54*. Many investigators have evaluated the effect of these deletions on the oncolytic characteristics of HF10 in different cell lines as well as tumor models of colon cancer, breast cancer, bladder cancer, pancreatic cancer, and melanoma. Preclinical studies have found that HF10: (a) has high innate tumor selectivity, (b) has high viral replication, (c) induces a complete cytopathic effect, (d) mediates a highly potent bystander effect, and (e) has potent antitumor efficacy against different malignancies. Consequently, preclinical studies have translated into successful clinical trials with promising results in different cancer types including recurrent metastatic breast cancer, recurrent head and neck squamous cell carcinoma (HNSCC), advanced pancreatic cancer, refractory and superficial cancers, and melanoma. Recently, there has been a lot of effort to establish the full layout of HF10 as an OV in cancer treatment. This review outlines a detailed approach for using HSV-HF10 as an OV. We will address the similarities and differences of the genomic structures of HF10, T-Vec, and other HSV OVs. Furthermore, we will describe the effect of the natural deletions in HF10 on its oncolytic efficacy in cancer treatment through a concise review of all preclinical studies and clinical trials, comparing it to genetically engineered viruses such as T-Vec. Finally, we will outline future directions for preclinical and clinical studies.

TABLE 1 | Families of oncolytic viruses (OVs).

	Herpes simplex virus-1 (HSV-1)	**Adenoviruses**	**Paramyxoviruses**		**Poxviruses**	**Picornaviruses**		**Reoviruses**	**Rhabdoviruses**	**Retrovirus**
Family	Herpesviridae	Adenoviridae	Paramyxoviridae		Poxviridae	Picornaviridae		Reoviridae	Rhabdoviridae	Retroviridae
Genus	Simplexvirus		Avulavirus	Morbillivirus	Orthopoxvirus	Enterovirus	Seneca virus	Orthoreovirus	Vesiculovirus	–
Nucleic acid	Double stranded DNA (dsDNA)	dsDNA	ssRNA	ssRNA	dsDNA	ssRNA	ssRNA	dsRNA	ssRNA	ssRNA
OVs	HF10 T-Vec G207 NV1020 HSV-1716 G47Δ M032	Telomelysin Ad5-CD/TKrep Ad5-D24-RGD Ad5-SSTR/TK Onyx-015 CGTG-102 INGN-007 (VRX-007) ColoAd1 CG7870/CV787 CG0070 Oncorine (H101) CG7060	**Newcastle Disease virus** PV701 MTH-68/H NDV-HUJ	**Measles virus (Edmonston)** MV-CEA MV-NIS	**Vaccinia (Wyeth strain)** JX-594 **Vaccinia (Western Reserve)** vvDD-CDSR **Vaccinia (Lister)** GL-ONC1 (GLV-h68)	**Coxsackie virus (CVA21)** CAVATAK **Poliovirus (Sabin)** PVS-RIPO	Seneca Valley virus	**Reovirus (Dearing)** Reolysin	**Vesicular stomatitis virus (Indiana)** VSV-hIFNβ	Toca 511

HF10 Virion Structure

HF10 was originally purified from the HSV-1 strain HF as HF clone 10 (HF10) (6). The HF10 virion is similar to other HSV-1 virions. Early studies revealed that the HSV virion consists of four elements as shown in **Figure 1**: (a) a core containing linear dsDNA wrapped as a toroid or spool with the negative charges of DNA neutralized by polyamines (spermine and spermidine); (b) an icosahedral capsid comprised of 162 capsomers arranged in a $T = 16$ symmetry containing a nucleocapsid in the outer layer composed of four viral proteins (VP) plus VP5 as the major capsid protein; (c) a tegument consisting of an unstructured proteinaceous layer surrounding the capsid composed of 18 VP with VP16 as the most notable; and (d) an envelope, consisting of glycoproteins gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM (7, 8). HF10 lacks *UL49.5* that encodes gN, which links with gM (UL10 protein) to form a disulfide-linked complex (9). Moreover, HF10 overexpresses *UL53*, which encodes gK, a regulator of the egression process of the HSV virion from infected cells (10).

GENOMIC STRUCTURES OF HF10 AND T-Vec

To date, there are 17 strains of HSV-1 that have been isolated (ICTV 2015 taxonomy). Seven genomes have been completely or partially sequenced. HSV HF17 (NC_001806, X14112) is often used as a reference for genome sequence comparison (11). The HF10 genome was the first HSV genome to be completely sequenced, while T-Vec has only been partially sequenced. The HF10 and T-Vec genomes have the following similarities (**Figure 2**). (a) Both genomes are made up of linear dsDNA. (b) Each genome is composed of two unique inverted sequences, a unique long sequence (UL) flanked by a terminally repeated long sequence [TRL = (a_L, b)], and an internally repeated long sequence [IRL = (b′a′)]. (c) Each genome also has a unique short sequence designated as (US) bracketed by a terminally repeated short sequence [TRS = (c, a)], and an internally repeated short sequence [IRS = (c′a′)] (12, 13).

HF10 differs from T-Vec in their strain origins and their genomic deletions and insertions panel (**Table 2**). T-Vec was genetically modified from the JS1 strain to improve tumor-selective replication and immune response. As shown in **Figure 2B**, both copies of the *ICP34.5* gene have been deleted from the parent virus genome to suppress its replication in normal tissues. The *ICP47* gene has also been deleted to increase the expression of MHC class I on infected cells. Moreover, the hGM-CSF cassette has been inserted in lieu of the *ICP34.5* gene loci to enhance the antitumor cytotoxic immune response. Expression of hGM-CSF is derived from the cytomegalovirus and polyadenylation signal (pA) (bovine growth hormone) immediate early promoters, respectively (13).

HF10 has natural deletions and insertions within the genome. The *UL56*/IRL junction has been deleted from 116.515 bp to 120.346 bp, leading to the lack of expression of *UL56* and LATs (**Figure 2A**). In addition, 2,295 bp of the TRL has also been deleted and replaced by 6,027 bp that express the $UL52_{partial}$, *UL53*, *UL54*, *UL55*, and *UL56* inverted sequences. Both deletions and insertions lead to duplicated copies of *UL53*, *UL54*, *UL55*, two incomplete copies of *UL56*, and one complete and one incomplete copy of *UL52*. Frame-shift mutations in the N-terminal region cause a loss of the functional expression of the *UL43* and *UL49.5* gene products (12).

As noted above, the deletions cause the gene products of *UL43*, *UL49.5*, *UL55*, *UL56*, and LAT to not be expressed, whereas duplication leads to *UL53* and *UL54* overexpression. The main question here is "What are the functions of the deleted and duplicated genes and their effect on HF10 antitumor efficacy?" To answer this question, the function of the deleted genes must be known.

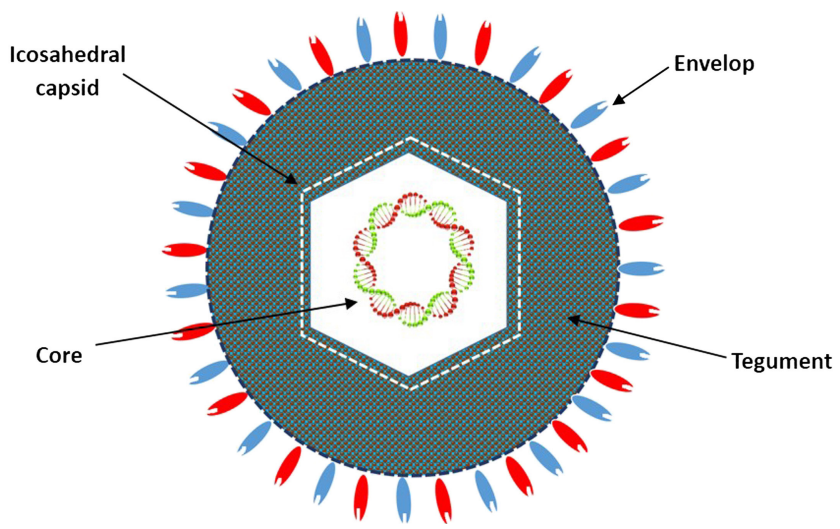

FIGURE 1 | Diagrammatic structure of the HF10 virion. The HF10 virion is composed of four elements: *envelope*, contains glycoprotein receptors; *Tegument*, consists of viral proteins; *Icosahedral capsid*, comprised of capsomers and a nucleocapsid in the outer layer; and *Core*, contains linear double stranded DNA.

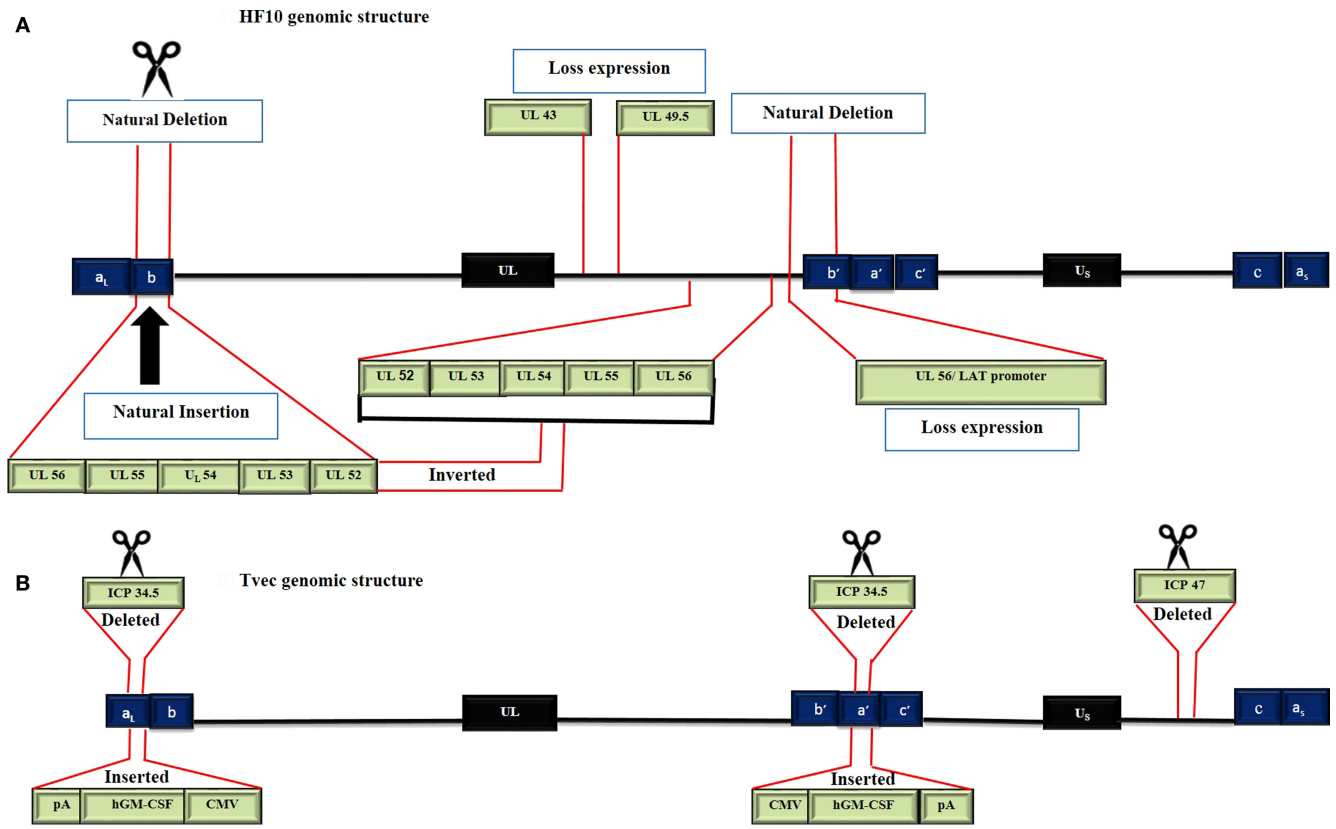

FIGURE 2 | Genomic structure of HF10 and talimogene laherparepvec. Each genome consists of two components: a Unique Long sequence (UL) flanked by a terminally repeated long sequence (TRL) and an internally repeated long sequence (IRL) and a Unique short sequence (US) flanked by a terminally repeated short sequence (TRS) and an internally repeated short sequence (IRS). **(A)** The HF10 genome has two deletions: 3,832 bp were deleted at the *UL56*/IRL junction and 2,295 bp were deleted in the TRL region and replaced by 6,027 bp of *UL56*, *UL55*, *UL54*, *UL53*, and *UL52*. A frame-shift mutation led to the loss of *UL43*, *UL49.5*, and *UL55* expression. **(B)** T-Vec genomic structure. *ICP47* was deleted. Both copies of *ICP34.5* were deleted and replaced with an h*GM-CSF* cassette that is composed of immediate early promoters from cytomegalovirus (CMV) and polyadenylation signal (pA) (bovine growth hormone).

TABLE 2 | Genomic comparison of HF10 and other herpes simplex virus-1 oncolytic viruses (OVs).

OVs	HF10	Talimogene laherparepvec	G207	NV1020	G47Δ	HSV-1716	Mo32
Strain	HF strain	Js1 strain	F strain	F strain	F strain	17+ strain	F strain
Isolation or generation year	1991	2003	1995	2002	2001	1991	2000
DNA sequencing	Completely	Partially	Partially	Partially	Partially	Partially	Partially
Genetic manipulation	Natural deletions and insertion	Genetically modified	Genetically modified	Genetically modified	Genetically modified	Genetically modified	Genetically modified
Deleted Genes	• UL56 • Latency-associated transcript (LATs)	• ICP 34.5 • ICP 47	• ICP34.5 • ICP6	• ICP34.5 • ICP4 • ICP0	• ICP34.5 • ICP6 • ICP 47	• ICP34.5	• ICP34.5
Inserted genes	UL52, UL53, UL54, UL55	hGM-CSF or mGM-CSF	LacZ		–	–	IL12
Overexpression	UL53, UL54	–	–	Thymidine kinase	–	–	–
Loss of expression	UL43, UL49.5, UL55, UL56, LAT	–	–	UL24	–	–	–

UL43: (γ Gene, Accessory Gene)

The HSV-1 *UL43* gene acts as a γ accessory gene. *UL43* mRNA encodes a hydrophobic transmembrane protein (14) that is conserved in the α and γ herpesviruses but absent in β herpesviruses (15–17). The UL43 protein is dispensable for viral growth in cell culture. Deletion of UL43 does not impair characteristics including virus entry, cell–cell fusion *in vitro*, viral replication *in vivo,* or neuroinvasiveness (18). Another study mentioned that

HSV17^{UL43-} has the ability to infect 40 to 60% of dendritic cells *in vitro* but the role of this deletion remains unclear (19). Thus, the lack of *UL43* expression may play a role in the direct interaction between HF10 and antigen-presenting cells (dendritic cells) to enhance the immune response.

UL49.5: (γ, Core Gene)

The *UL49.5* gene is a γ core gene that is conserved in all HSVs. It encodes a type 1 transmembrane glycoprotein N (gN). This gN forms a heterodimeric complex with glycoprotein M (gM) (20, 21). *UL49.5* homologs of HSV-1 have no effect on the transporter associated with antigen processing function (TAP) (20, 22). Hence, *UL49.5* deletion is likely involved in the syncytial (syn) phenotype of HF10 while the effect of this deletion on the oncolytic capacity of HF10 remains unclear.

UL53: (γ, Accessory Gene)

UL53 encodes glycoprotein K (gK) protein. gK regulates HSV egression from infected cells. gK is the most common locus of syn mutations. HF10 has duplicated *UL53*, which leads to gK overexpression, which causes accumulation of virus in the perinuclear space of infected cells as long as there are defects in viral egression (10). The accumulation of virus in cells accounts for a margin of safety when HF10 is inoculated into humans, as no shedding of virus to other organs has been observed. Previous studies have reported that gK prevents the formation of syncytia (23, 24). However, HF10 forms complete syncytia *in vitro* in different cell lines.

UL55: (γ, Accessory Gene)

UL55 acts as a γ accessory gene. *UL55* mRNA encodes a nonstructural protein that is associated with sites of virion assembly. Previous studies have shown that *UL55* is not necessary for intraperitoneal virulence and establishment of latency in mice (25, 26).

UL56: (γ, Accessory Gene)

The *UL56* gene is located at the right end of the unique long region of the HSV-1 genome (26). During acute infection, HSV-1 *UL56* is naturally expressed; it is considered a component of the HSV-1 virion (27). *UL56* is involved in the pathogenicity and latency of HSV-1. Lack of *UL56* expression may be involved in viral neuroinvasiveness (28). A previous study has reported that the deletion of *UL56* from the HSV-1 strain HFEM is pathogenic in tree shrews (29).

Latency-Associated Transcripts

Latency-associated transcripts are expressed during virus latency. LATs play a role in neuroinvasiveness and reactivation from latency. One study has reported a correlation between LATs and *ICP34.5* deletion compared with wild-type virus. LATs alone and *ICP34.5* alone each reduced spontaneous reactivation by 10–30% and 10%, respectively, compared to wild type. However, deletion of both LATs and *ICP34.5* led to undetectable levels of reactivation, even when the amount of virus was increased to 10^8 pfu (30). Therefore, the lack of LATs in the HF10 genome leads to suppression of reactivation from latency and supports the safety margin in the long-term, after treatment.

Genomic Deletions and Insertions in HF10 and Other HSV OVs

The identification of viral genes provides a strategy for genetically modifying OVs. To date, there are seven HSV-1 OVs (**Table 2**) being investigated in clinical trials. When we compare the genomic structure of OVs, we can see that the deletions and insertions of genes in HF10 are different from those in other OVs. *ICP34.5* is deleted in HSV-1 OVs used in clinical trials, but present in HF10. *ICP34.5* is thought to be involved in HSV neurovirulence. However, the exact mechanism by which HSVs induce encephalitis is unclear (31). HSV-1 OVs are classified according to the number of modified genes. First-generation OVs have only one modified gene (*ICP34.5* deletion), such as HSV-1716 [*ICP34.5*(−)] (32). Second-generation OVs have several gene deletions or insertions, and include OVs, such as HF10, NV1020 [*ICP34.5*(−), *ICP4*(−), *ICP0*(−), *TK*(+)] (33), G207 [*ICP34.5*(−), *ICP6*(−), *LacZ*(+)] (34), and G47Δ [*ICP6*(−), *ICP34.5*(−), *ICP47*(−)] (35). Third-generation OVs include therapeutic genes such as T-Vec [*GM-CSF*(+)/*ICP34.5*(−)/*ICP47*(−)] (13), and Mo32 [*ICP34.5*(−)/*IL12*(+)] (36).

PRECLINICAL STUDIES OF HF10 AS MONOTHERAPY

After investigating genomic changes in HF10, many investigators evaluated the oncolytic effect of HF10 in different malignant tumor models (**Figure 3**). Preclinical studies were conducted to evaluate the effect of HF10 replication on tumor selectivity and antitumor efficacy. HF10 was evaluated *in vitro* against Colon 26 and melanoma B16 cell lines, which showed that HF10 VP mediate cell–cell fusion to form enlarged multinucleated cells (syncytia formation). Furthermore, the therapeutic efficacy of HF10 was studied in murine and human breast cancer *in vitro* animal models. HF10 was also investigated in human and murine bladder cancer cell lines and in disseminated peritoneal metastasis.

To compare the antitumor effect and genomic structure of OVs, HSV-1 hrR3 *[ICP6(−)]* was chosen as a control due to the deletion of the *UL39* gene. *UL39* is also deleted in G207 and G47Δ HSV OVs. *UL39* encodes ICP6, the large subunit of ribonucleotide reductase, which is required for viral replication in non-dividing cells (37, 38). However, the deletion of *UL39* was postulated to increase the tumor selectivity of hrR3. HF10 induced complete syncytia formation in Colon 26 and melanoma B16 cell lines *in vitro*, while hrR3 induced a partial cytopathic effect. Furthermore, in a peritoneal tumor model, injection of 1×10^7 pfu of HF10 showed a more potent antitumor response, with a long-term survival rate over 90 days, than the same dose of hrR3 (39). Two studies have confirmed that HF10 replication was higher than hrR3 replication by 10-fold in CT26 cell and NfSa Y83 fibrosarcoma cells (40, 41). One limitation of hrR3 was elevated levels of neutralizing antibodies against hrR3 after 5 days of intraperitoneal inoculation (42). In addition, Luo et al. reported that HF10 has a greater bystander effect than hrR3 due

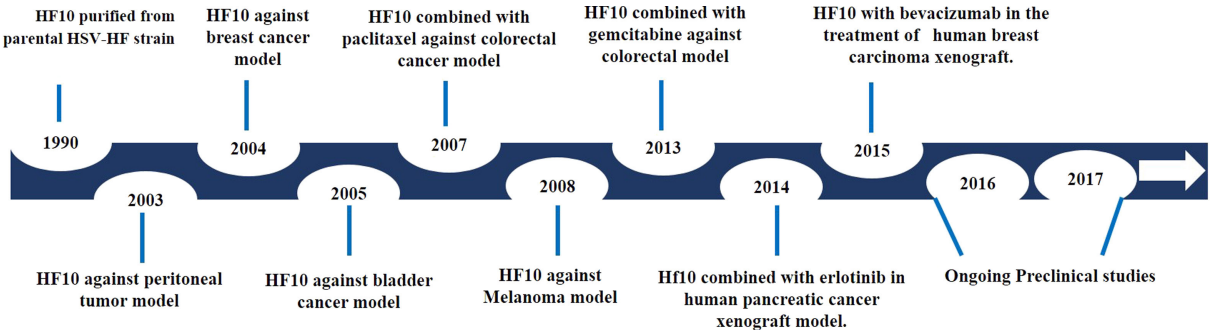

FIGURE 3 | History of HF10 preclinical studies. Antitumor efficacy of HF10 against tumor models in previous years.

to enhanced expression of connexin 43 subunits (43). Regarding other genetic deletions, Oncovex$^{ICP34.5(-),ICP47(-)}$ induced a classic cytopathic effect against human cell lines including HT1080 (fibrosarcoma), HCT 116 (human colonic carcinoma), CAPAN-1 (human pancreatic adenocarcinoma), and BHK (hamster normal baby kidney) cells (44). Therefore, natural deletions in HF10 may increase tumor selectivity, replication, cytopathic effect, and bystander effect compared with known deletions in other HSV OVs.

HF10 viral replication and cytotoxicity has also been studied in human and mouse melanoma cell lines (G-361 cells and clone M3 cells). HF10 induced 100% cell lysis in the clone M3 cell line after 48 h at MOI 3 and 0.3. Even at MOI 0.03, 92.6% of melanoma cells were lysed 72 h after infection (45). T-Vec was studied in the SK-MEL-28 melanoma cell line. At MOI 0.1 and 1, T-Vec caused 48 and 89% cell death, respectively, after 24 h. At 48 h after infection, T-Vec induced 84 and 100% cell death, respectively (EMEA/H/C/002771/0000). In an *in vivo* study, HF10 significantly reduced tumor growth in a subcutaneous melanoma model. Complete survival was shown in an intraperitoneal melanoma model without any obvious adverse effects. The antitumor efficacy and safety of HF10 were supported by detection of HF10 antigens with lymphoid cells and polymorphonuclear cells for at least 7 days after treatment (45). Recently, the B16F10 melanoma cell line, which lacks the expression of HSV entry receptors, was modulated to express the HSV-1 entry receptor Nectin1. A preliminary result with T-Vec showed sensitivity against Nectin1-expressing B16F10 *in vitro* and prolonged survival in an *in vivo* model (46).

Studies were extended to determine HF10 cellular tropism in other tumor models. The therapeutic efficacy of HF10 was studied in murine and human breast cancer animal models. HF10 efficiently replicated with high cytolytic effect in human and mouse breast cancer cell lines (MCF-7 and YMB-1, respectively). After 48 h, HF10 lysed almost all cells at MOI 3 and 0.3. However, HF10 replicated poorly in the MM102-TC mouse breast cancer cell line, even with increasing MOI titers. With inoculation of 1×10^7 pfu of HF10, there was suppression of tumor growth with prolonged survival rates up to 120 days without any neurologic or toxic side effects (47). In another study, HF10 and T-Vec were evaluated in a human breast adenocarcinoma cell line (MDA-MB-231). At MOI 1, HF10 caused approximately 50 and 90% cell death after 24 and 48 h post infection, respectively (48), while at the same MOI, T-Vec induced only 13.6 and 64.4% cell death after 24 and 48 h post infection, respectively (EMEA/H/C/002771/0000). In summary, HF10 with natural deletions had a significant oncolytic effect against human breast cancer cell lines.

Furthermore, the oncolytic effect of HF10 was investigated in human and murine bladder cancer cell lines (T24 and MBT-2) *in vitro* and also in a disseminated peritoneal metastasis model and a bladder cancer model. At MOI 3, HF10 replicated well in both T24 and MBT-2 cell lines and induced complete cell death by 48 h. In addition, serial HF10 treatments significantly prolonged survival rates in both models. HF10 safety and selectivity were supported by the presence of HSV antigens in the bladder on day 1 after intravesical treatment without shedding to other organs (49). These results suggested that HF10 has promising effects in a bladder cancer model and should be studied in a clinical trial. Chemotherapeutic or immunotherapeutic agents have not yet been approved for the treatment of bladder cancer due to the lack of effectiveness. Among OVs, only one adenovirus CG0070$^{GM\text{-}CSF(+)}$ phase II/III study is ongoing (NCT01438112); a durable response was observed in a phase I study (50).

Taken together, all the preclinical data on HF10, such as the loss of *UL56*, LATs, *UL43*, and *UL49.5* expression and *UL53* overexpression from the HF10 genome, lead to the following characteristics: innately high tumor selectivity, high viral replication, complete cytopathic effect, mediation of a highly potent bystander effect, and potent antitumor efficacy.

PRECLINICAL STUDIES OF HF10 AS COMBINATION THERAPY

To date, OVs have not shown serious toxicities or any therapeutic resistance, in contrast to chemotherapeutic drugs that cause severe dose-limiting toxicities and emerging cell resistance. As each approach has different mechanisms of action, combination therapy with OVs and chemotherapy enhances the antitumor effect with limited toxic side effects. A number of chemotherapeutic drugs are able to modulate the activities of myeloid-derived suppressor cells (MDSCs) and regulatory T cells in the suppressive tumor

microenvironment. Gemcitabine (GEM) inhibits MDSCs and enhances antitumor immune responses through T cell expansion (51). To date, GEM has been extensively investigated in combination with many OVs in different malignancies, including pancreatic cancer (52–56), renal cell carcinoma (57), and lung cancer (58, 59). Esaki et al. evaluated the synergistic effect between HF10 and GEM in a bilateral colorectal cancer model. After 3 days of GEM treatment, HF10 was injected at a dose of 1×10^7 pfu for 3 days to avoid possible interference with its replication. The study showed complete reduction of tumor size when HF10 was injected on the same side or even on the contralateral side. The oncolytic effect was enhanced by a significant decrease in $CD11b^+/F4/80^+$ macrophages and $CD11b^+/Gr\text{-}1^+$ MDSCs after GEM injection (60). GEM is one of the first-line therapeutic agents against pancreatic carcinoma with a median survival rate 4.4–5.6 months (61, 62). Unfortunately, combination therapy with other cytotoxic agents produced intolerable toxicities without any added benefits. In contrast, HF10 had a promising antitumor effect with a high safety margin in the investigator-initiated clinical studies for pancreatic cancer. Hence, HF10 will be an ideal agent to combine with GEM to achieve a high antitumor effect against pancreatic cancer with minimal side effects.

Regarding other chemotherapeutic drugs, paclitaxel induces cell death through mitotic arrest due to its effect on microtubule stabilization (63). HF10 has been combined with paclitaxel to enhance antitumor efficacy in *in vitro* and *in vivo* immunocompetent colorectal cancer models. Paclitaxel did not interfere with the replication or cytotoxicity of HF10 with CT26 cells *in vitro*. Paclitaxel and HF10 combination therapy resulted in superior survival rates in peritoneal colorectal cancer compared with either treatment alone (40). High proportions of mitotic and apoptotic cells were reported in combination with Reovirus type 3 Dearing strain (ReoT3D) OV and paclitaxel in non-small cell lung cancer cells (58). Another study investigated combination therapy with paclitaxel plus oncolytic Rhabdovirus Maraba MG1 virus in breast cancer, which showed controlled tumor growth and prolonged survival (64).

As with other OVs, the antitumor activity of HF10 depends on two mechanisms of action: selective replication within tumor cells causing tumor cell bursting and spreading and expression of tumor antigens, which induce an antitumor immune response (65). Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, binds to the ErbB-1 receptor, thus inhibiting tyrosine kinase activity and disrupting the activity of downstream pathways, including the Ras/Raf mitogen-activated protein kinase, phosphoinositide-3 kinase/Akt, and Jak2/STAT3 pathways (66). In addition to inhibiting cell proliferation, erlotinib also induced apoptosis and anti-angiogenesis of tumor cells (67). Previous studies have reported that human pancreatic cancer cell lines BxPC-3 and PANC-1 cells express EGFR (68, 69). Yamamura et al. evaluated the antitumor efficacy of HF10 combined with erlotinib in human pancreatic xenograft *in vitro* and *in vivo* using BXPC-3 and PANC-1 (70). The study reported that HF10 induced cell lysis in both cell lines; however, erlotinib was only sensitive in BxPC-3 cells. Combination treatment with HF10 and erlotinib resulted in a more significant cell lysis effect in BxPC-3 cells than with either HF10 or erlotinib alone. In BxPC-3 subcutaneous xenograft models, HF10 alone suppressed tumor growth more than erlotinib alone. However, in combination therapy, erlotinib caused high distribution of HF10, resulting in a significant tumor growth reduction compared with HF10 alone. Interestingly, the survival rate with HF10 alone was longer than with erlotinib alone (70).

The most important obstacle for OVs is the elevation of interstitial fluid pressure within tumors, which directly affects viral distribution (71). HSVs induce vascular endothelial growth factor (VEGF) production, which enhances angiogenesis in cells (72, 73). Bevacizumab is a monoclonal antibody suppressing tumor angiogenesis through inhibition of VEGF-A, which has been shown to be overexpressed in different solid tumors (74, 75). Tan et al. examined the oncolytic activity of HF10 in combination with bevacizumab in an experimental human breast carcinoma xenograft model (48). They showed that the MDA-MB-231 human breast cancer cell line has higher VEGF-A expression than the MC7 and T47D cell lines. By increasing MOI and time, HF10 alone induced cell cytotoxicity in the MDA-MB-231, MC7, and T47D cell lines. Bevacizumab did not induce any cell toxicity or interference with HF10 replication. In this study, two tumor models were established in BALB/c Slc-nu/nu mice bearing a single subcutaneous tumor or an advanced subcutaneous tumor. Intratumoral inoculation of HF10 (10^6 pfu) and bevacizumab (5 μg i.p.) significantly inhibited tumor growth in both models. In addition, immunohistochemical studies showed that the combination of HF10 and bevacizumab replicated more efficiently and with syncytia formation than HF10 treatment alone. More upregulation of VEGF-A with downregulation of CD31 was observed in endothelial cells after treatment with bevacizumab and HF10 compared with HF10 alone in both the single and advanced subcutaneous tumor models (48). A similar effect of bevacizumab was reported with other OVs, including adenoviruses (76), hrR3 (77), vaccinia virus (78), and reovirus (79).

HF10 CLINICAL TRIALS

Phase I Clinical Trial in Breast Cancer

HF10 has transitioned from preclinical to clinical trials to evaluate its therapeutic effect on human malignancies (**Figure 4**). The first clinical trial was performed from 2003 to 2006 by a team that included the surgery II, virology, and histopathology departments at the Graduate School of Medicine, Nagoya University, in Japan. The phase I clinical study evaluated the toxicity and efficacy of HF10 when directly injected intratumorally into cutaneous or subcutaneous metastatic nodules of recurrent breast cancers. All six patients had undergone mastectomy with recurrence after conventional therapies including chemotherapy, hormonal therapy, radiotherapy, and surgery. Patient age ranged from 48 to 76 years. They were seropositive for HSV and had metastatic recurrence in the skin (6/6), lymph nodes (4/6), lung (2/6), brain (1/6), and bone (1/6). In addition, all patients had more than 10 cutaneous and subcutaneous nodules. The first nodule was injected with diluted HF10, with doses ranging from 1×10^4 to 5×10^5 pfu/0.5 mL for 3 days. Another nodule was injected with sterilized saline as a control (**Table 3**). All patients

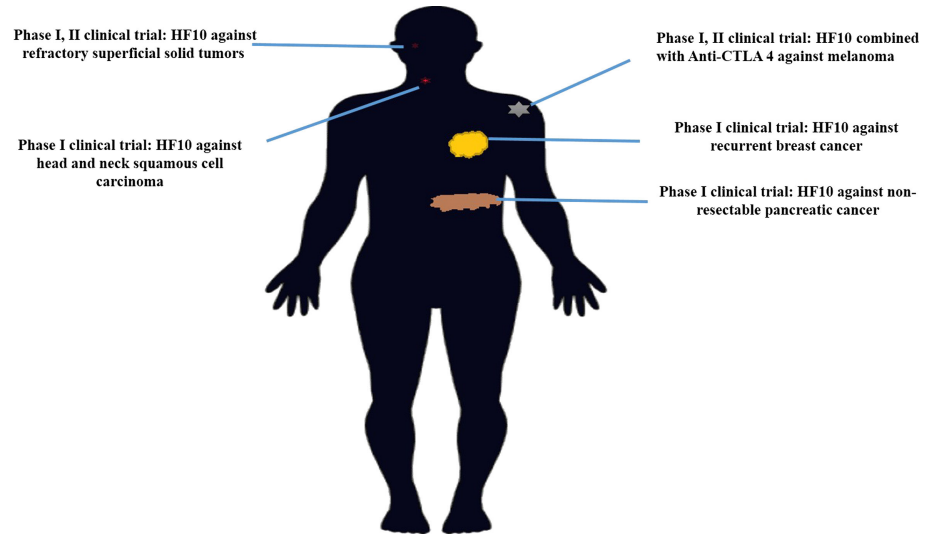

FIGURE 4 | History of HF10 clinical studies. Clinical trials of HF10 of various phases against different malignancies in previous years.

TABLE 3 | Profiles and responses of patients with metastatic breast cancer in a HF10 phase I clinical trial.

Patient No.	Age (years)	Recurrence region	Prior therapy	HF10 pfu/0.5 mL × 3 days	No. of Doses	Response	Side effects	Shedding
1	61	Skin, LN, lung, brain	CT-, HT-, RT-	1×10^4	1	Moderate response	None	No shedding into body fluids
2	62	Skin, LN	CT-, RT-	2×10^5	1	Mild response		
3	48	Skin, LN, lung, bone	SR	3×10^5	3	Marked response		
4	66	Skin, LN	CT-, HT-	5×10^5	1	Moderate response		
5	72	Skin	S-, CT-, HT-	5×10^5	3	Complete response		
6	76	Skin	CT-, HT-	5×10^5	3	Not applicable		

CT, chemotherapy; HT, hormonal therapy; RT, radiotherapy; SR, surgery.

tolerated the treatment well without any serious adverse effects. Histological examination showed nuclear viral inclusion bodies and adequate HF10 replication with high selectivity and distribution within malignant cells only. Tumor cell deformation was observed histologically, with 30 to 100% tumor death. Interestingly, a wide range of melting like fibrosis was observed after tumor cell destruction. There was considerable cytotoxic $CD8^+$ T cell infiltration around tumor islets. Moreover, there was no change in the count of blood cells such as white blood cells and natural killer (NK) cells, or in the levels of cytokines, such as IL10, IL12, IFNα, and IFNβ. These data supported HF10 safety through selective replication within tumor cells without any severe side effects. Furthermore, HF10 induced a cytotoxic immune response against breast cancer with $CD4^+$ and $CD8^+$ T cell infiltration (80, 81).

On the other hand, in the first clinical trial of T-Vec in 30 patients with cutaneous or subcutaneous metastases, the OV was injected into cutaneous or subcutaneous nodules in 14 breast cancer patients. Age ranged from 39 to 80 years; half of the patients were HSV seropositive and the other half were seronegative. T-Vec doses ranged from 10^6 to 10^8 pfu/mL in 1 or 3 injections. In this study, there was no complete or even partial responses, but stable disease was observed without significant differences between seropositive or seronegative patients. Most patients tolerated the treatment well, with some side effects such as grade I pyrexia, low-grade anorexia, nausea, fatigue, and vomiting (82).

Phase I Clinical Trial in HNSCC

An additional study demonstrated the safety and efficacy of HF10 in a phase I dose-escalation pilot study at the School of Medicine, Nagoya University, in Japan, on February 12, 2005. Two patients with advanced HNSCC were HSV seropositive. They were classified with aT2N1M0 disease, with several skin metastases and rT0N3M1 disease, with lymph node and skin metastasis, respectively (**Table 4**). Adverse effects, virus replication, and immunological response were evaluated after intratumoral injection of HF10 (1×10^5 pfu/1 mL or 0.5 mL for 3 days). In both patients, HF10 replicated well and induced tumor cell death with significant $CD4^+$ or $CD8^+$ cell infiltration. The patients had a low-grade fever after injection but no other obvious adverse effects. As no significant regression in tumor size was observed on days 13 and 15 after treatment, higher doses of HF10 might be used in another trial (83). Five patients with positive or negative HSV serotype that had metastatic head and neck cancer received three doses of Oncovex$^{GM\text{-}CSF}$ (10^6, 10^7, and 10^8 pfu/1 mL) for 3 days. Stable disease was observed without any complete or partial response. Some side effects such as pyrexia, low-grade anorexia, nausea, fatigue, and vomiting were observed (82).

TABLE 4 | Profiles and responses of patients with metastatic HNSCC in a HF10 phase I clinical trial.

Patient	Age (years)/sex	Clinical stage	Prior therapy	HF10 pfu/ mL × 3 days	Time	Response	Side effects
1	79/female	rT0N3M1	CT, RT	10^5 pfu/0.5 mL	1	No significant tumor regression on day 13 or 15	Low-grade fever after injection only
2	64/male	rT0N3M1	CT, SR	10^5 pfu/1 mL	1		

HNSCC, head and neck squamous cell carcinoma; CT, chemotherapy; RT, radiotherapy; SR, surgery.

TABLE 5 | Profiles and responses of patients with metastatic pancreatic cancer in a HF10 phase I clinical trial.

Patient	Age (years)	Clinical stage	HF10 PFU/0.5 mL/days	Time	Response	Survival (days)	Side effects	Shedding
1	68	Invasive ductal carcinoma	$1 \times 10^5 \times 3$	1	PD	200	None	No shedding into body fluids
2	61		$1 \times 10^5 \times 3$	1	SD	166		
3	60		$5 \times 10^5 \times 3$	3	SD	318		
4	52		$1 \times 10^6 \times 3$	1	PD	98		
5	73		$1 \times 10^6 \times 3$	3	PR	209		
6	76		$1 \times 10^6 \times 3$	3	SD	315		
7	49		$1 \times 10^6 \times 6$	6	PD	206		
8	64		$1 \times 10^6 \times 6$	6	PD	113		

PD, progressive disease; SD, stable disease; PR, partial response.

Phase I Clinical Trial in Pancreatic Cancer

A phase I clinical trial was performed in eight male patients with invasive pancreatic ductal carcinoma from 2005 to 2009 at the School of Medicine, Nagoya University, in Japan. All eight patients were HSV seropositive because of safety concerns. Six patients received one injection of HF10 (1×10^5/two patients, 5×10^5/one patient, and 1×10^6/three patients) per day for three consecutive days. After 3 days of injections, the patients were given no further treatment for 30 days and monitored for adverse and therapeutic effects. The first dose of 0.5 mL was injected in four sites or as 2.0 mL during laparotomy. The other two doses were injected using an intratumoral catheter inserted at the time of surgery. Moreover, the last two patients received an additional injection of 10^6 pfu/1.0 mL HF10 once a week for total of 3 weeks *via* endoscopic ultrasound (**Table 5**). All patients tolerated the treatment well without any observed adverse effects after treatment. Three patients showed declines in the tumor marker CA19-9. There was no HSV shedding into the blood or body fluids based on plaque-forming assays at this time. HF10 envelope protein was also detected in autopsy specimens with infiltrations of macrophages, $CD4^+$ and $CD8^+$ cells, and activation of NK cells, suggesting that HF10 enhances antitumor immunity. The response to treatment was classified as stable disease in three patients, partial response in one patient, and progressive disease in four patients. Survival time ranged from 98 to 318 days, with an average of 180 days. These results suggested that higher doses of HF10 can be used in future trials (84, 85).

Phase I and Phase II Clinical Trials in Refractory Superficial Cancers and Melanoma in the US

A phase I clinical trial in patients with refractory superficial cancers and melanoma was conducted at the University of Pittsburgh in the United States. This trial evaluated the tolerability and efficacy of HF10 therapy in 26 patients, including HSV seropositive and seronegative patients, with refractory superficial cancers and melanoma. The trial was divided into two stages. In Stage 1, patients received a single HF10 dose at 1×10^5, 3×10^5, 1×10^6, or 1×10^7 pfu. In Stage 2, patients received four injections of HF10 at 1×10^6 to 1×10^7 pfu. The results showed that adverse events of any kind occurred in 34.6% of patients overall. Drug-related adverse events included chills (11.5%), fatigue (7.7%), pyrexia (3.8), and injection site reaction (6%). In comparison, T-Vec caused pyrexia (52%), fatigue (48%), and nausea (30%) in 50 melanoma patients. Moreover, no significant difference was observed between HSV-1 seropositive and seronegative patients. In summary, HF10 was safe and well tolerated. The response rate was evaluated in 24 patients. Eight patients had stable disease. The reduction in tumor size in some patients ranged from 30 to 61%. Interestingly, one patient showed pathological complete response after 4 months of treatment (86).

A phase II clinical trial of HF10 combination therapy was conducted in the United States. HF10 was combined with ipilimumab (anti–CTLA-4) in patients with unresectable or metastatic melanoma in this study (NCT02272855). A total of 46 patients were enrolled in this clinical trial, and results were evaluated in 44 patients. Regarding tumor growth inhibition, the best overall response (BOR) was evaluated by Immune-Related Response Criteria at 24 weeks. BOR was 41% (irCR: 16%, irPR: 25%), clinical therapeutic efficacy was 68% (irCR + irPR + irSD), and irSD was 27%. Regarding survival rate, median progression-free survival was 19 months and median overall survival was 21.8 months. This combination showed a beneficial therapeutic effect as second-line therapy; in 20 patients, BOR was 30% (87).

FUTURE DIRECTIONS

Over 14 years ago, HF10 was being investigated in various preclinical models, including disseminated peritoneal colon cancer, melanoma, pancreatic cancer, breast cancer, and bladder cancer. These studies have translated into successful clinical trials in different cancer types including recurrent metastatic breast cancer,

recurrent HNSCC, advanced pancreatic cancer, refractory and superficial cancers, and melanoma. Although the data on HF10 in preclinical and clinical trials suggest that therapeutic applications can be developed with a high safety margin, combination therapies with either chemotherapy or immunotherapeutic agents are a promising approach in the near future. However, the ideal combination with HF10 still needs more investigation. As few OVs have shown efficacy against cancer stem cells and chemoresistant cells, more studies of HF10 against these types of cells are needed. For OVs in general, future studies must overcome physical tumor barriers that limit intravenous delivery.

AUTHOR CONTRIBUTIONS

IE: preparing manuscript, collecting data, designing, writing, and editing. YN: revising manuscript and providing critical considerations for manuscript design. TI: manuscript collecting data and editing. IB-V: revising manuscript and editing. MT, WZ, NMu, TM, NMi, HH, SS, BA, and YK: contributing to manuscript design and collecting data. HK: revising manuscript and providing critical considerations for manuscript design as well as final approval of the version to be published.

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Enhancing the Oncolytic Activity of CD133-Targeted Measles Virus: Receptor Extension or Chimerism with Vesicular Stomatitis Virus Are Most Effective

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Therapy resistance and tumor recurrence are often linked to a small refractory and highly tumorigenic subpopulation of neoplastic cells, known as cancer stem cells (CSCs). A putative marker of CSCs is CD133 (prominin-1). We have previously described a CD133-targeted oncolytic measles virus (MV-CD133) as a promising approach to specifically eliminate CD133-positive tumor cells. Selectivity was introduced at the level of cell entry by an engineered MV hemagglutinin (H). The H protein was blinded for its native receptors and displayed a CD133-specific single-chain antibody fragment (scFv) as targeting domain. Interestingly, MV-CD133 was more active in killing CD133-positive tumors than the unmodified MV-NSe despite being highly selective for its target cells. To further enhance the antitumoral activity of MV-CD133, we here pursued arming technologies, receptor extension, and chimeras between MV-CD133 and vesicular stomatitis virus (VSV). All newly generated viruses including VSV-CD133 were highly selective in eliminating CD133-positive cells. MV-CD46/CD133 killed in addition CD133-negative cells being positive for the MV receptors. In an orthotopic glioma model, MV-CD46/CD133 and MV^{SCD}-CD133, which encodes the super cytosine deaminase, were most effective. Notably, VSV-CD133 caused fatal neurotoxicity in this tumor model. Use of CD133 as receptor could be excluded as being causative. In a subcutaneous tumor model of hepatocellular cancer, VSV-CD133 revealed the most potent oncolytic activity and also significantly prolonged survival of the mice when injected intravenously. Compared to MV-CD133, VSV-CD133 infected a more than 10^4-fold larger area of the tumor within the same time period. Our data not only suggest new concepts and approaches toward enhancing the oncolytic activity of CD133-targeted oncolytic viruses but also raise awareness about careful toxicity testing of novel virus types.

Keywords: glioblastoma, hepatocellular carcinoma, prominin-1, virotherapy, tumorsphere

INTRODUCTION

Despite considerable progress in cancer therapy, relapse and dissemination of tumor cells remain a frequent therapeutic outcome, which is more and more ascribed to an insufficient targeting and killing of a small population of tumor cells with stem-cell like properties (1). Such cancer stem cells (CSCs) were initially detected in tumors of hematopoietic origin demonstrating that only a small fraction of cells in the tumor mass is capable of forming metastasis and new tumors (2). Among many putative markers for CSCs, CD133 was among the first to be discovered in carcinomas and since then became one of the most frequently studied and targeted marker (3, 4). Besides on tumor cells, CD133 is expressed on neuronal and endothelial progenitors as well as on hematopoietic stem cells (HSCs). While there is evidence for a role of CD133 in cell differentiation and epidermal–mesenchymal transition, its precise physiological function remains unknown (4).

The relevance of CD133 as universal marker for CSCs has frequently been challenged so that there is ample evidence for the presence of stemness properties also in CD133-negative tumor cells (5). Nevertheless, evidence for a strong correlation between high levels of CD133 expression and poor prognosis for patients suffering from various cancer types has increased, as well. In glioma, a systematic meta-analysis covering 1,500 patients revealed reduced overall survival for grade IV patients with high CD133 expression (6). Since further recent articles came to the same conclusion for glioma (7–9) as for hepatocellular cancer (HCC) (10, 11), targeting of CD133 remains an attractive therapeutic concept for these cancer entities and possibly also for others.

Oncolytic viruses have become a novel treatment option in cancer therapy with a first product based on herpes virus having recently obtained marketing approval (12). The antitumor concept relies on the selective infection and lysis of tumor cells resulting in the release of tumor antigens against which an effective immune response can be triggered (13). Oncolytic measles viruses (MVs) derived from attenuated strains are currently studied in various clinical trials assessing their antitumoral activity for different cancer entities (14, 15). Recently, clinical benefit was achieved for patients suffering from multiple myeloma upon systemic injection of a high MV dose (16). Supposedly, selectivity for tumor cells is on one hand due to overexpression of CD46, one of the receptors used by attenuated MV strains. On the other hand, attenuated MV strains are sensitive to much lower levels of interferon than wild-type MV strains, a phenomenon thought to be a consequence of mutations in the P/V/C gene that accumulated during attenuation (17, 18). Interestingly, the oncolytic activity of MV can be enhanced by exchanging the P gene from attenuated strains against that of wild-type MV without compromising safety (19).

Restriction of virus replication to tumor cells can moreover be achieved through rational engineering at the level of receptor recognition and cell entry. In particular, the glycoproteins of herpes simplex virus and MV have been shown to be amenable for engineering receptor usage (20, 21). In the case of MV, recognition of the natural receptors CD46, SLAM, and nectin-4 (22) can be destroyed by four point mutations and new receptor usage gained by fusing a single-chain antibody fragment (scFv) or other targeting domains to the hemagglutinin on the genetic level (23, 24). Such MVs only infect cells that express the cognate antigen of the displayed targeting domain on their surface (25). Interestingly, the MV glycoproteins can replace the glycoprotein G in the vesicular stomatitis virus (VSV) genome, resulting in chimeric VSV-MVs that replicate faster and to higher titers than the corresponding MVs (26).

By displaying CD133-recognizing scFvs, we have previously shown that oncolytic MV can be engineered to recognize CD133 as receptor for cell entry and can selectively destroy CD133-positive tumor cells (27). Remarkably, these viruses exhibited a stronger oncolytic activity *in vivo* than untargeted MV using the ubiquitously expressed CD46 receptor for cell entry. In a clinical setting, CSCs are rare in tumor tissue making it challenging for CD133-targeted viruses to hit and infect these cells. Here, we therefore aimed at further improving the oncolytic activity of CD133-targeted oncolytic viruses by assessing various strategies. We show that in glioma, MVs using CD133 and CD46 as receptors are particularly promising, while for HCC or other carcinomas not involving the central nervous system, VSV targeted to CD133 appears to be the best choice.

MATERIALS AND METHODS

Generation of the Viruses

Cloning of MV-CD133, previously termed MV-141.7, was described before (27). To generate MV^{Pwt}-CD133 the reading frame for H in the plasmid encoding MV-eGFP-Pwt (19) was exchanged against that of the engineered H protein encoded in the genome of MV-CD133 *via* PacI/SpeI restriction sites. MV^{SCD}-CD133 was generated by exchanging the GFP coding sequence in the genome of MV-CD133 against that of SCD using the MluII/AatII restriction sites. To reconstitute the N and P genes after SCD insertion, these were inserted *via* AatII restriction in a second step. The genome of MV-CD46/CD133 was cloned by first generating the expression plasmid pCG-H-scFvCD133-141.7-6His encoding the H protein C-terminally fused to the CD133-specific scFv 141.7 (27), but carrying no point mutations in the MV-receptor recognition sites. The H gene cassette in MV-CD133 was then exchanged against that of pCG-H-scFvCD133-141.7-6His *via* PacI/SpeI restriction sites. Interested researchers may request Miltenyi Biotec GmbH (Germany) to grant access to the plasmids under a Material Transfer Agreement.

Rescue of MV-CD133, MV^{Pwt}-CD133, MV^{SCD}-CD133, and MV-CD46/CD133 was performed using the T7 rescue system with 293-3-46 producer cells (28) overlaid onto Vero-αHis cells (25). Starting from a single virus syncytium, virus was propagated on Vero-αHis cells and stocks were generated from cell lysates.

For cloning of the genome plasmid of VSV-CD133, the sequence encoding the CD133-specific scFv was inserted into pMC11-VSV-FHaa-mUPA-eGFP (encodes the non-attenuated Indiana serotype) *via* SfiI/NotI restriction sites (29). To rescue VSV-CD133, in addition to the helper plasmids encoding

VSV-N, -P and -L, a plasmid encoding VSV-G was co-transfected into BHK-21 cells. The T7 RNA polymerase was provided by infection of the transfected BHK-21 cells with a modified vaccinia virus Ankara coding for the polymerase (MVA-T7-Pol) (30). Cell lysate was harvested, MVA was removed by filtration (0.2 µm pores), and single syncytia were isolated after overlay on Vero-αHis cells as described. VSV-MV was rescued from pMC11-VSVFH-eGFP as described previously (26). VSV-CD133 and VSV-MV were propagated on Vero-αHis cells. The 50% tissue culture infective dose ($TCID_{50}$/ml) was determined on Vero-αHis cells.

All viruses were handled under biosafety level 2 conditions as authorized by the Regierungspräsidium Giessen, Germany.

Cells

BHK-21 (ATCC CCL-10), Chinese hamster ovary (CHO)-K1 (ATCC CCL-61) cells, HuH7 cells (Japanese Collection of Research Bioresources Cell Bank, Japan), 293-3-46 cells (28), Vero-αHis cells (25), CHO-CD46 cells (31), and CHO-hSLAM cells (32) were all cultivated in DMEM (Sigma-Aldrich, Germany) supplemented with 10% FCS (Biochrom, Germany) and 2 mM L-glutamine (Sigma-Aldrich, Germany). CHO-CD133 cells were generated by stable integration of the human CD133 coding sequence into CHO-K1 cells (ATCC CCL-61). The cells were cultivated in DMEM supplemented with 10% FCS and 10 µg/ml puromycin.

Primary glioblastoma cells NCH644 and human HSCs were cultivated as described previously (27).

Immunoblotting

Virus stocks (5.0×10^5 $TCID_{50}$: MV-NSe, MV-CD133, MV^{Pwt}-CD133, MV^{SCD}-CD133, MV-CD46/CD133; 2.5×10^5 $TCID_{50}$: VSV-MV and VSV-CD133) were mixed with urea sample buffer (5% SDS, 8 mM urea, 200 mM Tris–HCl, 0.1 mM EDTA, 0.03% bromphenol blue, 2.5% di-thiothreitol, pH 8.0) in equivalent amounts and incubated 10 min at 95°C before separating them *via* SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane (GE Healthcare, Germany) and blocked with TBS-T containing 5% milk powder. Subsequently, membranes were incubated with rabbit sera recognizing MV-F (Abcam, Great Britain), the cytoplasmic tail of MV-H (27), MV-N (Novus Biologicals, USA), or rabbit-α-VSV serum (α-VSV) as described in Ref. (33). After three wash steps, membranes were incubated with polyclonal horseradish peroxidase-conjugated goat-α-rabbit secondary antibody (DakoCytomation, Germany). Protein signals were detected using the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, USA).

Colony-Forming Assays

Primary human CD34-positive cells derived from G-CSF mobilized peripheral blood of anonymous donors were obtained from the blood donation center in Frankfurt in accordance with the ethical standards of the responsible committee on human experimentation. An ethics approval was not needed for this type of research. The cells were purified and cultivated as described (34). After overnight stimulation with medium supplemented with StemSpan CC100 cytokine cocktail (Stemcell Technologies, Germany) and 2 mg/mL TPO (Peprotech, Rocky Hill, NJ), 5×10^4 cells were infected with virus at an MOI of 1. Lysate of uninfected Vero-αHis cells was added as control and to equilibrate all samples to identical amounts of cell lysate. 24 h postinfection, 0.1 mM 5-fluorocytosine was added to the cells. 48 h postinfection, cells were washed twice with medium without cytokines. Next, 1% of the cells were transferred into 3 ml MethoCult GF H4434 medium (Stemcell Technologies, Germany) and plated in triplicates. After 10 days in an incubator at 37°C and 95% humidity, clonal clusters (colonies) of maturing cells of the myeloid and erythroid lineage were enumerated and morphologically classified by light microscopy.

Monitoring Cell Viability

For analyzing the viability of virus-infected HuH7 cells, 1.0×10^4 cells were seeded per 96-well and infected at different MOIs (0.0001–10) to determine dose dependency. Cell viability of infected cells was determined using the premixed WST-1 Cell Proliferation Reagent (Clontech, USA) according the manufacturer protocol. To determine the EC_{50}, the MOI required to kill half of the cells was determined for each virus.

To analyze cell viability in glioma sphere cultures, 7.0×10^3 NCH644 cells were seeded per well in a 96-well plate and infected with different MOIs (0.001–10) to determine dose dependency. When using MV^{SCD}-CD133, different concentrations of 5-FC were added to the culture medium to determine the optimal dose response. Cell viability of infected cells was determined using the RealTime-Glo MT Cell Viability assay (Promega, Germany) according to the manufacturer's protocol.

To determine IFNα in the supernatant of infected cells, cell culture supernatants were collected at time points 0, 12, 24, 48, and 72 h postinfection. Cell free supernatants were obtained by centrifugation at 250 *g* and stored at −80°C until analysis by Human IFN-α pan ELISA (Mabtech, Sweden) according to the manufacturer's instructions.

Orthotopic Glioma Model

1×10^5 NCH644 tumor sphere cells either uninfected or infected at an MOI of 0.5 were dissociated and stereotactically implanted in 5 µl of PBS into the corpus striatum of the right hemisphere (1.7 mm lateral, 0.5 mm rostral to bregma at 3 mm depth) of 6- to 8-week-old NOD/SCID mice. After pausing for 5 min to allow the diffusion of the carrier fluid into the brain parenchyma, the injection needle was slowly extracted. Intracranial injection of virus was performed stereotactically in 5 µl PBS into the same coordinates. Survival and general condition of mice were monitored daily. The experimental end point was reached at the onset of neurological symptoms and/or weight loss of more than 20%.

For *ex vivo* culturing of glioma spheres, the whole tumor was excised and enzymatically dissociated using the Miltenyi Brain tumor dissociation Kit according to the manufacturers' instructions. 2×10^5 single cells were cultured in T25 flask (Sarstedt, Germany) in DMEM/F-12 medium containing 20% BIT serum-free supplement, basic fibroblast growth factor, and epidermal growth factor at 20 ng/mL (all Provitro, Germany). Cells were cultivated until spheres had formed, and a cell density of about 1×10^6 cells per T25 flask was reached.

HuH7 Tumor Model

To analyze the antitumoral effect of oncolytic viruses in the s.c. HuH7 xenograft model, 5×10^6 HuH7 cells were implanted into the flank of NOD/SCID mice (Charles River, Germany). The tumor development was monitored twice a week using a digital caliper. Once tumors had reached an average size of 100 mm^3 mice were assigned into the different treatment groups. A total dose of 4×10^6 $TCID_{50}$ of each virus in 50 µl Vero-αHis lysate was injected intratumorally split in four injections every other day. Control mice received virus-free Vero-αHis lysate. For intravenous administration 3×50 µl virus were injected *via* the tail vein every second day. Treatment and monitoring were performed in a blinded manner over the whole course of the study until sacrifice. The area under the curve (AUC) was determined for each animal and was normalized against the value obtained from the last survivor of the mock group (day 40 posttransplantation). To account for missing scores of the tumor size of sacrificed animals before the end point for the analysis, the values were carried forward until termination of the study (last observation carried forward). NOD/SCID mice were sacrificed once the tumor had reached a size of 1,000 mm^3 or mice had lost more than 20% of their body weight.

Quantification of Infected Areas in Tumor Sections

HuH7-derived tumors were explanted, cut into two halves and fixed in 4% formaldehyde in PBS for 24 h. Next, the tissue was dehydrated in 40% sucrose in PBS and then embedded in optical cutting temperature medium (Sakura Finetek, Germany) for snap freezing. Specimens were stored at −80°C. Slices of 8 µm thickness were obtained with a cryostat (Leica CM1900) and dried at room temperature overnight. Slices were permeabilized with 0.2% Triton X100/PBS for 10 min and blocked with 5% donkey serum for 30 min at room temperature. For staining against GFP slices were incubated with the rabbit anti-GFP antibody (1:200, Life Technologies, Germany) overnight at 4°C, followed by incubation with the donkey anti-rabbit Cy2-coupled secondary antibody (1:200, Dianova, Germany). Sections were mounted with Fluoroshield with DAPI containing mounting media (Sigma-Aldrich, Germany).

Up to 600 tiles per slice were acquired by a motorized Axio-Observer Z1 microscope equipped with an ApoTome optical sectioning unit (Carl Zeiss, Jena, Germany). For quantification of the GFP-fluorescent areas, computational analysis was conducted using the Cell Profiler software (35). Threshold levels were determined on mock-treated tumors. Autofluorescent tumor areas were excluded by applying a smoothing filter. For each tumor the total identified GFP-positive area was calculated by the Cell Profiler software.

RESULTS

Generation and Basic Characterization of CD133-Targeted OVs

To improve the antitumoral potency of the CD133-targeted oncolytic virus MV-CD133, we followed several strategies. First, we exchanged the P gene against that of a wild-type MV strain resulting in MV^{Pwt}-CD133 (19). Alternatively, the GFP reporter gene was exchanged against the suicide gene super cytosine deaminase (SCD), which converts the prodrug 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) resulting in MV^{SCD}-CD133 (36). To extend the tropism of MV-CD133 from CD133-positive cells also to CD133-negative tumor cells, MV-CD46/CD133 was generated, in which the CD133-specific scFv was fused to unmodified H protein. Finally, we transferred the CD133-targeting strategy to VSV by exchanging the VSV glycoprotein gene against the CD133-targeted MV-H and the MV-F genes resulting in VSV-CD133 (**Figure 1A**).

After rescuing the panel of oncolytic viruses, we first assessed the protein composition of each virus type by Western blot analyses. All MV-derived viruses contained comparable amounts of F1 and N proteins and showed the expected shift in the electrophoretic mobility of the H-scFv fusion proteins compared to unmodified H present in MV-Nse (**Figure 1B**). The protein composition of VSV-CD133 was compared to those of VSV-MV and VSV. Whereas the glycoprotein G was detected in the lane loaded with VSV, this signal was absent in lanes loaded with VSV-MV or VSV-CD133. Instead, the latter viruses showed the measles H and F1 proteins. There was no difference in the amounts of the VSV-N, P and M proteins detectable between the VSV-MV chimeras and VSV (**Figure 1B**).

To address usage of CD133 as entry receptors, CHO cells transgenic for the natural entry MV receptors CD46 or SLAM, or the target receptor CD133 were infected with MV-NSe, MV-CD133, MV^{Pwt}-CD133, MV-CD46/CD133, or VSV-CD133 at low multiplicity of infection. The parental CHO-K1 cell line served as negative control. As shown in **Figure 1C**, MV-NSe infected only those cell lines expressing CD46 or SLAM. While the CD133-targeted MV and VSV viruses exclusively infected CHO-CD133 cells, MV-CD46/CD133 was able to infect CD46- and CD133-positive CHO cells as well as SLAM-positive cells. Syncytia formation was comparably strong between all MVs. Lysis was most pronounced in cells infected with VSV-CD133. The parental cell line CHO-K1 showed no green fluorescence.

These results revealed that the cell tropism of the oncolytic viruses was selective and restricted to cell lines expressing the targeted surface receptors.

CD133-Targeted OVs Do Not Impair the Differentiation Potential of Human HSCs

Oncolytic viruses not only must be effective but also have to be safe. In particular, this means that healthy cells endowed with the target receptor should be spared by the targeted oncolytic virus. Besides tumor-initiating cells, CD133 is also a marker for early progenitors of the hematopoietic system. Infection of these cells by the CD133-targeted viruses could result in tremendous side effects such as myelosuppression. To determine the proliferative and differentiation capacity of hematopoietic progenitors incubated with the OVs generated here, we performed colony-forming assays (CFAs). Flow cytometric analyses of CD34-positive human hematopoietic cells confirmed high cell surface expression levels of both, CD46 and CD133 (Figure S1 in Supplementary Material).

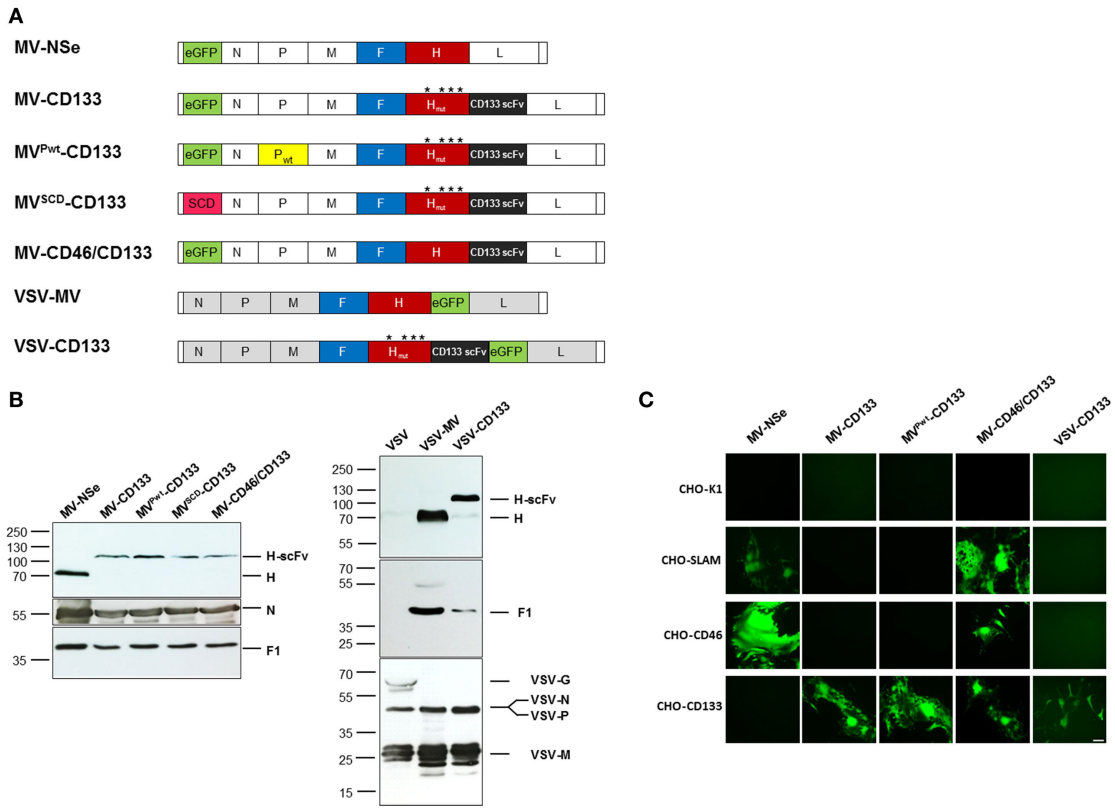

FIGURE 1 | Generation of CD133-targeted oncolytic viruses. **(A)** Schematic overview on the genomic organization of the OVs used in this study. Point mutations in H protein introduced to ablate natural receptor usage are indicated by asterisks. **(B)** Immunoblot showing the incorporation of measles virus glycoproteins into recombinant measles virus (MV) and vesicular stomatitis virus (VSV) particles. Supernatants of Vero-αHis cells infected with the indicated viruses were denatured followed by fractionation by SDS-PAGE. Viral glycoproteins were detected with polyclonal antibodies directed against the indicated proteins. The parental MV-NSe, respectively, VSV and VSV-MV served as unmodified controls. N blots were used as loading control in both cases. **(C)** A panel of receptor-transgenic Chinese hamster ovary (CHO) cells (as indicated) was infected with MV-NSe, MV-CD133, MV^{Pwt}-CD133, MV-CD46/CD133, or VSV-CD133 at an MOI of 0.03. CHO-K1 served as receptor-negative cell line. GFP-fluorescent images were taken 72 h postinfection; Scale bar, 200 µm.

The cells were plated into semisolid methylcellulose medium supplemented with cytokines and growth factors enabling the cells to proliferate and differentiate to produce clonal clusters of maturing cells of the hematopoietic lineage. Myeloid progenitors and committed progenitors of the erythroid, monocyte, and granulocyte lineages could then be enumerated and identified according to their morphology.

CD34-positive cells were subjected to the CFA 3 days postinfection with an MOI = 1 of each virus type. After 11 days, colonies derived from maturing hematopoietic progenitor cells were identified and quantified by light microscopy. In each sample, hematopoietic progeny of all lineages covered by this assay were detected. Colony numbers in total and subparts of all treatment groups were not decreased compared to mock-treated cells (**Figure 2**). However, a slight decrease of the total colony number of HSCs, which were infected with MV-NSe, was found. The difference in the proliferation and differentiation of cells derived from mock, cell lysate and virus-infected cells was not significant (**Figure 2**). Furthermore, none of the colonies contained any GFP-positive cells. The results demonstrate that the CD133-targeted viruses investigated here do not impair the hematopoietic capabilities of CD34-positive cells.

Infection and Killing of Tumor Cells

Next, the oncolytic activity of the newly established viruses was analyzed in cell killing assays, at first using the CD133-positive hepatocellular carcinoma cell line HuH7. In killing kinetics using an MOI = 1, MV^{Pwt}-CD133, MV-CD46/CD133, or VSV-CD133 killed nearly 100% of the cells within 72 hpi, while MV-CD133 lagged slightly behind (**Figure 3A**). Cell viability was reduced to less than 50% before 48 hpi in VSV-CD133 infected cells. VSV-CD133 was thus at least 15 h faster than the MVs. To further quantify the dose dependency of the killing capabilities, tumor cells were infected with different MOIs to determine the effective concentrations (EC_{50}) required to reduce the cell viability to 50% relative to untreated controls. Infection with all viruses resulted in substantial dose-dependent killing of HuH7 cells, with VSV-CD133 and MV^{SCD}-CD133 (in combination with 5-FC) being substantially more active than the other viruses at low MOIs (Figure S1 in Supplementary Material). Accordingly, the EC_{50} of VSV-CD133 and MV^{SCD}-CD133 (+5-FC) was reached at 31-fold and 26-fold, respectively, less infectious virus particles than for the other viruses (**Figure 3B**).

To determine the oncolytic activity of the viruses against primary tumor cells, we assessed the infection of the primary

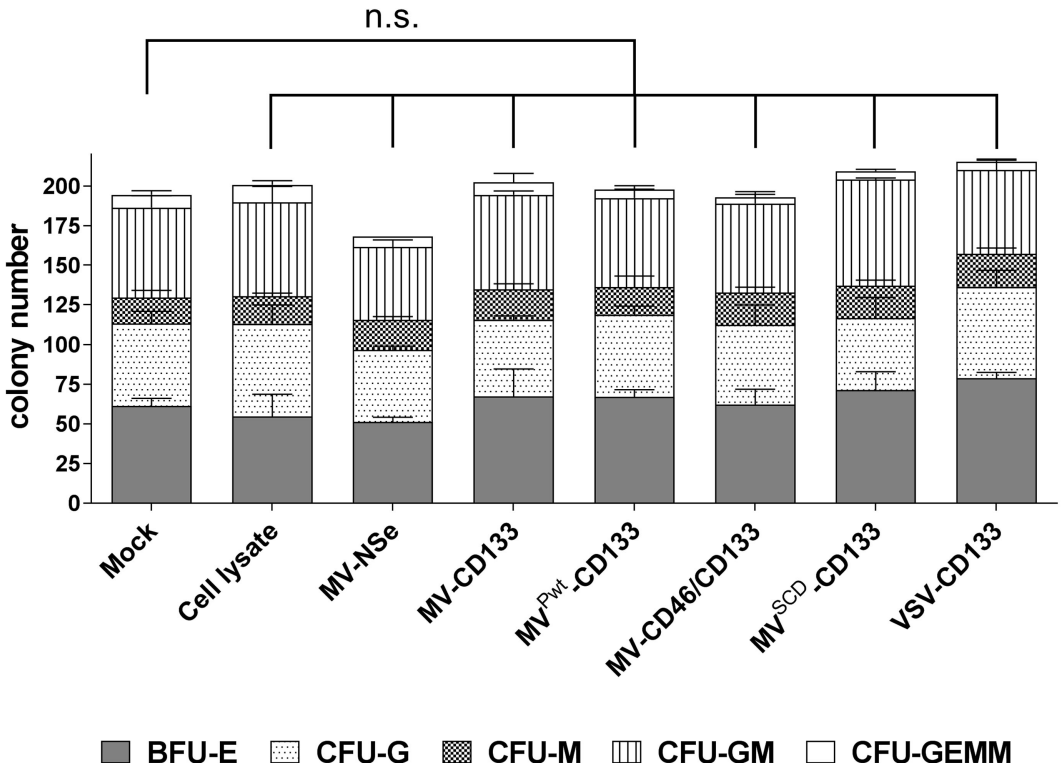

FIGURE 2 | Hematopoietic stem cell properties are not impaired by infection. A colony-forming assay was performed with human CD34-positive cells purified from G-CSF mobilized peripheral blood that were incubated with the indicated viruses (MOI of 1), lysate from uninfected Vero-αHis cells, or PBS (mock). After 11 days, the number of colonies derived from erythroid and myeloid progenitors was determined by light microscopy. The proportion of the respective progenitors is shown in relation to the total colonies. Mean distribution ± SD of all colonies derived from three technical replicates is shown as a bar. The statistical analysis was carried out by a descriptive-explorative data analysis. Differences between treatment groups and the mock control group were not significant according to one-way ANOVA followed by Sidak's multiple comparison test: $P = 0.9993$ (cell lysate), $P = 0.4103$ (MV-Nse), $P > 0.9969$ (MV-CD133), $P > 0.9999$ (MV^{Pwt}-CD133), $P > 0.9999$ (MV-CD46/CD133), $P = 0.9036$ (MV^{SCD}-CD133), and $P = 0.6528$ (VSV-CD133). BFU-E, burst-forming unit erythroid; CFU-G, colony-forming unit-granulocyte; CFU-M, colony-forming unit-macrophage; CFU-GM, colony-forming unit-granulocyte, macrophage; CFU-GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte; n.s., not significant; VSV, vesicular stomatitis virus; MV, measles virus.

glioma cells NCH644, which are about 90% positive for CD133 and form spheres under serum-free conditions (27). Tumor cells were infected as single cell suspension at an MOI of 1. Green fluorescent cells became detectable 24 h postinfection with strongest signals for MV^{Pwt}-CD133 and MV-CD46/CD133. Cytopathic effects were most prominent in cells infected with MV-CD46/CD133. However, all viruses were able to infect glioma tumor spheres and to induce syncytia formation (**Figure 4A**). To quantify cell killing we monitored the cells over time. All viruses killed more cells with increasing dose (Figure S2 in Supplementary Material). Over time, however, a steady reduction in cell numbers was observed for the MV-derived viruses but not for VSV-CD133 and VSV-MV (Figure S2 in Supplementary Material). In fact, cell killing reached its maximum by 24 h with these viruses. From then on cells started to propagate again (Figure S2 in Supplementary Material; **Figure 4B**). Supernatant from MV-CD133 and VSV-CD133 infected cells collected at time points 12, 24, 48, and 72 postinfection did not contain any evidence for the presence of IFNα in any of the samples as tested by ELISA with a sensitivity of 6.25 pg/ml ($N = 3$ biological replicates). Among the MVs, cell killing was most efficient with MV-CD46/CD133 and also with MV^{SCD}-CD133, after having identified the optimal prodrug concentration (Figure S3 in Supplementary Material; **Figure 4B**). These viruses were significantly more efficient in killing glioma tumor spheres than MV-CD133 or MV^{Pwt}-CD133 at 72 hpi.

MV-CD46/CD133-Treated Mice Show a Survival Benefit in an Orthotopic Glioma Model

In a next step, we intracranially implanted NCH644 glioma spheres infected shortly before injection and followed survival of the mice. With the exception of VSV-CD133, all mice treated with oncolytic viruses survived substantially longer than untreated mice (**Figure 5A**). MV-CD46/CD133 showed a tendency toward being most effective, since mice treated with this oncolytic agent survived on average longer than all others (median survival = 89 days). Interestingly, some individuals from the MV^{SCD}-CD133-treated group survived especially long (more than 100 days). However, the median survival of this group was below that of the MV-CD46/CD133-treated group. Unexpectedly, all

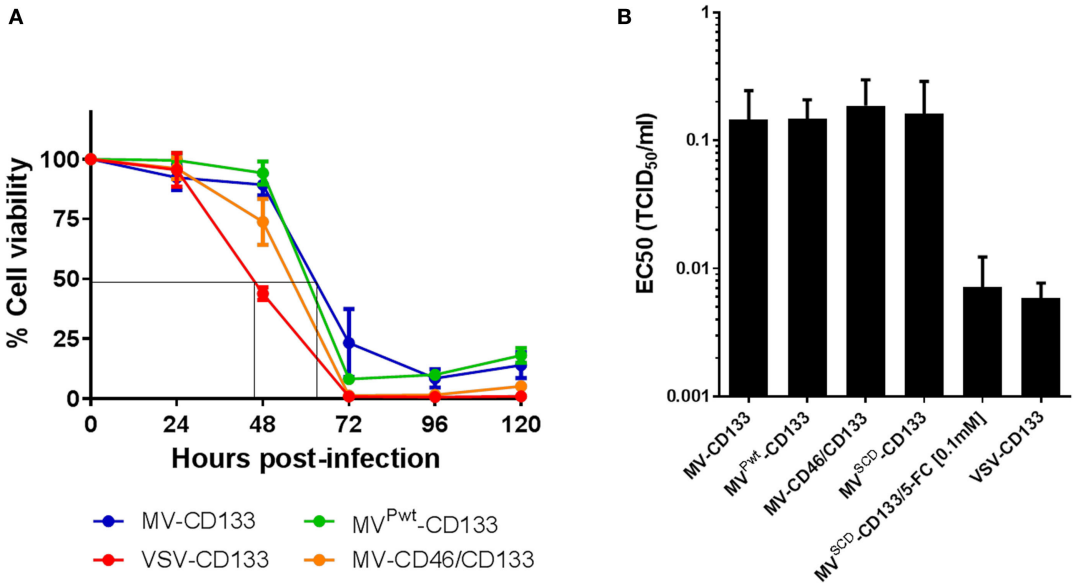

FIGURE 3 | Comparison of the *in vitro* killing performance of oncolytic viruses on Huh7 cells. Cells were infected with the indicated viruses, and cell viability was measured every 24 h postinfection by WST assays. **(A)** Cell viability after infection at an MOI of 1 at the indicated time points. Depicted is the percentage of living cells in relation to mock-infected control culture, which was set to 100%. The results are an average of two biological and four technical replicates. **(B)** EC_{50} values of the indicated viruses determined at 72 h postinfection relative to untreated controls. The results are an average of three biological and four technical replicates.

mice treated with VSV-CD133 had to be sacrificed much earlier than the control group (at day 8 post transplantation) due to severe peracute neurologic symptoms such as ataxia, tremor, or apathy.

To analyze the remaining stemness properties of the tumor cells present in the brains of sacrificed mice, we explanted some of the tumors and assayed the cells for sphere formation and proliferation. All samples derived from mock ($n = 4$), MV-CD133 ($n = 2$), and MV^{Pwt}-CD133 ($n = 4$) treated animals were able to produce spheres *ex vivo*, whereas this was the case for only three out of four tumor specimens of MV-CD46/CD133-treated animals ($n = 4$) and only half, i.e., two, of the tumors treated with MV^{SCD}-CD133 ($n = 4$). Moreover, there was a tendency for tumor cells that were removed from animals with longer survival times to require longer cultivation times to reach a defined cell number (**Figure 5B**). There were no signs for GFP expression in these cells that could point to a persistent infection with virus.

Direct Intracranial Application of the Oncolytic Viruses

We next evaluated the antitumoral activities of MV-CD46/CD133 and VSV-CD133, respectively, in the orthotopic glioma model in a clinically more relevant setup by direct intracranial injection of the viruses into pre-established tumors. 1×10^5 NCH644 cells were implanted intracranially into NOD-SCID mice. Five days later, 2×10^5 $TCID_{50}$ of the oncolytic viruses or PBS as control were stereotactically injected through the same hole, respectively. Mice treated with MV-CD46/CD133 revealed a tendency for longer survival (median survival = 28.5 days) over mock-treated animals (**Figure 6A**). All VSV-CD133-treated mice developed neurological symptoms within 15 days and thus had to be sacrificed earlier than PBS treated mice (**Figure 6A**). To assess a potential influence of the tumor cells on the observed neurotoxicity we intracerebrally injected VSV-CD133 or UV-irradiated VSV-CD133 into tumor-free mice. As control we included VSV-MV which does not use CD133 as entry receptor. Mice injected with UV-inactivated VSV-CD133 survived up to the end point of the study without developing any symptoms. In sharp contrast, animals from both other groups came down with neurological symptoms within two weeks (**Figure 6B**). This adverse event must thus have been caused by combining the MV glycoproteins with VSV but not by display of the CD133-specific scFv.

VSV-CD133 Is Superior to MV-CD133 in a Subcutaneous Xenograft Model

To test the oncolytic performance of the CD133-targeted viruses toward HCC, we established subcutaneous xenograft tumors in NOD/SCID mice using HuH7 cells. Initially, we intratumorally injected 1×10^6 $TCID_{50}$ of each virus in four administrations, respectively, once the tumor volume had reached 100 mm^3. Compared to mock-treated mice, all viruses reduced tumor growth, with VSV-CD133 being most effective (**Figure 7A**). Considering the area under the tumor growth curve (AUC) on day 40, by which all mice of the mock group had been sacrificed, the reduction in tumor growth was significant just for the VSV-CD133 treatment cohort (**Figure 7B**). This was also reflected by the Kaplan–Meier survival curves, which showed that treatment with VSV-CD133 resulted in the most pronounced survival benefit (**Figure 7C**).

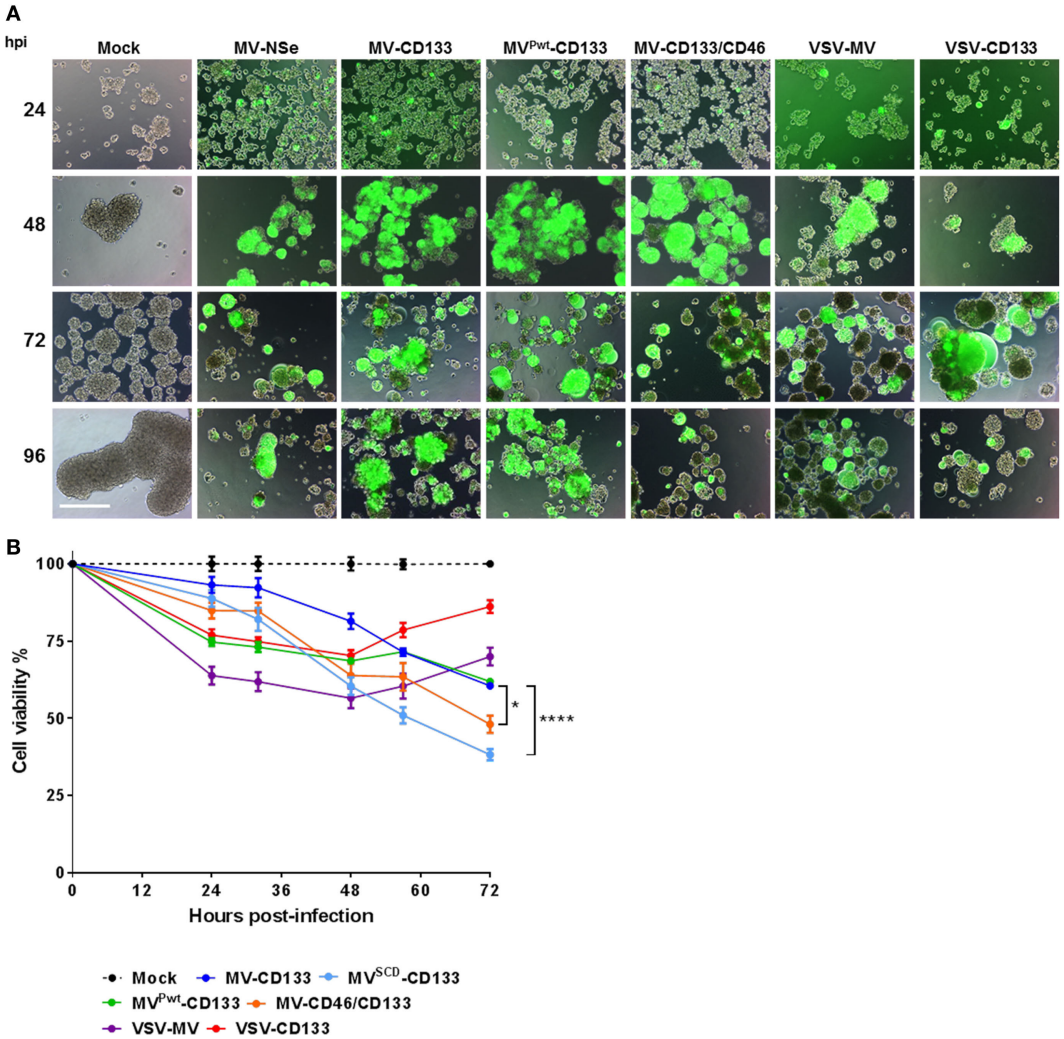

FIGURE 4 | Infection and killing of primary glioma spheres. Single cell suspensions of NCH644 cells were infected with the indicated viruses at an MOI of 1, respectively. **(A)** Cells were monitored microscopically for GFP expression up to 96 h postinfection. Scale bar, 500 µm. **(B)** Cell viability was determined using the RealTime-Glo MT Cell Viability assay twice a day for 72 h after virus addition. 1 mM 5-FC was added to MV^{SCD}-CD133-infected cells only, at time point 21 hpi. Average values of three independent killing assays are shown. One-way ANOVA with Dunnett's multiple comparison test, $^{*}P < 0.05$; $^{****}P < 0.0001$.

To quantify the intratumoral spread of VSV-CD133 or MV-CD133 we performed GFP immunostaining of a series of cryo-slices. Slices were prepared throughout two tumors of each virus-treatment group and of the mock-treated group as control by covering six different sites within each tumor, respectively (**Figure 8A**). There were no fluorescence signals detectable in slices from the mock group. For quantification of GFP-fluorescent areas, tile-by-tile acquisition of tumor cross-sections was performed using the AxioVision MosaiX software. In all viewed sections we observed a tremendous difference between tumors from VSV-CD133 and MV-CD133-treated mice (**Figure 8B**). Each slice from VSV-CD133-treated mice contained many GFP-positive spots, whereas more than half of the slices from MV-CD133-treated animals were GFP-negative. Taken all slices together, VSV-CD133 infected a 2×10^4 times larger area than MV-CD133 (**Figure 8C**).

After the intratumoral treatment setup, we performed a clinically more relevant approach by injecting VSV-CD133 or MV-CD133 intravenously into mice bearing subcutaneous HuH7 tumors. In this setting, both viruses prolonged survival, but significance was reached for the VSV-CD133 treatment only (**Figure 9**).

DISCUSSION

CD133-positive tumor cells represent a prime target for the development of novel cancer therapeutics. Conventional drugs as well as immunotherapies are under development including CD133-specific antibodies coupled to toxins, CD133-specific BITEs, or CD133-targeted chimeric antigen receptors (37–40). We have previously added oncolytic MV engineered to use CD133 as receptor for cell entry to this list (27). In the current

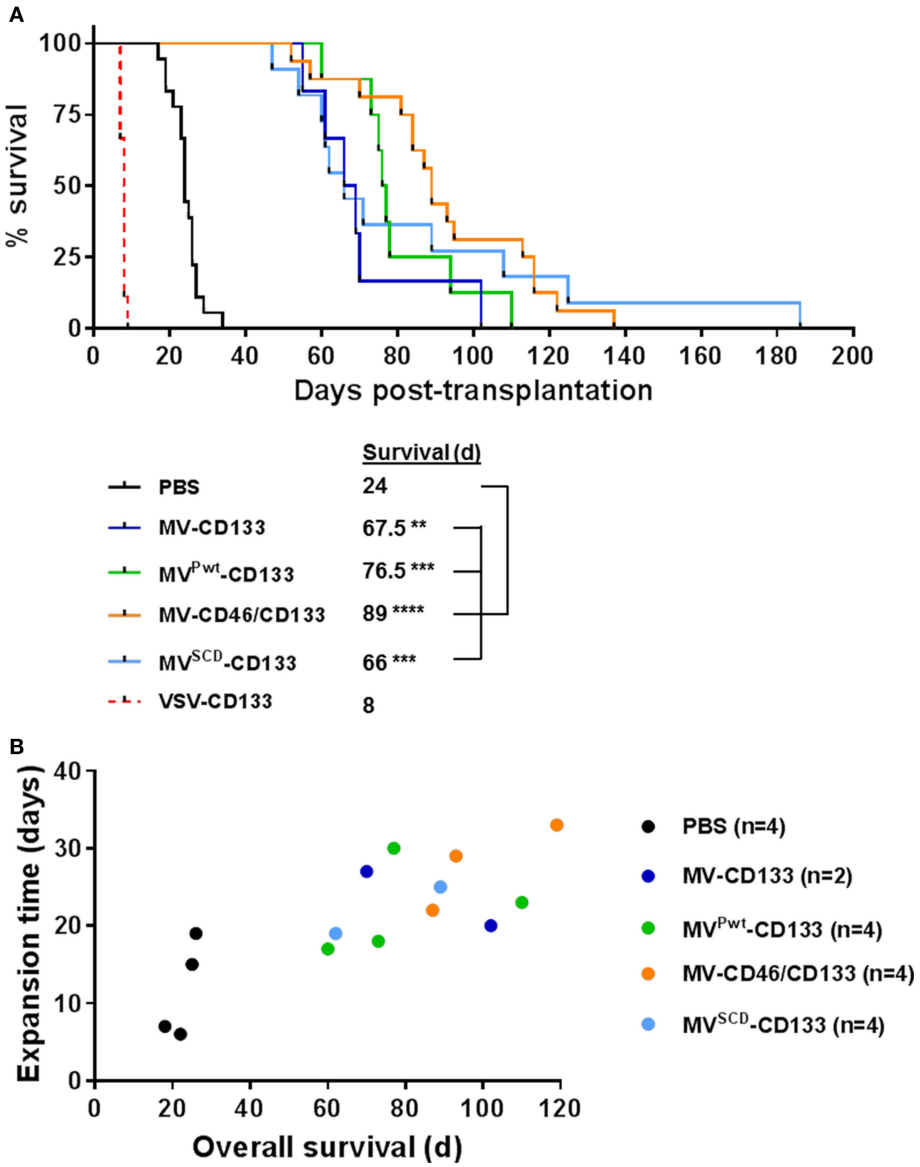

FIGURE 5 | Intracranial implantation of preinfected glioma spheres. Primary glioma spheres were infected with the indicated viruses at an MOI of 0.5 followed by stereotactically assisted implantation into the corpus striatum of NOD-SCID mice 16 h later. Mice having received MV^{SCD}-CD133-infected cells received 5-FC (200 mg/kg body weight) twice per day intraperitoneally initiated 3 day postimplantation for four consecutive days. **(A)** Health status and body weight were monitored daily. Mice were sacrificed with onset of neurological symptoms and/or weight loss of more than 20%. Based on the defined end points, a Kaplan–Meier survival curve was generated. Comparison of the mean survival periods between the mock control and the treatment groups was conducted by a log-rank test followed by a Bonferroni correction for multiple comparisons. Log-rank test, $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; PBS, $n = 18$; measles virus (MV)-CD133, $n = 6$; MV^{Pwt}-CD133, $n = 8$; MV-CD46/CD133, $n = 16$; MV^{SCD}-CD133, $n = 11$; vesicular stomatitis virus (VSV)-CD133, $n = 9$. **(B)** At the experimental endpoint, tumors were explanted and identical numbers of cells cultivated. The time required to expand to a cell count of 1×10^6 was plotted against the median survival time of the respective animal.

study we aimed at enhancing the oncolytic activity of MV-CD133 through arming either with the suicide gene SCD or the P gene from wild-type MV, through extending receptor usage from CD133-only to the natural MV receptors, and by transferring the CD133-targeted MV envelope to VSV. The later approach resulted in VSV-CD133, the by now second example of a fully replication-competent, receptor-targeted VSV. VSV-CD133 and all the other newly generated viruses entered cells *via* CD133 and were highly selective for CD133-positive cells with the exception of MV-CD46/CD133, which, as expected, also infected CD133-negative but MV-receptor positive cells.

We performed a careful side-by-side comparison of the whole panel of viruses toward their capability to lyse the hepatocellular carcinoma derived tumor cell line HuH7 or glioma derived tumor sphere cells, both *ex vivo* and *in vivo*. Since all viruses use the same surface protein for entry, the comparison was unbiased through

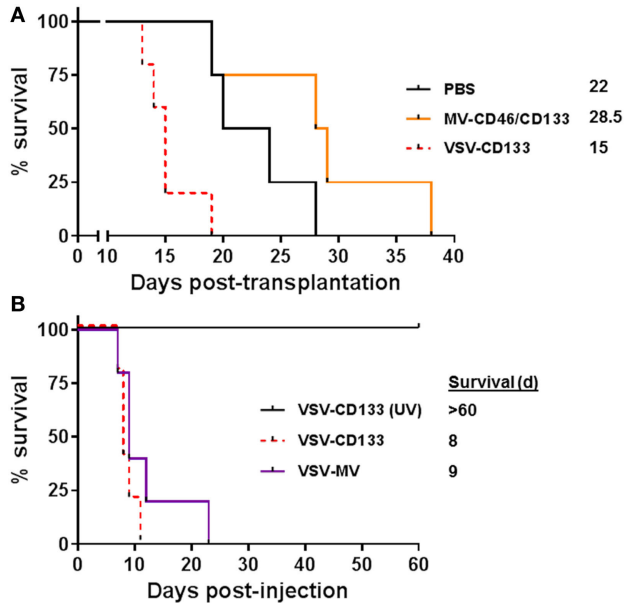

FIGURE 6 | Intracranial injection of oncolytic viruses. **(A)** 1×10^5 primary glioma sphere cells were stereotactically implanted into the corpus striatum of NOD-SCID mice. After 5 days, 2×10^5 $TCID_{50}$ of the indicated viruses in 5 µl PBS or PBS were stereotactically injected into the same coordinates. Health status and body weight were monitored daily. Mice were sacrificed with onset of neurological symptoms and/or loss of weight by more than 20%. Based on the defined end points Kaplan–Meier survival plots were generated. PBS, $n = 4$; measles virus (MV)-CD46/CD133, $n = 4$; vesicular stomatitis virus (VSV)-CD133, $n = 5$. **(B)** 2×10^5 $TCID_{50}$ of VSV-CD133, UV-inactivated (UV) VSV-CD133, or VSV-MV in 5 µl PBS, respectively, were stereotactically injected into the corpus striatum of NOD-SCID mice. Health status and body weight were monitored daily. Mice were sacrificed with onset of neurological symptoms and/or weight loss of more than 20%, $n = 5$.

usage of different receptors as it is usually the case when oncolytic viruses are being compared. Injection of the viruses resulted in reduced tumor growth, but not a complete elimination of tumor cells. Complete eliminations of tumors are in general rarely seen in preclinical models after single round treatments with oncolytic viruses, especially in immunodeficient mice, where an antitumoral immune response triggered by the virus infection cannot add to the oncolytic effect. Reasons for this can, e.g., be inaccessibility of the tumor cells, low virus dose or resistance against the virus in a subset of tumor cells. While in our system the latter could have occurred through loss of CD133 expression, also MV-CD46/CD133 with its expanded tropism was unable to completely clear tumors in the given setting, arguing against a major contribution of CD133 downregulation. Future experiments assessing multiple dosing cycles, the oncolytic activity in immunocompetent animal models and infection experiments with tumor cells re-isolated from treated animals will clarify this.

Interestingly, we identified different virus types as best option for each tumor type. On HuH7, VSV-CD133 was by far most efficient in infection and tumor cell lysis. This was also the case *in vivo* in subcutaneous HuH7 tumors. After intratumoral injection VSV-CD133 reached basically all parts of the tumor tissue thus covering an area more than 10^4 times larger than that reached by MV-CD133 during the same time period. This impressive capacity in spreading through tumor tissue, which was reflected by our observation that VSV-CD133 was also efficacious when injected intravenously, can be regarded as important property to reach and destroy CSCs in a clinical situation of HCC, where CD133-positive CSCs make up a low percentage of all cells present in tumor tissue (4, 41). In such a situation, it may be worth considering to also extend the receptor usage of VSV-CD133 to CD46, since our data show that MV-CD46/CD133 was superior over MV-CD133 in both tumor models. Being capable of using both these surface proteins as entry receptors should increase the likelihood to reach and infect rare CSCs.

The situation was different on glioma tumor spheres. Here, MV-CD46/CD133 and MV^{SCD}-CD133 turned out to be the most promising viruses. VSV-CD133 was more effective and more rapid in infection and cell killing *in vitro* only within the first 24 h, and even when used at an MOI of 10 was unable to kill more than about 60% of the cells. In fact, 2–3 days after infection tumor spheres started expanding again. The MV-derived viruses in contrast caused a continuous decrease in cell number, most efficiently with MV^{SCD}-CD133 and MV-CD46/CD133. It is rather unlikely that this was due to loss of CD133 expression. CD133-negative cells can indeed be killed by MV-CD46/CD133 or MV^{SCD}-CD133 because of CD46 receptor usage or the SCD-mediated bystander activity. However, NCH644 tumor spheres contain more than 90% CD133-positive cells and infection with VSV-MV, relying on CD46 for cell entry, showed the same recovery of the cells observed for VSV-CD133. It is therefore more likely that the cells became resistant through a postentry mechanism. Since VSV is known to be highly sensitive toward the IFN-mediated innate immune response, an intact type-I IFN response in some of the cells could be an explanation. However, absence of IFN-α in the supernatant of the infected cells rather argues for an alternative mechanism. This is further supported by the absence of any oncolytic advantage of the Pwt-armed MV^{Pwt}-CD133, which was expected to be especially pronounced in presence of a type-I IFN response. The resistance of NCH644 cells must therefore be the subject of further studies. Despite the negative results obtained for MV^{Pwt}-CD133 in our study, preclinical testing of this virus may still be worthwhile on other tumor types, especially those with an active type-I IFN system.

A postentry block triggered by the IFN system is most likely also the reason for the unimpaired differentiation of HSCs infected with the panel of viruses generated in our study into the hematopoietic lineages. Independently from the molecular mechanism, this is an important result with respect to the safety of CD133-targeted viruses. We had previously shown that HSCs appear to be protected from infection with MV-CD133 but did not exclude any influence on their differentiation capability (27).

An unexpected outcome of our study was the severe neurotoxicity exerted by VSV-CD133. All mice intracerebrally transplanted with glioma tumor spheres infected with VSV-CD133 came down with neurological symptoms within 10 days. VSV is indeed known for its distinct neurotoxicity. However, strategies have been developed to attenuate the virus to become applicable as oncolytic agent, and first clinical trials have been initiated (42).

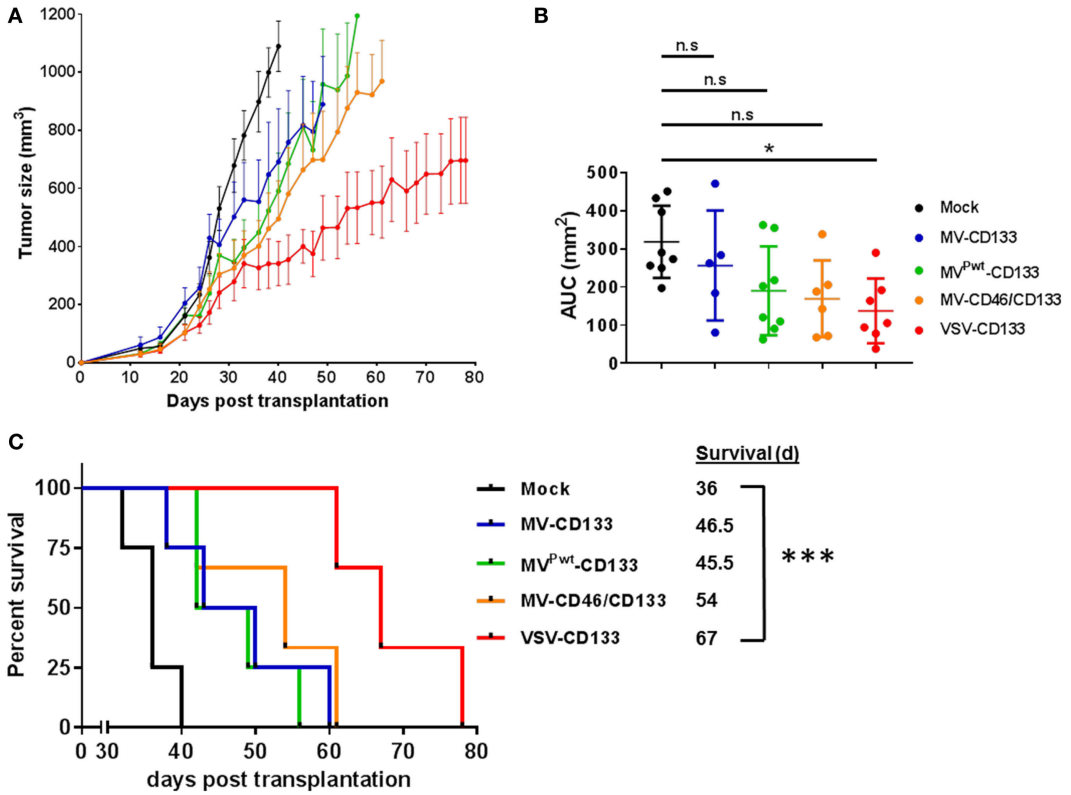

FIGURE 7 | Oncolytic activities of intratumorally applied oncolytic viruses in a subcutaneous xenograft model of hepatocellular carcinoma. HuH7 cells were implanted subcutaneously into the flank of NOD/SCID mice. Tumor size was monitored using a caliper. Intratumoral treatment was initiated at an approximate tumor volume of 100 mm^3 with four applications of 1×10^6 $TCID_{50}$ of each of the viruses on four consecutive days. Virus samples were blinded prior application, and the monitoring was carried out in a blinded fashion over the whole course of the study until sacrifice. **(A)** Tumor dimensions of each individual animal were measured twice a week. The mean tumor volume (mm^3) of each treatment group was calculated and plotted against the course of the observation point. **(B)** Group comparisons were performed by determining the area under the curve (AUC) for each individual animal normalized against the value obtained from the last survivor of the mock group (40 days after treatment). The values were plotted as box and whiskers. One-way ANOVA with Dunnett's multiple comparison test, $^*P < 0.05$. **(C)** Survival data were depicted as Kaplan–Meier survival curves. Comparison between the group with the lowest median survival (MV^{Pwt}-CD133) and the mock group was conducted by a log-rank test with Bonferroni adjustment for multiple comparisons. Mock, $n = 4$; measles virus (MV)-CD133, $n = 4$; MV^{Pwt}-CD133, $n = 4$; MV-CD46/CD133, $n = 3$; vesicular stomatitis virus (VSV)-CD133, $n = 3$. $^{***}P < 0.001$.

Our observation is especially astonishing, since the glycoprotein G had been identified as main neurotoxic component and mutating or exchanging it against envelope proteins of other viruses usually abolishes neurotoxicity (42–44). Neurotoxicity of vaccine strain-derived oncolytic MV has by now only been observed in CD46 transgenic and IFN receptor deficient mice after intracerebral injection (24, 45), while intracerebral injections into non-human primates did not reveal any signs of neurotoxicity (46).

The NOD/SCID mice we applied here do neither express primate CD46 that can be utilized by MV for cell entry, nor do they have a defect in innate defense although being B and T cell deficient. Moreover, VSV-CD133 is deficient in natural MV-receptor recognition and the displayed CD133-specific scFv only recognizes human CD133. Indeed, when we assessed VSV-MV, a non-targeted virus, in which VSV-G had been replaced by MV-F/H, the same extent of neurotoxicity was observed. Notably, there were no tumor cells present in this setting, thus excluding virus burst from preinfected spheres as potential trigger for the fatal neurological signs. First hints for neurotoxicity of VSV-MV were published by Ayala-Breton et al. (47). Here, however, virus was injected intravenously and resulted in neurotoxicity only when mice expressed human CD46 (47). Since we observed neurotoxicity in mice neither expressing human CD46 nor any of the other known MV receptors in their brains, neurotoxic infection must have been mediated either by an as of yet unidentified neuronal "receptor X" contacted by H (48), or a membrane fusion process that occurred independently from H protein receptor contact. Such a process has recently been suggested for F proteins found in AIDS patients suffering from MV-induced encephalitis (49). These F proteins, like those found in SSPE patients, however, carried mutations that enhanced their fusogenic activity (50). Since VSV-MV encodes the original F protein sequence, fusogenicity in this setting would have to be enhanced through being placed in the context of VSV. Indeed, absence of the MV matrix protein is known to result in enhanced cell-to-cell fusion activity of MV glycoprotein complexes (51) In addition, the much faster replication machinery and the apoptosis-inducing VSV matrix protein (M) likely contributed to the neurotoxicity observed with the VSV-MV chimeras.

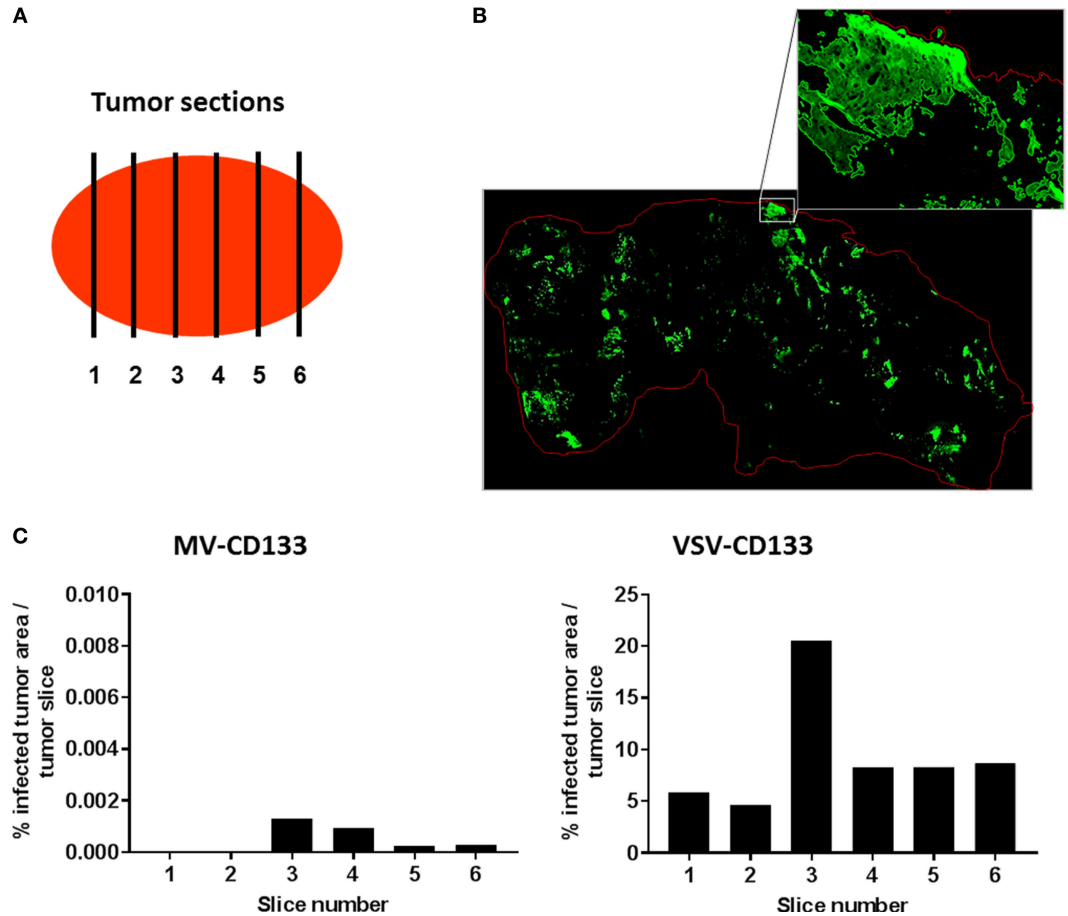

FIGURE 8 | Quantification of infected areas in explanted tumors. Cryosections of explanted tumors from vesicular stomatitis virus (VSV)-CD133 or measles virus (MV)-CD133-treated mice shown in **Figure 7** were immunostained against GFP to visualize virus infection centers. (**A**) Schematic illustration of the distribution of sections chosen to cover the complete tumor. (**B**) Representative overview image of a cross-section of a tumor from mice injected with VSV-CD133 (top) or MV-CD133 (bottom). Shown is a composite image that was generated by tile-by-tile acquisition using the AxioVision MosaiX software. The zoom-in represents the output image of one tile reconstructed by the CellProfiler software. The red line indicates the tumor border. (**C**) Bar graph showing the quantification of infected tumor area per slice of one tumor. The percentage of infected tumor area within the whole tumor area is plotted for each tumor site indicated in panel (**A**).

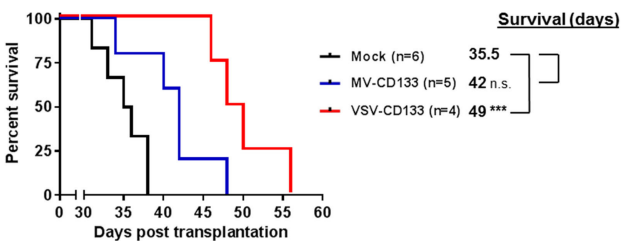

FIGURE 9 | Oncolytic activity of intravenously administered viruses in a subcutaneous xenograft model of hepatocellular carcinoma. HuH7 cells were implanted subcutaneously into the right flank of NOD/SCID mice. Intravenous injection of 1×10^6 $TCID_{50}$ of vesicular stomatitis virus (VSV)-CD133 ($n = 4$) or measles virus (MV)-CD133 ($n = 5$), respectively, or PBS as control (mock; $n = 6$) was initiated at an approximate tumor volume of 100 mm^3 and performed three times every second day. Survival data were depicted as Kaplan–Meier survival curves. Comparisons of both treatment groups with the mock group were conducted by a log-rank test with Bonferroni adjustment for multiple comparisons. ***$P < 0.001$.

With respect to translation into clinical applications, our data further underline that careful toxicity testing of chimeric VSV-MV viruses is an essential requirement for any new type of chimeric virus. For VSV-CD133, it will have to be assessed if the neurotoxicity we observed in NOD/SCID mice will be similarly severe in immunocompetent mice. That mice with a deficient innate immune system are especially prone to neurotoxicity caused by VSVs carrying heterologous envelope glycoproteins was recently observed for chimeras containing the glycoproteins of chikungunya or influenza virus (52). IFN-deficient mice are most sensitive toward VSV, even more than NOD/SCID mice, which were safe against a VSV-Lassa virus chimera in contrast to IFN-deficient mice (53). However, certain types of VSV chimeras can also be neurotoxic in immunocompetent mice as recently observed for VSVs carrying the Nipah virus glycoproteins (52). It is currently impossible to predict which combination may be crucial. Experimental testing is therefore unavoidable.

In this context, it is also important to stress that we did not see any signs of toxicity after systemic injection of VSV-CD133. The impressive spreading of VSV-CD133 in tumor tissue and the significant prolongation of survival after systemic administration therefore warrant further testing of VSV-CD133 toward applications in cancers of the gastrointestinal tract with CD133-positive CSCs being involved. Enhancing the safety of VSV-CD133 can for example be achieved by replacing its M gene by the MΔ51 variant (42). A negative outcome of a careful neurotoxicity testing provided, hepatocellular or pancreatic cancer could then be prime candidates (4).

With respect to glioblastoma, it will be worth further exploring the therapeutic activities of MV^{SCD}-CD133 and MV-CD46/CD133. As next step it will be important to assess the viruses on primary tumor material from patients suffering from these cancer types to find out if CSCs will be infected and potentially eliminated. Lysis of CSCs may, however, not be necessary. Only entering into CD133-positive CSCs and triggering a type-I interferon response may be sufficient to sensitize these cells for treatment by chemotherapy or radiation, or to induce their differentiation (54–56). Since vaccine strain-derived oncolytic MVs have lost at least part of their capacity to suppress IFN-responses and induce higher amounts of IFNs than wild-type strains (57), CSC-targeted MVs may be especially suited for such parallel modes of action.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the German animal protection law. The protocol with the reference number F107/1002 was approved by the Regierungspräsidium Darmstadt.

AUTHOR CONTRIBUTIONS

DK designed and performed experiments and contributed to the writing of the manuscript. JH performed experiments and S-KK provided advice. CA-B and K-WP were involved with the protocols and reagents. K-MH evaluated data. MM supplied reagents and contributed in the writing of the manuscript. AM and TA supervised work and performed experiments. CB conceived and designed the study, acquired grants, supervised work and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fonc.2017.00127/full#supplementary-material.

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Conflict of Interest Statement: CB and TA are inventors of a patent on tumor-stem cell targeted oncolytic viruses that has been out-licensed. All other authors declare no conflict of interest.

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The Oncolytic Virotherapy Era in Cancer Management: Prospects of Applying H-1 Parvovirus to Treat Blood and Solid Cancers

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Non-Hodgkin lymphoma (NHL) and leukemia are among the most common cancers worldwide. While the treatment of NHL/leukemia of B-cell origin has much progressed with the introduction of targeted therapies, few treatment standards have been established for T-NHL/leukemia. As presentation in both B- and T-NHL/leukemia patients is often aggressive and as prognosis for relapsed disease is especially dismal, this cancer entity poses major challenges and requires innovative therapeutic approaches. In clinical trials, oncolytic viruses (OVs) have been used against refractory multiple myeloma (MM). In preclinical settings, a number of OVs have demonstrated a remarkable ability to suppress various types of hematological cancers. Most studies dealing with this approach have used MM or B- or myeloid-cell-derived malignancies as models. Only a few describe susceptibility of T-cell lymphoma/leukemia to OV infection and killing. The rat H-1 parvovirus (H-1PV) is an OV with considerable promise as a novel therapeutic agent against both solid tumors (pancreatic cancer and glioblastoma) and hematological malignancies. The present perspective article builds on previous reports of H-1PV-driven regression of Burkitt's lymphoma xenografts and on unpublished observations demonstrating effective killing by H-1PV of cells from CHOP-resistant diffuse large B-cell lymphoma, cutaneous T-cell lymphoma, and T-cell acute lymphoblastic leukemia. On the basis of these studies, H-1PV is proposed for use as an adjuvant to (chemo)therapeutic regimens. Furthermore, in the light of a recently completed first parvovirus clinical trial in glioblastoma patients, the advantages of H-1PV for systemic application are discussed.

Keywords: oncolytic virotherapy, oncolytic H-1 parvovirus, glioblastoma, pancreatic ductal adenocarcinoma, oncolytic (parvo)virotherapy of hematological malignancies, diffuse large B-cell lymphoma, cutaneous T-cell lymphoma

Abbreviations: ALL, acute lymphoblastic leukemia; BL, Burkitt's lymphoma; OV(s), oncolytic virus(es); H-1PV, parvovirus H-1; PDAC, pancreatic ductal adenocarcinoma; MM, multiple myeloma; CV, coxsackievirus; VSV, vesicular stomatitis virus; NHL, non-Hodgkin lymphoma; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; DLBCL, diffuse large B-cell lymphoma; CTCL, cutaneous T-cell lymphoma; HDACi, histone deacetylase inhibitor.

INTRODUCTION

Viruses and Human Health, a Two-Edged Sword: Chronology of Virus Rehabilitation

1898: viruses are discovered as "minute living things capable of reproducing themselves." After the pioneering work of Adolf Eduard Mayer, Dmitri Ivanovsky, and Martinus Beijerinck, two German researchers, Friedrich Loeffler and Paul Frosch, were the first to contradict the "contagium vivum fluidum" (contagious living fluid) hypothesis to define a virus (the foot-and-mouth disease virus) as a tiny particle and to suggest that "the causative agents of numerous other infectious diseases of man and animals may also belong to this group of minute organisms" (1). Thus, at the beginning of the 20th century, the door opened to a new and exciting research area: virology.

Twentieth century: viruses as triggers of human infectious diseases. In the course of the 20th century and as predicted by Loeffler and Frosch, viruses were identified as the unquestionable causative agents of many human infectious diseases, from yellow fever (2), rabies (3), and poliomyelitis (4) to the acquired immunodeficiency syndrome (5). And this was not the end of the story: new disease-causing viruses, such as human bocaviruses, continue to emerge (6). It never rains but it pours…

Further bad news: viruses and human cancer. In addition to their vicious role as causative agents of numerous human infectious diseases, viruses are also involved in cancer development. This was first demonstrated at the beginning of the 20th century. Some 15–20% of all human cancers are attributed to viruses, notably Epstein–Barr virus, papilloma viruses, hepatitis B and C viruses, human herpesviruses, and human T-lymphotropic virus 1 (7). The molecular mechanisms underlying virus-induced carcinogenesis are diverse and complex. In addition to causing direct effects such as induction of genomic instability, DNA damage, and viral oncogene-triggered cell transformation (8, 9), oncogenic viruses can establish a chronic infection allowing them to escape from the host's immune system while producing proteins that control cell death and proliferation. Chronic infection also leads to inflammatory reactions promoting cancer development (10). In nasopharyngeal cancer, certain lymphomas, cervical cancer, liver cancer, Kaposi's sarcoma, and human adult T-cell leukemia/lymphoma, malignant transformation is likely to be initiated by host cell infection by an oncogenic virus. And yet…

Two sides to every coin: viruses have a bad side and an "oncolytic" side. Breakthrough observations at the start of the 20th century and findings peaking in the 1950s made it clear that "severe (virus) infections may on occasion favorably modify the course of far-advanced neoplastic disease…" (11). A significant drop in leukocyte counts associated with some clinical improvement was documented in children diagnosed with acute lymphoblast leukemia (ALL) having simultaneously acquired a varicella virus infection (11). At least five cases showing Hodgkin's disease regression after measles virus infection were described (12–14). Similar observations were made in patients having developed viral hepatitis during Hodgkin's lymphoma progression (15). In 1971, Bluming and Ziegler published a case report on Burkitt's lymphoma (BL) regression associated with measles virus infection (16). Today, more than a century after the first report on virus infection-associated clinical remission in cancer patients, virotherapy with oncolytic viruses (OVs) is the focus of a rapidly growing research field. Studies in this field have brought convincing evidence that oncolytic virotherapy, alone or in combination with surgery, chemotherapy, or radiotherapy, may significantly impact cancer mortality and improve patients' quality of life.

Oncolytic Viruses As Anticancer Tools: From Bench to Clinical Trials

Oncolytic viruses form a diverse biological group whose members belong to at least 10 different virus families, contain either an RNA or a DNA genome, and vary considerably as regards genome size, particle complexity, and natural host preferences (17). OVs naturally possess? or are engineered to acquire the capacity to selectively infect, replicate in, and destroy tumor cells (oncolysis) while sparing their normal counterparts (17, 18). Multiple factors explain this oncoselectivity: altered expression by tumor cells of virus entry receptors and/or intracellular permissiveness factors, rapid tumor cell division and high metabolic activity, deficient antiviral type I interferon responses in tumor cells, etc. (19). Furthermore, there is mounting evidence that OV infection of tumor cells induces an immunogenic process, with neo-antigen recognition and establishment of specific antitumor immune responses (20). The remarkable potential of OVs as cancer therapeutics has been well documented in a number of preclinical studies, and the resulting knowledge has been translated into an expanding wave of clinical trials (21, 22). In 2015, talimogene laherparepvec was the first OV to receive FDA approval as an anticancer drug (23) based on the fact that this herpes simplex virus type 1-based oncolytic immunotherapy has demonstrated therapeutic benefit against metastatic melanoma in a phase III clinical trial (24). In 2016, there were about 40 OV-based clinical trials recruiting cancer patients (19).

H-1 PARVOVIRUS (H-1PV) AGAINST PANCREATIC CANCER AND GLIOMA: THE BRAVE LITTLE CANCER FIGHTER

With a particle diameter of only 22 nm, the non-enveloped ssDNA-containing H-1 parvovirus is the smallest of the OVs. Its natural host is the rat. Humans are not naturally infected with H-1PV, no firm association between this virus and any human disease has been established, and no preexisting H-1PV immunity has been detected in the human population (25). Failure to observe any virus-related pathogenic effects in two early studies of H-1PV administration to human cancer patients (26, 27) prompted further therapy-oriented H-1PV research. Considerable preclinical evidence has accumulated over the last 30 years [reviewed in Ref. (28–30)] providing straightforward proof that H-1PV has broad oncosuppressive potential. In particular, pancreatic carcinoma and glioblastoma have attracted major attention as parvovirotherapy targets. In the respective preclinical models, efforts have been made to unravel the mechanisms and improve the efficacy of H-1PV treatment.

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor, often unresectable at the time of initial diagnosis. Median overall survival is only 6–9 months. As current therapies for PDAC patients fail to improve significantly their quality of life and to prolong survival (31), it is urgent to develop novel curative strategies. Extensive work by our team on H-1PV-based virotherapy for PDAC has yielded the following key findings: (i) H-1PV efficiently kills PDAC cells, including gemcitabine-resistant ones (32); (ii) H-1PV infection of pancreatic cancer cells results in active cathepsin B translocation to the cytosol (32) and in extracellular HMGB1 danger signaling (33); (iii) some predictive markers of PDAC permissiveness to H-1PV infection and lysis, e.g., SMAD4, have been identified (34); (iv) in an orthotopic PDAC model, H-1PV causes tumor regression and prolongs animal survival, without affecting bone marrow activity, liver function, or kidney function (32); (v) H-1PV-induced tumor suppression is potentiated under conditions of gemcitabine pretreatment (the current gold standard in pancreatic cancer therapy) (32); (vi) H-1PV oncosuppressive effects involve the participation of immune cells, which become activated either after an abortive infection with the virus (35) or through induction of immunogenic factors such as NK cytotoxicity receptor ligands (36) in H-1PV-infected PDAC cells; (vii) the vaccination potential of H-1PV, in combination with IFN-γ, extends to the treatment of peritoneal carcinomatosis, an untreatable condition traditionally managed with palliative measures only (37). Current preclinical achievements and prospects for pancreatic cancer parvovirotherapy are summarized in Ref (38, 39).

Glioma

Glioblastoma is the most aggressive human primary brain tumor. Life expectancy remains very poor, despite standard and alternative therapeutic attempts (40). Our team has shown that oncolytic H-1PV infection of human glioma cells results in efficient cell killing (41). High-grade glioma stem cell models are also permissive to lytic H-1PV infection (42). The cellular mechanism of virus-induced glioma cell killing has been elucidated and is based on active lysosomal cathepsin B translocation and accumulation in the cytosol of H-1PV-infected glioma cells but not normal cells (astrocytes) (43). Enhanced glioma cell killing has been observed when the virus was applied shortly after tumor cell irradiation, suggesting that this protocol might be translated to cases of non-resectable recurrent glioblastoma (44). In animal models, local, systemic, or intranasal administration of H-1PV has been found to cause regression of advanced tumors, virus replication being restricted to tumor tissues (45, 46). The favorable safety profile of local or systemic treatment with medical-grade GMP-produced H-1PV has been confirmed in a study using a permissive animal model (47, 48).

On the basis of the above preclinical evidence, the first phase I/IIa clinical trial (ParvOryx01) of an oncolytic parvovirus (H-1PV) in recurrent glioblastoma patients was launched in 2011 (49) and successfully completed in 2015. This trial, in addition to confirming the excellent safety and tolerability of H-1PV, yielded valuable observations, which strongly encourage further clinical development of this virus as an anticancer therapeutic. Particularly essential is the evidence suggesting that H-1PV (i) crosses the blood–brain barrier after systemic administration and (ii) may induce immunogenic conversion of the tumor microenvironment. In 2015, a second phase I/IIa trial was launched in inoperable metastatic PDAC patients. The outcome of this study is eagerly awaited.

Glioblastoma and pancreatic cancer are far from being the only tumor types sensitive to H-1PV-induced oncotoxicity, since it has also been demonstrated in preclinical models of breast, gastric, cervical (29), and colorectal (50, 51) cancer. H-1PV thus has the potential to treat not only brain and pancreatic but also a variety of other tumors.

ONCOLYTIC (PARVO)VIRUSES AGAINST HEMATOLOGICAL MALIGNANCIES

Preclinical Experience

Lymphoma and leukemia are the two cancer types tightly associated with the dawn of the oncolytic virotherapy era. Later, however, they were superseded as oncolytic virotherapy targets by solid tumors, such as breast, ovarian, bladder, skin, colon, and lung carcinomas. Nevertheless, a substantial set of preclinical data shows that several OVs can selectively lyse hematopoietic stem cells or downstream blood cell lineages (**Table 1**). As shown in the table, the predominant preclinical model is multiple myeloma (MM), followed by leukemia/lymphoma of B-lymphoid, myeloid, or T-lymphoid origin. Myxoma virus, a poxvirus whose natural tropism is restricted to European rabbits and is non-pathogenic for other vertebrates, has been demonstrated to selectively induce apoptotic death in MM cells (52–54). MM has also been successfully targeted by a double-deleted vaccinia virus (55), adenovirus

TABLE 1 | Oncolytic viruses (OVs) targeting hematological malignancies: preclinical evidence.

OV	Malignancy	Malignant cell type	Reference
DNA viruses			
Myxoma virus (*Poxviridae*)	MM, AML	Plasma, myeloid	(52–54)
Vaccinia virus (*Poxviridae*)	MM	Plasma	(55)
Adenovirus (*Adenoviridae*)	MM, lymphoma	Plasma, B-L	(56, 67, 68)
Herpes virus (*Herpesviridae*)	Lymphoma	B-L, T-L	(69)
RNA viruses			
CVA21 (*Picornaviridae*)	MM	Plasma	(57)
Reovirus (*Reoviridae*)	MM, lymphoma	Plasma, B-L	(58–60, 70)
VSV (*Rhabdoviridae*)	MM, AML, CLL	Plasma, myeloid	(61, 66, 71)
Measles virus (*Paramyxoviridae*)	MM, lymphoma, leukemia	Plasma, B-L, T-L	(62–65, 72–76, 87)
H-1PV (*Parvoviridae*)	Lymphoma, leukemia	B-L, T-L, myeloid	(77–79)

MM, multiple myeloma; AML, acute myeloid leukemia; B-L, B-lymphocyte; T-L, T-lymphocyte; CVA21, coxsackie virus A21; VSV, vesicular stomatitis virus; CLL, chronic lymphocytic leukemia.

serotype 5 (56), coxsackievirus A21 (57), reovirus (58–60), vesicular stomatitis (VSV) virus (61), and measles virus (62–65). Furthermore, myxoma and VSV infections are oncotoxic to acute myeloid leukemia cells (66), while adeno- (67, 68), herpes- (69), reo- (70), VSV (71), and measles virus (72–76) are reported to induce killing/suppression of B- and T-lymphoma or leukemia-derived cells/xenografts.

First proofs of the capacity of H-1PV to infect and destroy human blood cancer-derived cells date back to the 1980s, when Faisst et al. screened for H-1PV permissiveness and killing a panel of BL, adult T cell leukemia-derived, and *in vitro*-transformed lymphoblastoid cell lines (77, 78). Further proof-of-concept was provided by Angelova et al., who showed that African and European BL cells, including those lacking CD20 and hence resistant to the CD20-targeting therapeutic rituximab, are highly susceptible to H-1PV-induced killing, in contrast to normal B lymphocytes from healthy donors. In a SCID mouse lymphoma model, a single intratumoral H-1PV injection was sufficient to cause full tumor suppression and disease-free survival for the whole period of observation (70 days). This striking oncosuppression was observed even when the virus was applied late after tumor initiation, so as to mimic an advanced disease stage (79).

Clinical State of the Art

The rapid development of gene therapy and immune modulation approaches in recent years has led to greatly improving the management of many hematological cancer types. Several clinical trials are currently examining the effects of RNA interference, suicide gene therapy, and immune modulation in myeloma, lymphoma, and leukemia patients (80). In the development of new therapies, the most progress has been made in the treatment of B-cell leukemia/lymphoma. These account for over 80% of all non-Hodgkin lymphomas (NHL). The current standard treatment is a combination of the anti-CD20 antibody rituximab and chemotherapy, e.g., the CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) (81, 82). In contrast, NHL/leukemia of T-cell origin remains a therapeutic challenge, and treatment advances lag behind those for B-NHL. For example, treatment outcome is worse in pediatric T-ALL patients than in pediatric B-ALL patients (83). Adult T-ALL poses even greater treatment difficulties and no current option prolongs survival satisfactorily (84). In both B- and T-NHL/leukemia patients, outcomes of relapsed disease are usually dismal. Late effects and systemic toxicities related to conventional strategies (chemo- and radiotherapy) must also be considered. This spells out a continuing need for innovative approaches, especially for patients with relapsed B-NHL or newly diagnosed/relapsed T-NHL. Targeted therapies (85), immunotherapy (86), and oncolytic virotherapy have triggered growing interest and are the focus of much attention. Two OVs, the wild-type reovirus and an engineered measles virus, have successfully reached the clinical testing phase (87).[1,2]

In particular, a non-randomized phase I study conducted in Switzerland and involving cutaneous T-cell lymphoma (CTCL) patients with accessible lesions allowing intratumoral measles virus application has already yielded promising results as regards both the safety and efficacy of this OV treatment (87). One should note, however, that OV trials currently recruiting hematological cancer patients are restricted to refractory MM and that they are strikingly fewer than, for instance, melanoma or glioma OV trials. Given the promising preclinical data that demonstrate the potential of several other OVs to induce oncolytic effects in myeloid, B- and T-cell lymphoma/leukemia models, further clinical development of this anticancer approach is to be expected, and also hoped for, in the case of hematological malignancies. A recent study by Kishore and Kishor, comparing mortality rates between parvovirus-B19-infected and uninfected pediatric ALL patients has raised the intriguing hypothesis that natural B19 infection may exert unexplored oncolytic effects (88).

ONCOLYTIC H-1PV AS A CANDIDATE FOR FURTHER DEVELOPMENT IN ONCOHEMATOLOGY

After the first demonstration that H-1PV could induce efficient BL cell killing *in vitro* (77, 78) and BL regression in animal models (79), the question arose: might H-1PV be used against other types of hematological cancers? This question is of general interest, since BL is mostly seen in Uganda and Nigeria and is a rare condition outside Africa (89). It prompted us to conduct further studies to assess the capacity of this virus to target cells derived from other hematological malignancies. A panel of commercially available ATCC cell lines derived from aggressive or indolent lymphomas/leukemias of B- or T-cell origin was tested *in vitro* (A. Angelova, Z. Raykov, J. Rommelaere, unpublished data). First, encouraging results were obtained as shown in **Table 2**. Only one mixed type B-cell lymphoma and one Sézary syndrome CTCL were resistant to H-1PV-induced cell death. This resistance was associated with either the absence (Hut78 cells) or a low level (Farage cells) of progeny virion production and was not due to blockage of virus entry. In contrast, large B-cell-lymphoma-derived cells supported high levels of H-1PV progeny virion production and were almost totally eradicated by very low virus doses. Notably, DLBCL cell lines (e.g., Pfeiffer) with upregulated expression of aldehyde dehydrogenase 1A1 conferring CHOP resistance (90) were among the most sensitive H-1PV targets. These results suggest a potential use of H-1PV in chemoresistant DLBCL cases. Furthermore, H-1PV was able to replicate in T-ALL and some CTCL cells, with striking cytopathic effects. Although CTCL is a relatively rare condition, its incidence has increased about threefold over the last 2–3 decades in the United States (91) and in other regions of the world (92). Advanced disease stages with blood involvement require systemic therapies and, in general, the quality of life of CTCL patients is greatly affected. We are, therefore, now expanding the panel of *in vitro* models to test the antineoplastic potential of H-1PV in several, mostly T-cell-derived, types of hematological cancers, including CTCL. The failure of CHOP-based chemotherapies in CTCL patients has led to the development

[1] *Oncolytics Biotech Inc. – Clinical Trials*. Available from: http://www.oncolyticsbiotech.com/reolysin/clinical-trials/

[2] *ClinicalTrials.gov: Vaccine Therapy With or Without Cyclophosphamide in Treating Patients With Recurrent or Refractory Multiple Myeloma*. Available from: https://clinicaltrials.gov/ct2/show/NCT00450814

TABLE 2 | Responsiveness of lymphoma- and leukemia-derived cell lines to oncolytic H-1PV infection.

Cell line		Disease	H-1PV-induced killing/sensitivity[a]	H-1PV progeny virion production[b]
B-cell malignancies				
Farage	ATCC® CRL-2630™	B-lymphoblast NHL (mixed type)	Resistant	+
Toledo	ATCC® CRL-2631™	DLBCL	++	++
Pfeiffer	ATCC® CRL-2632™	DLBCL	+++	++
DB	ATCC® CRL-2289™	B-lymphoblast large cell lymphoma	+++	+++
RL	ATCC® CRL-2261™	B-lymphoblast NHL	+	++
T-cell malignancies				
CCRF-CEM	ATCC® CCL-119™	T-ALL	++	++
Loucy	ATCC® CRL-2629™	T-ALL	+	+
SUP-T1	ATCC® CRL-1942™	T-lymphoblast NHL	+	++
Hut78	ATCC® TIB-161™	CTCL (Sézary syndrome)	Resistant	No
HH	ATCC® CRL2105™	CTCL	+++	++
Myeloid malignancies				
HL-60	ATCC® CCL240™	Acute promyelocytic leukemia	++	+
Malignancies of undetermined cellular origin				
SR	ATCC® CRL-2262™	Large cell immunoblastic lymphoma	+++	n.a.

[a]*Sensitivity to H-1PV-induced killing is scored as +++, ++, and + when the virus dose required to cause death of 50% of the cells was <5, 5–10, or 10–50 plaque-forming units (pfu)/cell, respectively. Cells were considered "conditionally resistant" when the virus dose required to achieve 50% cell death exceeded 50 pfu/cell.*

[b]*The capacity for H-1PV progeny virion production was scored as +++, ++, or +, when the ratio of the virus titer 72 h postinfection to the titer 12 h postinfection was >100, 10–100, or <10, respectively.*

NHL, non-Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; T-ALL, T-cell acute lymphoblastic leukemia; CTCL, cutaneous T-cell lymphoma; n.a., not analyzed.

and subsequent FDA approval of two histone deacetylase inhibitors (HDACis), vorinostat and romidepsin (93–95). As patients often fail to reach or sustain a 50% partial response to these drugs, other agents have to be added in a combinatorial manner, in order to overcome resistance to HDACi (94). OVs, notably H-1PV, appear as potential candidates, as it was recently shown by Li et al. that another HDACi, valproic acid, when combined with oncolytic H-1PV, increases parvovirus-mediated cytotoxicity toward cervical and pancreatic cancer cells, thus resulting in synergistic killing (96). Further preclinical studies are worth conducting to determine whether these findings can be extended to CTCL and other clinically challenging T-cell malignancies such as T-ALL. Interestingly, it was recently reported that expression of the transcription factor TAL-1 (associated with poor prognosis in T-ALL) is markedly downregulated upon HDAC inhibition (97).

CONCLUSION AND PERSPECTIVE

In conclusion, oncolytic H-1PV has shown outstanding oncosuppressive activity in preclinical models of various solid tumors. Data from the first H-1PV clinical trial in recurrent glioblastoma patients have confirmed the excellent safety and tolerability of this virus upon local or systemic application. Accumulating preclinical evidence shows, furthermore, that H-1PV can efficiently kill, *via* productive infection, cancer cells derived from different hematological malignancies. These include both rituximab- and chemotherapy-resistant B-cell lymphomas and T-cell leukemia/lymphoma, which currently pose a major therapeutic challenge. These first results strongly encourage further preclinical studies aimed at substantiating the oncolytic and adjuvant potential of H-1PV against hematological cancers, both as single agent and as a component of combination treatments. These studies should pave the way toward innovative improvements of current standard therapies, for the benefit of chemotherapy-resistant and relapsing patients.

AUTHOR CONTRIBUTIONS

AA, MW-H, JR, and AG contributed to writing the present article. AA contributed to generating, analyzing, and presenting the unpublished data.

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REVIEW
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Genetically Engineered Vaccinia Viruses As Agents for Cancer Treatment, Imaging, and Transgene Delivery

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Despite advances in technology, the formidable challenge of treating cancer, especially if advanced, still remains with no significant improvement in survival rates, even with the most common forms of cancer. Oncolytic viral therapies have shown great promise for the treatment of various cancers, with the possible advantages of stronger treatment efficacy compared to conventional therapy due to higher tumor selectivity, and less toxicity. They are able to preferentially and selectively propagate in cancer cells, consequently destroying tumor tissue mainly *via* cell lysis, while leaving non-cancerous tissues unharmed. Several wild-type and genetically engineered vaccinia virus (VACV) strains have been tested in both preclinical and clinical trials with promising results. Greater understanding and advancements in molecular biology have enabled the generation of genetically engineered oncolytic viruses for safer and more efficacious treatment, including arming VACVs with cytokines and immunostimulatory molecules, anti-angiogenic agents, and enzyme prodrug therapy, in addition to combining VACVs with conventional external and systemic radiotherapy, chemotherapy, immunotherapy, and other virus strains. Furthermore, novel oncolytic vaccinia virus strains have been generated that express reporter genes for the tracking and imaging of viral therapy and monitoring of therapeutic response. Further study is needed to unlock VACVs' full potential as part of the future of cancer therapy.

Keywords: oncolytic viral therapy, vaccinia virus, molecular imaging, gene therapy

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INTRODUCTION

Replication-competent oncolytic viral therapies have shown great promise preclinically and in clinical trials for the treatment of various cancers. They are able to preferentially and selectively propagate in cancer cells, consequently destroying tumor tissue mainly *via* cell lysis, while leaving non-cancerous tissues unharmed (1). Oncolytic vaccinia virus (VACV) strains have been of particular interest due to several advantages, including large genomic capacity, fast and efficient replication,

Abbreviations: CT, computed tomography; GFP, green fluorescent protein; hNIS, human sodium iodide symporter; hNET, human norepinephrine transporter; hSSTR2, human somatostatin receptor 2; HSV1-tk, herpes simplex virus 1-thymidine kinase; ^{124}I, iodine-124; ^{125}I, iodine-125; ^{131}I, iodine-131; MIBG, meta-iodobenzylguanidine; MRI, magnetic resonance imaging; MSPT, multi-spectral tomography; PET, positron emission tomography; $^{99m}TcO_4$, 99m-technetium pertechnetate; SPECT, single photon emission computed tomography; VACV, vaccinia virus.

and impressive safety profile (2, 3). In this review, an overview of replication-competent oncolytic vaccinia viruses is presented, with particular focus on its potential for cancer treatment, imaging, and transgene delivery.

WHY VACCINIA FOR ONCOLYTIC VIRAL THERAPY

There are several advantages of using vaccinia virus as an agent for oncolytic viral therapy. VACVs' large 192-kb genome enables a large amount of foreign DNA to be incorporated without significantly reducing the replication efficiency of the virus, facilitating genetic engineering for safer attenuated viruses and transgene delivery (2). Cytoplasmic replication of the virus lessens the chance of recombination or integration of viral DNA into normal cells, and its DNA-based genome also makes it more stable than RNA-based viruses. It has been shown to be capable of evading the immune system and of infecting a wide variety of cells, enabling more effective systemic delivery. Perhaps most importantly, VACVs' safety profile after its use as a live vaccine in the World Health Organization's smallpox vaccination program in more than 200 million people makes it particularly attractive as an oncolytic agent and gene vector (3). Furthermore, vaccinia immunoglobulin and antiviral drugs are available if needed (4).

Vaccinia virus has a natural selectivity to tumors, with suggestion that leaky vasculature found in tumors being one of the major determinants of tropism (5, 6). It has also been shown that oncolytic viruses target cancers that overexpress proteins such as ribonucleotide reductase, DNA repair enzymes, and anti-apoptotic proteins; characteristics that tend to make tumor cells resistant to chemotherapy and radiation therapy (7, 8). Further selectivity of VACV has been achieved though the deletion of the thymidine kinase (TK) gene, involved in nucleotide synthesis, limiting viral replication to nucleotide rich cancer cells (6, 9–11). More investigation is needed to elucidate the exact mechanisms rendering vaccinia viruses highly selective and oncogenic in tumors, with more recent studies also utilizing microarray analysis and pathway analysis for further understanding (12).

In addition to their oncotropic and oncolytic effects, replication-selective vaccinia viruses can be used for transgene delivery to facilitate imaging of viral replication and enhance the probability of tumor eradication through multiple avenues (13). Replication-selective viral systems can employ endogenous viral gene expression control signals (promoter/enhancer, polyadenylation, and splice signals) for transgene expression. The use of endogenous viral promoters may also allow more predictable and controlled transgene expression (14).

HISTORY OF VACCINIA VIRUSES AS ONCOLYTIC VIROTHERAPIES

Levatidi's laboratory was the first to discover that VACVs were naturally oncolytic (15). Cassel and Garrett followed this by successfully treating murine malignant ascites (16). In a case report by M.D. Anderson, inadvertent administration of the vaccinia virus resulted in remission of chronic lymphocytic leukemia (CLL) in a patient (17). Another patient had remission of his CLL for more than 3 years, although becoming ill from his vaccinia vaccination and was successfully treated with immunoglobulin therapy (18). A different patient with multiple myeloma had a partial response after intravenous administration of vaccinia virus (19). Partial remissions have also been reported in patients with metastatic renal or pulmonary carcinomas (19, 20). These findings lead to several clinical trials including treating melanoma with a potent vaccinia virus encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) (21, 22). Ongoing clinical trials are discussed in the following sections in this review.

While early studies and trials were considered ground breaking, interest in viruses as anti-neoplastic therapies was abandoned due to unimpressive and short-lived success, as well as unacceptable side effects that ended some trials (23). It is only in the past 3 decades that the fervor of viruses as a strategy against cancer has been reignited with the advancement in scientific knowledge and technology. We now possess tools that enable us to develop more targeted and effective viruses (24).

DEVELOPMENT OF NEWER VACV GENERATIONS

Due to the advantages of vaccinia viruses, several preclinical trials have been performed in a variety of cancer origins. The use of oncolytic vaccinia viruses derived from several strains, including WR, LIVP, Wyeth, Copenhagen, revealed that WR-derived strains were able to colonize tumors in human xenografts in nude as well as syngeneic tumors in immunocompetent wild-type animals (25, 26).

Greater understanding and advancements in molecular biology have enabled development of a generation of genetically engineered oncolytic viruses for safer and more efficacious treatment. One of the earliest examples of the development and use of a recombinant VACV was given by Timiryasova and colleagues, who investigated the use of VACV and recombinant derivatives, recVV2, rVV-p53, on the growth of C6 rat glioma cells in an athymic nude mice model. They found that VACV effectively infected C6 cells *in vitro*, inducing high level of foreign gene expression, including rW-p53-mediated expression of the tumor suppressor p53 protein. In C6-implanted nude mice, injection of VACV or rVV-p53 induced effective inhibition of tumor growth in comparison to control groups, with a greater effect with rVV-p53, apparently due to overexpressed p53 and p53-mediated cell apoptosis. These results, and others, paved the way for the use of vaccinia-mediated delivery of therapeutic genes represent novel potential strategies for tumor therapy (27). Since then, the successful use of vaccinia virus as an oncolytic agent has been so far published in at least 50 human tumor models (**Table 1**). Moreover, systemic treatment with vaccinia virus was shown to reduce metastatic burden, demonstrated with an aggressive PC-3 prostate cancer model (28) and in rabbits bearing VX2 liver tumors (29, 30).

Several strategies have been investigated with vaccinia viruses for the treatment of human cancers, including arming VACVs with immunostimulatory molecules and anti-angiogenic agents, utilizing VACVs as delivery agents for targeted enzyme prodrug

TABLE 1 | Vaccinia virotherapy in preclinical human tumor models.

Vaccinia virus	Tumor type	Tumor model	Reference
LIVP	Prostate	PC-3	(31, 32)
		DU-145	(30–32)
	Pancreatic	Mia-Paca2	(30, 32–34)
		PANC-1	(32, 33, 35)
		Suit-2	(36)
	Breast	GI-101A	(32, 37)
	Lung	A549	(30, 32)
	SCC	MSKQLL2	(38)
	Mesothelioma	MSTO-211H	(39)
	Thyroid	8505C	(40)
		DRO90-1	(40)
	Ovarian	OVCAR-3	(41)
		ES2	(42)
	Melanoma	1858-MEL	(41)
		888-MEL	(41)
WR	Renal	786-O	(43)
		ACHN	(43)
		769P	(43)
		Renca	(43)
	Multiple myeloma	My5	(44)
		RPMI8226	(44)
	Colorectal	HCT116	(29, 45)
	Ovarian	HT29	(46)
		UCI-101	(47)
		SKOV-3	(47)
		A2780	(48, 49)
WR (vvDD)	Brain	U87MG	(50)
		U118	(50)
Copenhagen	Colorectal	LoVo	(51)

therapy and reporter gene expression for imaging and combining VACVs with other cancer treatments including immune-, chemo- and radiotherapy (**Figure 1**).

VACVs Armed with Cytokines/ Immunostimulatory Molecules

Combining viral therapy with cytokines has been attempted with the aim of harnessing the host's own immunity to assist tumor rejection and destruction. One of the earliest examples of this was the development of vaccinia virus strains encoding human GM-CSF, JX-549, and JX-963, which were shown to enhance antitumor immunity due to the expression of the GM-CSF transgene *in situ* (46, 52). Direct oncolysis plus GM-CSF expression stimulated the shutdown of tumor vasculature and antitumoral immunity, significantly reducing tumor burden and increasing median survival. Tumor-specific virus replication and gene expression, systemically detectable levels of GM-CSF, and tumor-infiltrating cytotoxic T-cells (CTLs) as well as significant increases in neutrophil, monocyte, and basophil concentrations in the peripheral blood were also demonstrated.

Vaccinia expressing co-stimulatory cytokines have even also been shown to help overcome the tumor microenvironment's immune suppressive characteristics. The melanoma microenvironment in particular leads to local T-cell tolerance in part through down-regulation of co-stimulatory molecules, such as B7.1 (CD80). A 2-dose-escalation phase I clinical trial was conducted with 12 patients using a recombinant vaccinia virus expressing B7.1 for monthly IT vaccination of accessible melanoma lesions. The approach was well tolerated with only low-grade fever,

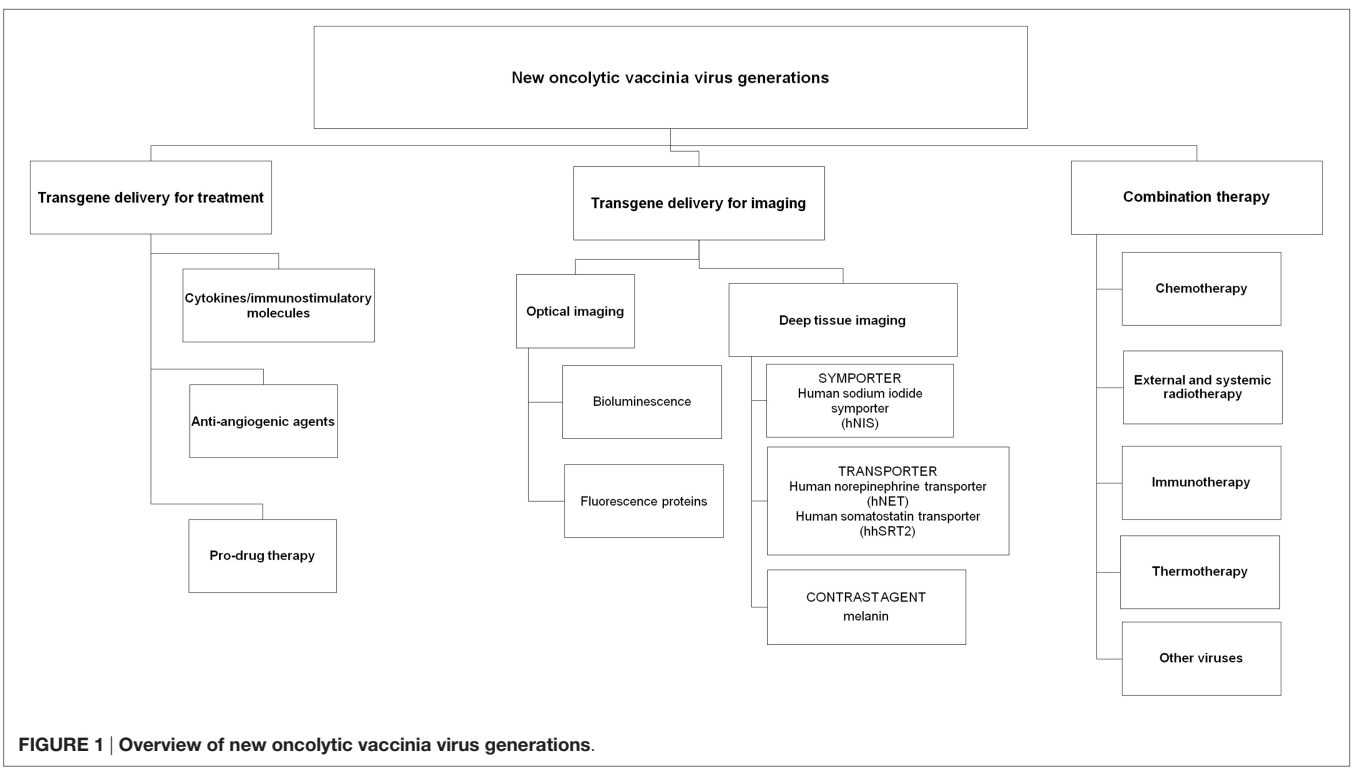

FIGURE 1 | Overview of new oncolytic vaccinia virus generations.

myalgias, and fatigue reported, with two patients experiencing vitiligo (28). An objective partial response was observed in one patient and disease stabilization in two patients, one of whom was alive without disease 59 months following vaccination. All patients demonstrated an increase in post-vaccination antibody and T-cell responses against vaccinia virus.

Cytokines have also been utilized to increase the tumor selectivity. Kirn et al. developed a vaccinia virus strain expressing the cytokine IFN-β, JX-795, which is incapable of responding to this cytokine to have the dual benefits as a cancer therapeutic with increased anticancer effects and enhanced virus inactivation in normal tissues (29). The virus was based on a vaccinia B18R deletion mutant backbone for IFN-β expression, as the B18R gene product neutralizes secreted type-I IFNs. JX-795 had superior tumor selectivity and systemic efficacy when compared to the TK-/B18R- control or wild-type vaccinia in preclinical models. The authors concluded that by combining IFN-dependent cancer selectivity with IFN-β expression to optimize both anticancer effects and normal tissue antiviral effects, tumor-specific VIRAL replication, IFN-β gene expression, and treatment efficacy were achieved following systemic delivery in preclinical models.

VACV Delivering Anti-Angiogenic Agents

Further improvement of oncolytic potential was studied by attempting to inhibit tumor vasculature *via* expression of an endostatin/angiostatin fusion gene, targeting the vasculature endothelial growth factor (VEGF) (30, 36, 43). VEGF binds to specific receptors on epithelial cells and is a major player in tumor angiogenesis. Inhibition of VEGF has been extensively studied in several cancer models (43, 53–56), with Avastin being one of the most successful immunotherapeutic proteins to date. This drug has been approved by the US Food and Drug Administration (FDA) for use in combination with chemotherapy for the treatment of metastatic colorectal cancer and most forms of metastatic non-small cell lung cancer (57, 58). Vaccinia-mediated blocking of VEGF was achieved by either fusing the VEGF receptor 1 to the Fc tail of human IgG antibody (VEGFR-1-Ig) or secretion of a single-chain antibody (GLAF-1) to VEGF. In both cases, VEGF was bound and thus prevented interaction to its natural receptors on endothelial cells resulting in lower blood vessel densities within the tumor tissue. The reduced tumor vascularity was accompanied by faster regression of tumors; although in one study, this depended on the dose of virus injected (43). In the same study, the VEGFR-1-Ig encoding vaccinia virus strain was found to be more lethal to mice than the parental strain. For the GLAF-1 encoding virus strains, no changes in toxicity were described.

Use of VACVs in Gene-Directed Enzyme Prodrug Therapy

Another approach to enhance the oncolytic effects caused by vaccinia virus strains is the so-called gene-directed enzyme prodrug therapy (GDEPT). In this article, a relatively non-toxic prodrug is enzymatically converted to toxic drugs which result in killing of the enzyme-producing tumor cells. Moreover, the so-called bystander effect caused by diffusion of the drug into neighboring cells results in killing of cells in close proximity to the enzyme-producing cell even if they were not made to express the prodrug converting protein.

The most prominent enzyme type in vaccinia virus-mediated GDEPT is cytosine deaminase, which is absent in mammalian cells and used in combination with 5-fluorocytosine (10, 48, 51, 59, 60). This prodrug is converted to 5-fluorouracil, whereby the efficiencies depend on the specific cytosine deaminase (e.g., bacterial and fungal) and the presence of uracil phosphoribosyltransferase (61, 62). When using this system in combination with oncolytic vaccinia virus strains, the reported results indicate better therapeutic effects when compared to the oncolytic virus alone. However, the therapeutic benefit was expected to be higher (48). In other studies, similar results were found when using a β-galactosidase-expressing vaccinia virus strain in combination with an inducible prodrug seco-analog of duocarmycin SA (63). Several reasons might be responsible for these observations: first, the rapid kinetics of oncolytic vaccinia virus replication might functionally overlap with the used prodrug system; and second, the administration of prodrug may have inhibited the viral replication, thus reducing the antitumoral cytotoxicity induced by the oncolytic virus itself. This effect has already been reported by McCart et al. (64) but was not observed in all prodrug systems (63). Different dosing schemes or other GDEPT systems should still be considered and might cause stronger synergistic effects between the oncolytic virus strain and prodrug therapy.

VACCINIA VIRUSES FOR CANCER IMAGING

Oncolytic vaccinia virotherapy has shown success in preclinical trials and much promise in completed and ongoing human clinical trials. However, biopsy is the current gold standard for monitoring the therapeutic effects of viral oncolysis (65). This may be feasible in preclinical trials, or early clinical trials; however, a non-invasive test facilitating ongoing monitoring of therapy is needed for human studies (66). This would enable the assessment of the biodistribution of oncolytic viruses to ensure safety and correlation with treatment efficacy, as well as the potential for a more sensitive and specific diagnostic technique to detect tumor origin and, more importantly, the presence of metastases (67).

Consequently, novel oncolytic vaccinia virus strains have been generated that express reporter genes such as green fluorescent protein (*GFP*), *RLuc* for optical imaging, and the human somatostatin receptor type 2, the human norepinephrine transporter (*hNET*), and the human sodium iodide symporter (*hNIS*), which selectively bind radiotracers and therefore should also be detectable in deep tissues of humans (35, 59).

Several non-invasive imaging methods are already in clinical use, including optical methods using fluorescence and bioluminescence, as well as deep tissue imaging modalities utilizing instrumentation such as positron emission tomography (PET) and single photon emission computed tomography (SPECT).

Optical Imaging

Optical detection methods such as fluorescence and bioluminescence have the advantage of short acquisition times

(for fluorescence imaging, few milliseconds to several seconds, and for bioluminescence, a few seconds to several minutes), and high spatial resolution. The major disadvantage of optical imaging is the inability to perform deep tissue imaging due to autofluorescence, light scattering, and the opacity of tissues to light below 600 nm due to absorbance by hemoglobin. Nevertheless, optical imaging in small animals has been and still is a very important tool to follow the distribution of oncolytic vaccinia viruses equipped with genes for luciferases (25, 26, 36, 42, 46, 47, 68–70) or fluorescent proteins such as GFPs (26, 31, 33, 37, 39, 44, 47, 50, 68, 70). Moreover, a GFP encoding vaccinia virus strain, GLV-1h68, is currently in clinical phase I and II trials in which this fluorescent protein can be used to monitor the colonization of near-surface tumors and metastases (71). The discovery of new fluorescent proteins in the near-infrared spectrum will probably result in the ability to detect oncolytic viruses in somewhat deeper tissues (72).

Deep Tissue Imaging

In contrast to optical imaging, deep tissue imaging modalities can be used for non-invasive deep tissue imaging utilizing radiotracers with differing properties. These radiotracer imaging technologies are able to measure the distribution of radiotracers in the human body (73). They are widely available and have a wide range of clinical and research applications. Two classes of clinical nuclear imaging systems exist: those designed to image single gamma-emitting radionuclides such as 99m-technetium pertechnetate ($^{99m}TcO_4$) and Iodine-131 (^{131}I) and those designed to image positron-emitting radionuclides such as fluorine-18, carbon-11, and Iodine-124 (^{124}I). The single gamma-emitting imaging system is referred to as single photon imaging or, when performed tomographically, single photon emission computed tomography (SPECT). The positron-emitting imaging system is known as PET. PET has greater spatial resolution and higher sensitivity and is easier to quantify than SPECT.

Viral gene expression during the lytic phase of the viral life cycle of vaccinia virus is highly regulated and can be broadly classified into three serially activated phases: immediate-early (IE), early (E), and late (L) (14). Based on the expression of endogenous viral genes, it may be possible to predict the expression kinetics (timing and expression levels) of the transgene(s) carried by the replicating agent. Furthermore, when multiple transgenes are inserted into a single virus, their expression may be orchestrated to occur simultaneously or serially, at levels that will maximize their therapeutic benefit. Expressing transgenes serially at different times in the viral lytic cycle is of greatest value early in treatment when the infection may be more synchronized. As a viral infection spreads and encounters a heterogeneous tumor cell mass, it will likely become asynchronous, although the relative expression of different transgenes may still be maintained.

Human Somatostatin Receptor 2 (SSTR2)

The SSTR2 is targeted by the high-affinity synthetic peptide pentetreotide, which is commonly used for receptor imaging after being radiolabeled with indium-111 (74). This receptor is expressed in normal human kidney cells and neuroendocrine tumors, and gene therapy approaches have also been attempted to deliver the SSTR2 to non-expressing tumors using adenoviral vectors (74, 75). In a study by McCart et al., nude mice bearing subcutaneous murine colon CA xenografts were injected intraperitoneally with an *SSTR2*-expressing VACV or control and imaged 6 days later with ^{111}In-pentetreotide-mediated SPECT. Tumors infected with the SSTR2-expressing VACV accumulated higher concentrations of radioactivity compared to tumors in animals receiving the control virus. Further, SSTR2-infected tumors were visible on imaging 6 days after VACV injection and could be visualized for up to 3 weeks post viral injection using repeat radiotracer injections (59). Limitations of the SSRT2 receptor are that radiotracers for SSRT2 require prior radiolabeling for accumulation of radioprobes and the 1:1 binding relationship with radiolabeled limiting signal amplification.

Human Norepinephrine Transporter

Another deep tissue reporter gene investigated in oncolytic viral strains is the *hNET*. hNET is a cell surface human protein mediating the transport of norepinephrine, dopamine, and epinephrine across the cell membrane. It can be imaged by SPECT or PET using the radiotracer meta-iodobenzylguanidine (MIBG) (76, 77). The use of the hNET-MIBG reporter imaging is attractive since it is of human origin and will unlikely induce an immune response, as well as its limited expression in the central and peripheral sympathetic nervous systems (78). An oncolytic vaccinia virus carrying *hNET*, GLV-1h99 derived from GLV-1h68, mediated the expression of the hNET protein on the cell surface of infected tumor cells, resulting in specific uptake of the radiotracer [^{131}I]-MIBG (35). In mice, GLV-1h99-infected pancreatic tumors were readily imaged by [^{124}I]-MIBG-PET. This virus further mediated imaging of an orthotopic mouse model of human malignant mesothelioma using both ^{123}I-MIBG-mediated SPECT imaging and ^{124}I-MIBG-mediated PET imaging (79).

Human Sodium Iodide Symporter

The *hNIS* is an intrinsic plasma membrane protein which mediates the active transport and concentration of iodide in the thyroid gland cells and some extra thyroidal tissues, in particular, the lactating mammary gland, as well as in the stomach, salivary glands, skin, brain, spleen, small intestine, ovaries, prostate, and testes (80).

hNIS gene transfer *via* viral vector may allow infected tumor cells to concentrate several easily attainable, commercially available, and relatively inexpensive, carrier-free radioisotopes such as ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{99m}TcO_4$, rhenium, and astatine for non-invasive imaging of *NIS* expression, all of which have long been approved for human use. The first vaccinia virus carrying the hNIS was GLV-1h153, also a derivative of GLV-1h68 (81). The virus also encoded for the *GFP* and the *RLuc* genes, and it was found to be successful in fluorescence, bioluminescent, and deep tissue image monitoring of viral replication and therapy (**Figure 2**) (82, 83). Moreover, GLV-1h153 successfully regressed several tumor types in preclinical models including pancreatic cancer, triple negative breast cancer, gastric cancer, malignant pleural mesothelioma, and most recently, prostate cancer (84–87).

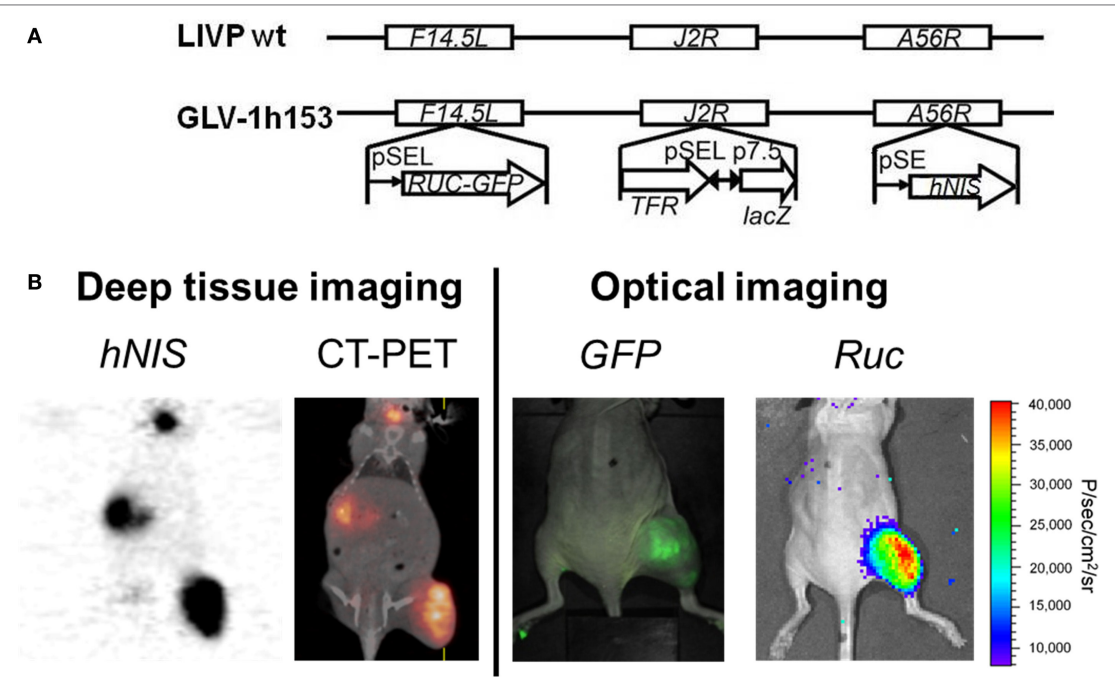

FIGURE 2 | **Molecular imaging of oncolytic vaccinia virus GLV-1h153**. **(A)** GLV-1h153 construct. GLV-1h153 was derived from LIVP-wt virus, by replacing the gusA expression cassette at the A56R locus with the human sodium iodide symporter (hNIS) expression cassette through homologous recombination. The virus also contains RUC-green fluorescent protein (GFP) and lacZ expression cassettes at the F14.5L and J2R loci, respectively. PE, PE/L, P11, and P7.5 are the vaccinia virus synthetic early, synthetic early/late, 11K, and 7.5K promoters, respectively. TFR is a human transferrin receptor inserted in the reverse orientation with respect to the promoter PE/L. **(B)** GFP, bioluminescence, and hNIS signal could be detected in GLV-1h153-infected tumors. Fusion of PET and CT images correlated hNIS-mediated uptake signal anatomically to location of thyroid and stomach due to intrinsic hNIS expression, bladder due to radiotracer excretion, and tumor due to virus-mediated hNIS expression. Virally-mediated GFP and bioluminescence signals located only to tumor, demonstrating tumor-specific viral replication.

Melanin

A recent study by Stritzker et al. explored the use of a VACV encoding for the production of a contrast agent, melanin (88). The oncolytic virus-mediated production of melanin and its optical absorption in the near-IR spectrum enabled the imaging of A549 tumors and metastases *via* the utilization of magnetic resonance imaging and multi-spectral tomography. The ubiquitous presence of melanin in all kingdoms of life suggests that the introduction of melanin synthesis as a diagnostic and theranostic marker is possible in most species.

COMBINATION THERAPIES WITH VACVS

Although the therapeutic effect of vaccinia virus shows promise, combining conventional therapies may enhance oncolytic viral treatment and help circumvent the immune system for optimal delivery of viruses to tumors.

Chemotherapy

Combination of oncolytic vaccinia virus with classical chemotherapeutic agents such as gemcitabine and cisplatin led to accelerated tumor size reduction compared to monotherapy using VACV alone (89, 90). At the same time, each of the chemotherapeutics could only slow down tumor growth but did not result in complete tumor regression. For example, combination treatment with VACV GLV-1h68 and cyclophosphamide significantly improved the antitumor efficacy of GLV-1h68 and led to an increased viral distribution within the tumors (89). Pro-inflammatory cytokines and chemokines were distinctly elevated in tumors of GLV-1h68-treated mice. Factors expressed by endothelial cells or present in the blood were decreased after combination treatment. A complete loss in the hemorrhagic phenotype of the PC14PE6-RFP tumors and a decrease in the number of blood vessels after combination treatment could be observed.

In another study by Ottolino-Perry and colleagues, a VACV expressed the human somatostatin receptor and red fluorescent protein, vvDD-SR-RFP, with oxaliplatin or SN-38 (active metabolite of irinotecan) in colorectal cancer cell lines *in vitro* (91). Utilizing the Choui–Talalay method for determining drug–drug interactions, they were able to show that combination therapy induced additive and synergistic effects in different cell lines, which also depended on doses of treatment utilized. The VACV was then combined with irinotecan in an orthotopic model of metastatic colorectal cancer. Combination therapy was well tolerated in tumor-bearing mice, with a significant increase in the median survival compared to control groups, including either treatment alone. Increased apoptosis following combination therapy was also observed. Combination of oncolytic VACV with other chemotherapeutic agents in future studies will provide useful data as to which combination therapies are best suited for each type of cancer.

Radiation Therapy

Radiation therapy has also been explored as a possible strategy against malignancies in combination with oncolytic viral therapy (90, 92, 93). Radiotherapy can either be local in the form of external beam radiation therapy (EBRT), or systemically administered. OVs may act as radiosensitizers by affecting pathways that render tumors resistant to treatment. Further, the selective cytotoxicity of viruses to tumors may enable more targeted radiotherapeutic strategies especially with systemically administered radiotherapies. In preclinical models, the combination of VACV and radiotherapy significantly delayed tumor growth and prolonged survival compared to single agent therapy in several cancers such as prostate and sarcoma (94–96), with data suggesting that virally mediated down-regulation of anti-apoptotic proteins may increase the sensitivity of tumor cells to the cytotoxic effects of ionizing radiation (96).

Vaccinia viruses encoding transporter genes such as *hNIS* have also been found to have a synergistic antitumor effect when combined with systemic ionizing radiation, such as ^{131}I (85, 97). One mechanism for such synergy appears to be radiation-induced upregulation of certain cellular DNA repair genes that result in promoting viral replication (7, 98). Furthermore, a bystander effect may be possible as ^{131}I undergoes alpha particle decay with a path length of 0.2–2.4 mm (99). If additive or synergistic effect is found, patients may be more safely treated with combinations of lower doses of virus and radioiodine. Application of carrier-free radioiodine would thus be extended, and the extensive experience with radioiodine in thyroid cancer management will undoubtedly be helpful in the treatment of other *NIS*-transfected tumors. Our laboratory demonstrated an enhanced effect of oncolytic viral therapy with GLV-1h153 when combined with radiotherapy ^{131}I in both pancreatic and breast cancer xenografts (97).

Immunotherapy

The mechanisms in which tumors 'escape' immune surveillance have long been a topic of much investigation. The immune surveillance theory, also referred to as "cancer immunoediting," is typically characterized by three main phases: elimination; equilibrium; and eventually, escape (100). Tumors are believed to escape surveillance when the adaptive immune system fails to recognize tumor cells as foreign or dangerous to the host (101). Evidence in murine models has shown that tumors that do not enter the lymph nodes (or are compartmentalized from T-cells) failed to alert adaptive responses, and thus are 'ignored' by the immune system (102). However, CTL responses were induced by direct interaction between tumor cells and T-cells. Therefore, mechanisms thought to enable tumor cells to be 'ignored' are mainly through alterations in the antigen-processing and presentation pathway. In particular, dendritic cells (DCs) are believed to be major characters in this immune response (103). They are considered the most potent of antigen-presenting cells, with antitumor effects due to their ability to induce CTL responses. Several studies utilizing dendritic cell vaccines have been conducted to understand the triggers of activation and maturation, as well as functioning mechanisms; however, limited success was yielded in clinical trials (47, 104). This may be due to the inability of CTLs to efficiently traffic to and disseminate into the tumor, or a suppressive local environment leading to loss of their cytotoxic potential or conversion into regulatory T-cells (104).

This suppressive environment may be mediated by immune checkpoints (105, 106). Immune checkpoints refer to inhibitory pathways crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses to minimize damage. It is now known that certain tumors exploit immune checkpoint pathways as a major mechanism of immune evasion, particularly against T-cells that are specific for tumor antigens. Many of these immune checkpoints are initiated by ligand-receptor interactions, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) (107), as well as proteins such as programmed cell death protein 1 (PD-1/PDL-1) (108), which can be readily blocked by antibodies or modulated by recombinant forms of ligands or receptors (105).

Virotherapy may have the ability to harness the benefits of the host immune response while inhibiting undesirable components. It is hypothesized that under certain conditions, a strong local host immune response at the site of infection within the tumor can support and enhance antitumor potential of the virus (109), so in addition to direct oncolysis, it may be possible to induce an immune response against the virus and subsequently against the tumor itself. This may even lead to systemic clearance of tumor metastases expressing specific antigens.

Several groups have looked into the combination of VACVs with several forms of immunotherapy, including cancer vaccines and immune checkpoint blockade (104). Strategies to date include the combination of DC vaccination with oncolytic viruses expressing chemokines known to attract the T-cells produced into the tumor (110), or the combination of chimeric antigen receptor T-cells with oncolytic virus strains expressing both chemokines and cytokines to attract both these cells into the tumor and subsequently maintain their phenotype (111).

Furthermore, since CTLA4 antibodies were approved by the FDA, studies have also explored the potential of combining vaccinia virus with immune checkpoint blockade (105, 112). For example, one group showed that combination therapy with oncolytic vaccinia virus and anti-CTLA4 can effectively treat several cancer types (106). However, the benefits of combination therapy were dependent on the viral strains, in addition to timing of the treatment. Administering both treatments simultaneously resulted in loss of therapeutic benefit, probably due to early induction of anti-viral immunity, dampening the effects of oncolysis. When the antibody was administered 3 days post viral treatment, synergy was observed. Therefore, timing of administration of oncolytic virus and immunotherapy combinations will need to be refined for progression to clinical trials. More recently, an oncolytic virus encoding for CXCL11, a chemokine known to attract T-cells, was used in combination with an anti-PDL-1 agent against a murine model of peritoneal carcinomatosis (113). The study demonstrated that vvDD-CXCL11 markedly upregulated PDL-1 in the tumor microenvironment due to enhanced T-cell infiltration, and reduced tumor burden when combined with anti-PDL-1. Furthermore, antitumor immunity was observed, with primary tumors growing more slowly in those treated with combination therapy after tumor rechallenge.

TABLE 2 | Clinical trials with oncolytic vaccinia viruses.

Condition	Intervention	Phase	Sponsor	Status	Reference
Solid cancers	vvDD-CDSR	Phase I	University of Pittsburgh	Completed	(119)
Hepatocellular carcinoma	JX-594	Phase II	Jennerex Biotherapeutics	Completed	(117, 118)
Metastatic refractory colorectal cancer		Phase I and II			(120)
Refractory solid tumors in pediatric patients		Phase I			(121)
Refractory solid tumors in adults		Phase I			(71)
Malignant melanoma		Phase I and II			(122)
Head and neck cancers	GL-ONC1	Phase I	Genelux Corporation	Completed	(71)
Solid organ cancers with or without Eculizumab		Phase I		Recruiting	(71)
Advanced peritoneal carcinomatosis		Phase I and II		Completed	(71)
Recurrent ovarian cancer		Phase I		Recruiting	(71)
Advanced solid organ cancers		Phase I		Completed	(71)
With Ipilimumab metastatic/advanced solid tumors	Pexa-Vex	Phase I	Centre Leon Berard	Recruiting	(71)
Hepatocellular carcinoma with Sorafenib vs Sorafenib alone		Phase 3			(71)

Source: www.clinicaltrials.gov.

Thermotherapy

The contrast agent, melanin, may facilitate near-IR-assisted thermotherapy in addition to oncolytic virotherapy (88). A near-IR laser was utilized to specifically transfer energy to melanin-induced cells, with the transferred energy consequently converted to thermal energy, eventually heating the melanin-producing cells and cells in their vicinity to temperatures causing protein denaturation and cell death, therefore, enabling thermotherapy. Stritzker et al. demonstrated that aliquots containing cells infected with VACV encoding melanin achieved a higher temperature exposed to laser light, with near-complete kill of all cells within that aliquot as compared to mock-infected cells. They also demonstrated that lung cancer xenografts on tumor-bearing mice treated with the melanin-inducing VACV had significantly enhanced regression when using a single 2-min laser treatment, compared to tumors that were not exposed to the laser light, demonstrating an additive effect.

Combining VACV with Other Viruses

In another innovative strategy, complementary oncolytic vesicular stomatitis virus (VSV) was combined with oncolytic vaccinia virus to improve therapeutic outcome (70). The two recombinant viral strains synergistically enhanced each other, resulting in better tumor tissue penetration and prolonged survival of tumor-bearing mice. The synergistic effect was, on the one hand, dependent on the VACV B18R gene product which locally antagonizes the innate cellular, antiviral response initiated by type-I IFNs (114–116) and, therefore, supports VSV growth. On the other hand, recombinant expression of the fusion-associated small transmembrane by VSV resulted in enhanced spreading of the VACV. Further studies are needed combining VACVs with other strains of oncolytic viruses to further elucidate potential additive and synergistic treatment effects.

VACCINIA VIRUSES IN CLINICAL TRIALS

Due to the success of vaccinia viruses in preclinical models, there are several ongoing clinical phase I and II studies for human cancer therapy following the treatment with oncolytic vaccinia virus strains including GLONC-1, JX-594, and Pexa-Vex, with promising safety profiles and therapeutic results (**Table 2**). For example, in a phase I trial using JX-594 in patients with hepatic carcinoma, 3 of 10 patients had a partial response and six had stable disease (117). The primary goals were to determine the maximum-tolerated dose and safety of JX-594 treatment. IT injection of JX-594 into primary or metastatic liver tumors was generally well tolerated, with grade I–III flu-like symptoms reported by all patients, and four patients experiencing transient grade I–III dose-related thrombocytopenia. Grade III hyperbilirubinemia was dose-limiting in both patients at the highest dose. JX-594 replication-dependent dissemination in blood was shown, with resultant infection of non-injected tumor sites. Safety was, therefore, acceptable in the context of JX-594 replication, GM-CSF expression, and systemic dissemination, which led to a phase II trial in patients with unresectable primary hepatocellular carcinoma with promising results (118).

CONCLUSION

Vaccinia virus has been shown to be a safe and promising anti-cancer agent, facilitating therapy, imaging, and combination treatment, which may help overcome cancer resistance to standard therapy regiments. VACVs' advantages of a large genomic capacity, fast and efficient replication, and strong safety profile make it an ideal candidate for genetic engineering. Several future generations of oncolytic vaccinia viruses are under investigation, including those armed with immune-stimulating, anti-angiogenic, and prodrug therapy, those encoding reporter genes for the imaging and serial monitoring of oncovirotherapy. Moreover, VACVs are being investigated in combination with various other anti-cancer strategies, including chemo-, radio-, and immunotherapies as well as other oncolytic VACVs. Further study is needed to unlock VACVs' full potential as part of the future of cancer therapy.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Oncolytic Viral Therapy for Mesothelioma

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The limited effectiveness of conventional therapy for malignant pleural mesothelioma demands innovative approaches to this difficult disease. Even with aggressive multimodality treatment of surgery, radiation, and/or chemotherapy, the median survival is only 1–2 years depending on stage and histology. Oncolytic viral therapy has emerged in the last several decades as a rapidly advancing field of immunotherapy studied in a wide spectrum of malignancies. Mesothelioma makes an ideal candidate for studying oncolysis given the frequently localized pattern of growth and pleural location providing access to direct intratumoral injection of virus. Therefore, despite being a relatively uncommon disease, the multitude of viral studies for mesothelioma can provide insight for applying such therapy to other malignancies. This article will begin with a review of the general principles of oncolytic therapy focusing on antitumor efficacy, tumor selectivity, and immune system activation. The second half of this review will detail results of preclinical models and human studies for oncolytic virotherapy in mesothelioma.

Keywords: mesothelioma, oncolytic, virotherapy, novel, measles virus, adenovirus, herpes simplex virus type 1, vaccinia virus

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INTRODUCTION: STANDARD THERAPY FOR MESOTHELIOMA

Mesothelioma is an uncommon malignancy of the parietal and visceral mesothelium, with about 3,300 new cases each year in the United States (1). Malignant pleural mesothelioma (MPM) accounts for 90% of cases, as inhalation asbestos exposure is the major risk factor. Most of the remaining cases arise from the peritoneum, with only 1–2% of cases occurring in the pericardium or tunica vaginalis testis (2). In the western world, incidence peaked in the early 21st century and has since leveled off in the US, while in Europe estimates are for a decrease in new cases (3–5). This is the result of concerted efforts over the last several decades to reduce asbestos exposure. Unfortunately, less developed countries that are slower to control asbestos exposure likely will continue to see an increase in incidence because of the prolonged latency period of at least 20 years before development of mesothelioma (6, 7).

The typical presenting symptoms of MPM are non-specific and include shortness of breath, chest pain, and weight loss. Characteristic findings on chest imaging are pleural abnormalities such as a unilateral effusion, calcified plaques, thickening, or masses (8). Diagnosis often requires a full-thickness pleural biopsy *via* pleuroscopy or video-assisted thoracoscopy. Pleural fluid cytology, although more easily obtained, is usually not sufficient. Even with adequate tissue, the pathologic evaluation can be challenging as mesothelioma is not frequently seen in most centers and has a number of different subtypes—epithelioid, sarcomatoid, biphasic—that must be differentiated from reactive processes in the pleura (9).

The management of mesothelioma is to the extent possible multimodality strategy incorporating chemotherapy, surgery, and/or radiation. The initial step is evaluating whether the disease

is surgically resectable, with the goal of macroscopic complete resection. The two main surgical techniques are extrapleural pneumonectomy (EPP) or the less radical pleurectomy/decortication (P/D). Comparisons of EPP and P/D are limited to observational studies, with the largest cohort showing a survival advantage to P/D (10). A recent meta-analysis found lower short-term mortality for P/D, although the 2-year survival was not significantly different than EPP (11). In the absence of randomized trial data, the surgical approach is determined on a patient-specific basis.

Chemotherapy for MPM is recommended for all patients undergoing active therapy, with either cisplatin or carboplatin combined with pemetrexed as the standard of care. In patients not eligible for surgical resection, cisplatin/pemetrexed was shown to have a superior median overall survival compared to cisplatin monotherapy of 12.1 vs. 9.3 months (12). Carboplatin is equally efficacious to cisplatin in combination with pemetrexed, providing an alternative for older patients and those with borderline renal function (13). The addition of bevacizumab to cisplatin/pemetrexed may offer further benefit, pushing median overall survival to 18 months (14). For those patients having surgical resection, chemotherapy is given either preoperatively or postoperatively with no studies comparing the two approaches.

The role of radiation therapy is less clear, with most studies evaluating its use in the postoperative setting to reduce the risk of local recurrence (15). Trimodality therapy of preoperative chemotherapy, surgical resection, and postoperative radiation has been evaluated in small studies with variable success (16, 17). More detailed reviews of standard therapy for pleural and peritoneal mesothelioma are available elsewhere (8, 18, 19).

Despite the application of multimodality therapy to MPM, most patients are candidates for only palliative chemotherapy and have a median overall survival of 1–2 years (20). These limitations in current treatment highlight the importance of investigational therapies that may improve the prognosis of an otherwise highly fatal disease. This review will focus on the use of oncolytic viral therapy for mesothelioma.

THE PRINCIPLES OF ONCOLYTIC VIRAL THERAPY

Background

The fact that viruses may inflict damage not only in healthy human tissue but also in tumor cells was first observed in the early 21st century (21). The first formal studies utilizing viruses as anticancer therapy were performed in the 1950s and documented transient tumor response in a small number of patients (22–24). However, these intriguing early results were tempered by technical and methodological constraints, and investigation declined for the next few decades (25).

A renewed interest began in the late 20th century as scientific advances in virology and molecular genetics allowed greater viral manipulation and the potential for increased efficacy (26). Many viruses have now been studied in this context, including adenovirus, herpes simplex virus (HSV), vaccinia, measles virus, and others, applied against a number of malignancies such as glioma, breast, head and neck, and lung (27–30). In 2015, a genetically modified HSV type 1 (HSV-1) (T-VEC) became the first FDA-approved oncolytic viral therapy, for use against melanoma (31).

The ideal oncolytic viral therapy is based on three basic principles (32, 33): (1) antitumor efficacy, the ability to directly infect and lyse tumor cells; (2) tumor selectivity, to preferentially infect tumor cells and minimize toxicity of infection to healthy tissue; and perhaps most importantly, (3) stimulation of the immune system, to provoke an antitumor response that will amplify the viral-directed cell death and provide ongoing tumor cell killing (**Figure 1**). Genetically engineering viruses to optimize tumor cell toxicity and selectivity has found success, while attaining a sustained immunotherapeutic response has proven a more difficult task.

Antitumor Efficacy

The concern for toxicity of wild-type viruses led to the first recombinant viruses being engineered as replication-incompetent strains, with the goal of delivering gene therapy but not necessarily propagation of viral infection (34). The development of techniques to enhance viral selectivity for tumor cells allowed a shift back toward using replication-competent oncolytic viruses. These virulent models allow the natural viral mechanisms to infect, replicate, and lyse tumor cells. As virions are released from lysed tumor cells, the infection spreads within the local tumor mass (33). This potentiates tumor cell killing compared to the initial input dose of viral particles and may lead to a more robust antitumor response from the immune system (32). Gene therapy with replication-incompetent viruses has a bystander effect that also may amplify cell death in the local tumor environment, although to a lesser extent than actively replicating viruses (25).

The mechanisms of tumor cell killing after viral infection are varied (25, 32). The most straightforward method is viral replication and shedding leading to eventual cell lysis. A second method of direct oncolysis is the production of cytotoxic viral proteins. Altering production of viral proteins is a target of genetic engineering to improve antitumor efficacy. For example, the adenovirus death protein (ADP) is produced during the normal adenovirus replication cycle to induce host cell death (35); a modified adenovirus designed to overexpress ADP has increased cytolytic activity in a mouse model of lung cancer (36).

A third method of antitumor efficacy is insertion of transgenes into the viral genome, so-called "armed" viruses. An early model of transgene insertion is the HSV thymidine kinase gene, which metabolizes ganciclovir into a toxic byproduct (37). Cells infected with a virus carrying this gene are rapidly lysed in the presence of ganciclovir. Both replication-deficient and replication-competent adenoviral vectors with the HSV thymidine kinase gene have been studied in humans against a number of tumors including mesothelioma, with encouraging results (38–41). Transgenes encoding cytokines such as IL-2 or TNFα to augment immune system response are also utilized (42, 43). With improved methods for oncolytic viruses to specifically target tumor cells, the use of replication-competent viruses armed with transgenes now has become common.

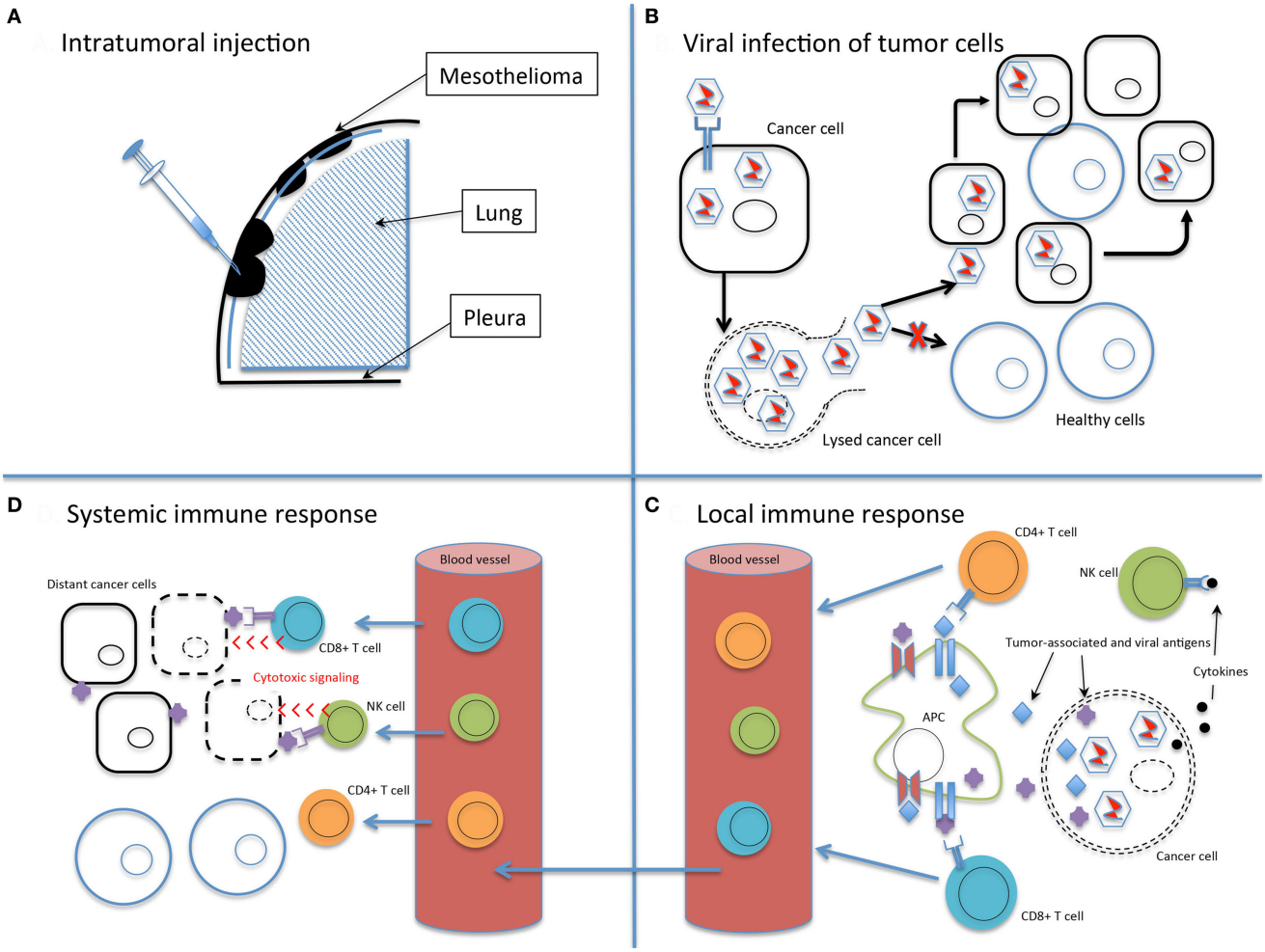

FIGURE 1 | The basic principles of oncolytic virotherapy. **(A)** Administration is most commonly *via* direct intratumoral injection rather than systemic intravenous route to avoid viral inactivation in the bloodstream and minimize off-target infection. The pleural location of mesothelioma is particularly amenable to direct injection. **(B)** Viral infection of cancer cells, followed by replication, leads to cell lysis and dissemination of infection. The use of non-replicating viruses results in lysis to a lesser extent than replicating viruses. Acquired defects of the cancer cells and engineered modifications of the viral genome drive infection selectively toward cancer cells. **(C)** Viral infection and lysis exposes tumor-associated and viral antigens to the immune system. Antigen-presenting cells process these novel antigens *via* the major histocompatibility complex for presentation to CD4+ and CD8+ T cells. Cytokine release attracts NK cells. Local tumor cell death is augmented by the immune response. **(D)** Activated T and NK cells circulate throughout the body and recognize distant tumor cells that express the previously uncovered tumor-associated antigens. Note that the systemic immune response is not dependent on viral oncolysis.

Tumor Selectivity

Engineering viruses to selectively target tumor cells has proven especially productive. By minimizing infection of and resulting toxicity to normal cells, larger viral doses can be administered and the therapeutic index widened. Mechanisms for engineering viruses for tumor selectivity include modification of the viral coat, exploiting abnormal signaling pathways, insertion of tumor or tissue specific promoters, and partial or entire gene deletions (**Figure 2**) (26, 34, 44).

Achieving tumor selectivity does not always require a recombinant virus, as a wild-type virus may already exhibit a preference for replicating in tumor cells. This can occur through overexpression of cell surface proteins that facilitate viral entry into the tumor cell (26). Specific viruses have natural tropism for these aberrant proteins, such as HSV-1 for overexpressed herpesvirus entry mediator and nectins on carcinoma cells, measles virus for CD46, and echovirus for an integrin domain on ovarian cancer cells (45–48).

When a natural viral tropism for tumor cell surface proteins is not present, viral coat protein expression can be modified. Ligands unique to the tumor cell surface are identified and the virus engineered for uptake specifically by these ligands (34). This is used in adenoviral vectors by modification of the Ad5 fiber knob domain (49). Another example is a measles virus designed with a surface antibody targeting carcinoma embryonic antigen expressed on adenocarcinoma (50).

Wild-type viruses can also preferentially infect tumor cells by exploiting altered signaling pathways in the tumor cell (44).

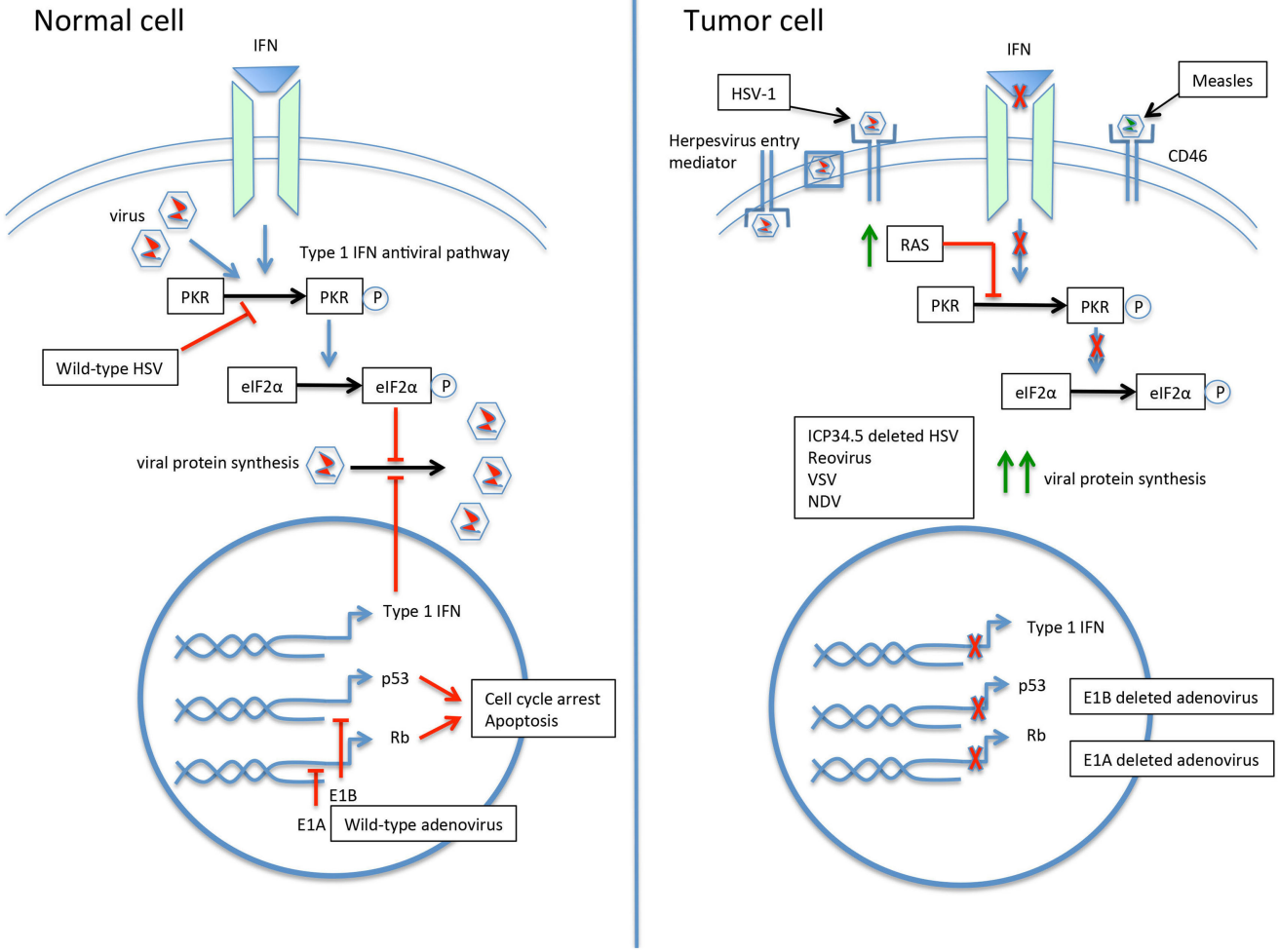

FIGURE 2 | The selective infection of tumor cells by oncolytic viruses. In the normal cell, the response to viral infection involves activation of the type 1 interferon (IFN) and protein kinase R (PKR) pathways, resulting in upregulation of eIF2α and inhibition of viral protein synthesis. The p53 and Rb pathways are also activated. Wild-type viruses are able to inhibit various steps of the antiviral response to allow ongoing replication. For example, the herpes simplex virus (HSV) gene ICP34.5 blocks PKR signaling, and the adenovirus genes E1A and E1B inactivate Rb and p53, respectively. The tumor cell may have a number of acquired defects that allow for preferential infection by oncolytic viruses. An increased expression of cell surface proteins facilitates viral entry, such as herpesvirus entry mediator for HSV type 1 (HSV-1) and CD46 for measles virus. Defective IFN and PKR pathways lead to unimpeded viral protein synthesis. Upregulation of RAS in tumor cells results in PKR pathway inhibition. Modification of viruses can further drive tropism and minimize infection of normal cells. Deletion of the HSV gene ICP34.5 renders the virus unable to inhibit PKR in healthy cells and drives infection toward PKR-deficient tumor cells. Similarly, deletion of the adenovirus E1A or E1B genes leads to preferential infection of p53- and Rb-deficient tumor cells.

This illustrates how cellular changes defining malignancy, such as resistance to apoptosis and loss of p53, often overlap with virally induced cellular changes (51, 52). The environment of a tumor cell then may be advantageous by supplying cell processes necessary for viral replication. Two key antiviral pathways present in normal cells are often implicated here—protein kinase R (PKR) and interferon (IFN) signaling (44). A dysfunctional PKR pathway enhances reovirus replication, and defects in the type 1 IFN response potentiate the replication of vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) (53–55).

Altered tumor cell signaling pathways provide opportunities for viral genetic engineering. Gene deletions can remove viral genes necessary for replication in normal tissue but not required for replication in tumor cells (34). Viral gene products block the normal antiviral response through the PKR, IFN, and p53 pathways. Deletion of these viral genes restores the ability of healthy cells to prevent viral replication, while cancer cells already deficient in the antiviral pathway remain susceptible. HSV-1 modified for deletion of the ICP34.5 gene is such an example. Lacking this gene, the virus no longer blocks PKR signaling in healthy cells, leaving PKR-deficient tumor cells to be preferentially infected (26, 56). Similarly, a modified adenovirus with a gene deletion for the protein E1B no longer inactivates p53. This allows healthy cells to initiate p53-mediated apoptosis prior to

viral replication; p53-deficient cancer cells are then selected for viral spread (57).

The goal of most viral gene deletions is to attenuate viral pathogenesis in normal cells. In fact, nearly all oncolytic viruses being studied for clinical use are attenuated in same manner. The first study of a virus modified specifically to improve oncolytic activity, by Martuza et al. in 1991, employed HSV-1 with deletion of the gene encoding the enzyme thymidine kinase (58). This deletion results in attenuated neurovirulence (59).

Just as genes are deleted from the viral genome to increase tumor specificity, insertion of gene promoter regions that are tumor or tissue restricted are frequent additions to achieve the same goal of specificity. This relies on the overexpression of tumor-specific proteins for activation of the promoter region of a gene that is necessary for viral replication and/or cell death. Healthy cells become relative life cycle dead ends for the virus by lacking the proteins needed to activate regulatory viral genes (44). The adenovirus E1A gene has been modified with various gene promoters including an alpha-fetoprotein gene promoter for tumor-specific replication in hepatocellular cancer cells and a prostate-specific antigen gene promoter with tissue-specific replication in prostate cancer (60, 61).

Immune System Activation

The concepts of viral antitumor efficacy and selectivity can be linked together as the first part of a two-step process necessary for successful oncolytic viral therapy. The initial viral-directed tumor cytotoxicity then must be followed by a sustained antitumor response carried out by the immune system (32). This critical second phase has been recognized for many years (62), although only recently have the mechanisms to make oncolytic viruses a more effective immunotherapy begun to be elucidated (63–65).

Tumor-induced immune tolerance is a critical part of the malignant process. This is accomplished through alteration of the tumor microenvironment by recruitment of immune-inhibitory cells and exclusion of immune-stimulating cells (66). Viral-mediated tumor cell death works to reverse this tolerance by exposing tumor-associated antigens previously restricted from presentation to the immune system, known as neoantigens, and provoking inflammatory cytokine release. Antigen-presenting cells activated by these neoantigens then direct an antitumor response by CD8+ T cells and NK cells (**Figure 1**) (26).

Prior to arriving at the current paradigm of immune stimulation as an essential part of virotherapy, a major concern was a robust antiviral response limiting the extent of oncolysis (67). In an effort to thwart the immune response, initial murine studies used immunocompromised models to allow adequate viral replication and cytolysis (68). The move to immune-competent models was accompanied by suppression of the immune response, such as dampening T cell response through gene deletions or administering cyclophosphamide prior to viral administration (69, 70). Current approaches aim for a balance between permitting both initial viral replication and the subsequent robust antitumor, and inevitably antiviral, immune response.

Viral genetic engineering now includes modifications to boost immune antitumor activity, often through insertion of cytokine genes. HSV expressing granulocyte macrophage colony-stimulating factor (GM-CSF) increases antigen presentation by dendritic cells and improves tumor reduction of lymphoma in a murine model (56). VSV expressing IFNβ decreased T-regulatory cells, increased CD8+ T cells, and prolonged survival in a murine lung cancer model (65). The HSV-1 protein ICP47 decreases antigen presentation on infected cells, and deletion of this gene augments antitumor effects (56, 71).

The recognition of immune stimulation by oncolytic viruses and the simultaneous development of the immune checkpoint inhibitors raise the possibility of synergy between these distinctive mechanisms of immunotherapy. A number of studies have already been completed in this new area with promising results (72–74). The remarkable success of checkpoint inhibition likely indicates the future role for oncolytic therapy as an adjunct to other more clinically advanced forms of immunotherapy.

Administration and Safety

The administration of oncolytic viral therapy must account for the setting of metastatic disease that requires a systemic immune response. Intravenous delivery of virus, while having the potential for rapid viral infection at all locations of disease, is problematic for several reasons. An immediate innate humoral immune response may lead to viral inactivation in the bloodstream, prior to infection of tumor cells. In the case of previous environmental exposure or vaccination, antiviral antibodies will provide effective at viral clearance (75–77). Even without preexisting immunity, repeated intravenous administration of virus results in production of antiviral antibody titers that quickly render vascular delivery ineffective (78).

The delivery of oncolytic viruses has predominantly been *via* direct intratumoral (IT) injection. IT administration has its own limitations, most apparent being the requirement of an accessible solid mass. Early viral inactivation by the innate immune system is also an issue with IT injection, although probably to a lesser extent than intravenous therapy (69, 79). Both systemic and direct viral administration must overcome a harsh local tumor environment that limits viral biodistribution (80, 81).

The main advantage of IT administration is ensuring local tumor delivery while also inducing distant tumor responses. This is true in some preclinical models and also in the phase III trial leading to approval of T-VEC (31, 65, 73). With the immune system able to provide a systemic response after local administration, any hypothetical advantage of intravenous delivery is no longer relevant.

Any effective oncolytic therapy needs to take into account effects on surrounding non-cancerous tissues. Of primary concern is the viral infection spreading to healthy cells, given the fact that cancer patients are already immunosuppressed and susceptible to infection. As previously discussed, the concept of selecting and designing a virus with tumor cell selectivity is the key to minimizing toxicity. Additional safety concerns include environmental shedding and reversion to wild-type virus. In general, studies have shown favorable toxicity profiles although perhaps at the expense of efficacy, as the field is now moving toward the use of less attenuated viruses with improved selectivity. A recent review covers safety concerns in more detail (82).

The Ideal Oncolytic Virus

In addition to optimizing antitumor efficacy, tumor selectivity, and immune system activation, a number of other viral characteristics are taken into account when choosing an oncolytic virus. These include viral genetic stability, non-integrating viruses that cannot incorporate into the host genome, a safety mechanism to inactive the virus after administration, and amenability to high titer production (44). A detailed discussion of these additional factors is beyond the scope of this review.

The ability to non-invasively image viral infectivity is of particular interest. These molecular imaging techniques allow localization of viral replication in tumor or healthy tissue, an important measure of toxicity. The viral dose and route of administration may be correlated with the level of infectivity without the need for repeat tissue biopsies (83). Two viral modifications for molecular imaging are insertion of the gene encoding green fluorescent protein (GFP) or the human sodium iodide symporter (hNIS) protein. The hNIS also offers the potential for radioiodide therapy like that used for ablation of thyroid tissue (84). Many of the mesothelioma studies detailed in the next section utilize addition of these reporter genes, allowing for monitoring of efficacy and toxicity.

With the expanding ability to genetically engineer oncolytic viruses, the use of viruses with multiple modifications is readily available. This is illustrated by T-VEC, an HSV-1 with three separate modifications—deletion of gene ICP34.5 to attenuate neurovirulence, deletion of ICP47 to increase antigen presentation on infected cells, and insertion of the gene for GM-CSF to attract antigen-presenting cells (31, 56, 85–87). This combinatorial approach to maximize efficacy through various mechanisms is now standard, as we will describe with oncolytic virotherapy for mesothelioma.

ONCOLYTIC VIRAL THERAPY FOR MESOTHELIOMA

Malignant pleural mesothelioma provides an optimal model for the study of oncolytic virotherapy for several reasons (88). The pleural location is accessible for direct IT injection, the preferred method of administration for most viral platforms. Although distant metastases can occur, complications and death usually stem from local disease spread. Also, limited improvement in outcomes with multimodality therapy lends more urgency to experimental approaches. Given these characteristics, despite being an uncommon malignancy, an extensive amount of preclinical data with oncolytic viruses in mesothelioma models is available. This work has progressed to early phase clinical MPM studies for a number of different viruses (**Table 1**).

Studies using replication-incompetent viruses are most accurately classified as gene therapy with a viral vector rather than oncolytic virotherapy, since "oncolytic" implies active viral replication. In the context of cancer, gene therapy is the transfer of genetic material to induce tumor cell death, as defined by Sterman (88). This can be accomplished in a number of ways, and oncolytic virotherapy is a subtype of gene therapy using actively replicating viruses. Given the significant overlap, studies of both replication-competent and replication-incompetent viruses are reviewed here.

Adenovirus

A non-enveloped virus with a linear, double-stranded DNA genome, adenovirus is one of the most extensively studied oncolytic viruses, rivaled only by HSV. A moderately sized genome of ~38 kilo base pairs (kb) allows for multiple modifications (26, 32). Other favorable characteristics are a stable genome, non-integration, and high-titer production. Most humans are exposed and asymptomatic upon infection, although susceptible individuals can develop upper respiratory symptoms or conjunctivitis (33).

Studies with oncolytic adenovirus have advanced to many early phase human trials including prostate, pancreatic, and colorectal carcinomas (60, 103, 104). Notably, a phase III trial for head and neck squamous cell carcinoma conducted in China combined an E1B-deleted adenovirus (H101) with chemotherapy (29). This led to approval in China of H101 for treatment of nasopharyngeal carcinoma in combination with chemotherapy.

Adenovirus for mesothelioma includes both preclinical and human studies. The first *in vitro* studies out of the University of Pennsylvania focused on gene therapy with the HSV-thymidine kinase suicide gene inserted into a replication-deficient adenoviral vector (Ad.HSV*tk*) (105, 106). This same adenovirus then proved successful in animal models (107, 108). For example, Elshami and colleagues in 1996 used a rat model of MPM to intratumorally administer Ad.HSV*tk* followed by systemic ganciclovir, which is metabolized into toxic byproducts by the HSV*tk* gene product (109). The experimental rats showed tumor regression at 20 days (average tumor weight 0.6 vs. 5.4 g) and improved mean survival (34 vs. 26 days) compared to controls.

Replication-deficient adenovirus has also been studied as a vector for cytokine gene therapy to counter the immune tolerance characteristic of mesothelioma (88). After passive immunotherapy with intrapleural or systemic delivery of IL-2, IFNα, and IFNγ for mesothelioma met with some success in phase I/II trials, administration of cytokines *via* gene therapy was proposed to improve efficacy (110–112). In several murine experiments, IT injection of an adenovirus with insertion of the IFNγ gene resulted in tumor regression and a CD8+ T cell-mediated response (113, 114). By using a different mechanism to induce antitumor immunity, a replication-deficient adenovirus engineered to express the costimulatory molecule CD40L showed regression of both directly injected and distant tumors, indicative of a systemic immune response (115).

More recently, a series of preclinical studies using conditionally replicating adenoviruses (CRAds), replication-competent oncolytic viruses with modifications to improve tumor selectivity, have shown antimesothelioma activity. Instead of the E1 gene being deleted to produce replication-incompetent viruses, the gene is placed under control of tumor-specific promoters. An *in vitro* study inserted a midkine promoter overexpressed in tumor cells and demonstrated effective oncolysis in human MPM cell lines (116). *In vivo* studies with murine models have used a number of CRAd modifications—promoters linked to E1 gene expression, viral capsid alterations, and insertion of

TABLE 1 | Human clinical studies of virotherapy for malignant pleural mesothelioma (MPM).

Strain	Modification(s)	Study design	Results
Adenovirus			
Ad.HSV*tk* (replication incompetent)	Insertion of thymidine kinase suicide gene	21 patients in single-arm, dose-escalation study received single intrapleural dose followed by ganciclovir (89)	Gene transfer documented in 11 patients, minimal toxicity, no tumor responses
		5 patients given high-dose vector in same method as above study, with addition of systemic steroids (90)	Decreased inflammatory response but no improvement in gene transfer
		Long-term follow-up of 21 patients who received high-dose vector (41)	Good safety profile, two patients lived >6.5 years
Ad.IFNβ (replication incompetent)	Insertion of interferon (IFN)β gene	Phase I dose-escalation study, 7 patients given single intrapleural dose (91)	Clinical response in three patients at 60 days, IFNβ detectable in fluid of eight patients
		Follow-up phase I study, 10 patients given 2 intrapleural doses (92)	Repeated dosing safe, response by CT scan at 60 days in two patients
Adenovirus expressing IFNα2b (replication incompetent)	Insertion of IFNα2b gene	Pilot and feasibility study with 9 patients given 2 intrapleural doses of vector (93)	Five patients with stable disease or tumor regression at 60 days, gene transfer augmented by second dose
		Phase II trial of two intrapleural doses of vector combined with chemotherapy in 40 patients (94)	Partial response in 25%, stable disease in 62.5%, median survival 13 months, six patients lived >2 years
Ad5-D24-GM-CSF (replication competent)	Partial deletion of E1A, insertion of granulocyte macrophage colony-stimulating factor (GM-CSF) gene	20 patients with advanced solid tumors (2 with MPM) given 1 intratumoral dose (64)	47% overall clinical benefit rate, one MPM patient with stable disease
ONCOS-102 (Ad5/3-D24-GM-CSF)	Insertion of Ad3 fiber knob, partial deletion of E1A, insertion of GM-CSF	12 patients with advanced solid tumors (2 with MPM) given multiple intratumoral injections combined with oral cyclophosphamide (95)	Clinical response rate 40% at 3 months, one MPM patient with stable disease, increased PD-L1 in both MPM patients
Ad5/3-D24-GM-CSF		21 patients with advanced tumors (1 with MPM) given one intratumoral and one IV dose, with oral cyclophosphamide (96)	Evidence of efficacy in 13 of 21 patients, MPM patient with stable disease, no grade 4/5 adverse events
Herpes simplex virus type 1 (HSV-1)			
HSV-1716 (replication competent)	Deletion of $\gamma_1$34.5 gene	Phase I/IIa study of inoperable MPM with single or multiple intrapleural doses (97)	Pending, expected completion in 2016 (NCT01721018)
Vaccinia virus			
VV–IL-2 (replication competent)	Insertion of interleukin-2 gene, deletion of thymidine kinase gene	Small pilot study with six patients receiving multiple intratumoral injections (98)	Well-tolerated, viral gene expression detected for up to 3 weeks after administration, no tumor responses
JX-594 (replication competent)	Deletion of thymidine kinase gene, insertion of GM-CSF gene	Phase I trial, 23 patients with metastatic solid tumors (1 MPM patient), given single IV dose (99)	No dose-limiting toxicities, MPM patient with partial response for >10 weeks
Measles			
Measles virus (MV)–NIS (replication competent)	Edmonston strain with insertion of NIS gene	Phase I trial enrolling patients with MPM confined to single pleural cavity, given q28 days for up to six cycles (100)	Pending, currently enrolling patients (NCT01503177)
Newcastle disease virus			
PV701 (replication competent)	Naturally attenuated, non-recombinant	Phase I trail of 79 patients with advanced solid malignancies (2 with mesothelioma), virus given intravenously at various doses and intervals (101)	9 patients with objective responses, 1 *peritoneal* mesothelioma patient with 35% tumor reduction and received 30 total doses, 1 MPM patient with progressive disease
Reovirus			
Type 3 Dearing strain (replication competent)	Wild-type, non-recombinant	Phase 1 trial in 25 patients with advanced malignancy (1 MPM patient), given IV q3 weeks at escalating doses, combined with docetaxel (102)	Disease control rate 88%, MPM patient with minor response

GFP for viral imaging (117, 118). For example, Watanabe and colleagues in 2010 used an adenovirus engineered to express a telomerase-driven promoter linked to the E1 gene (119). This was co-administered with a replication-incompetent adenovirus with insertion of the heparanase gene to improve viral penetration through the dense extracellular matrix. The study showed significant tumor regression compared to controls as well as improved survival.

Human studies using adenoviral vectors began relatively quickly following preclinical experiments. Consecutive phase I dose-escalation studies used the replication-incompetent Ad.HSV*tk* gene therapy followed by ganciclovir (89, 90, 120). The response rate was low, with 1 of 26 patients having evidence of tumor regression. Seventeen patients had evidence of IT gene transfer on repeat pleural biopsy, although this was limited to the outermost tumor layers. A follow-up study of 21 patients who received "high-dose" therapy reported a good safety profile and 2 patients surviving >6.5 years (41). Although these studies proved to be safe, the low response rate indicated a need for improved gene transfer within the tumor and a more robust antitumor immune response.

Focusing on stimulating an immune response rather than delivering a suicide gene, several human trials have been completed using an adenoviral vector for gene transfer of IFNβ (Ad.IFNβ). These studies, like those for Ad.HSV*tk*, were with replication-incompetent virus. The initial phase I dose-escalation trial enrolled seven patients with MPM and three patients with metastatic pleural effusions due to other malignancies, administering a single intrapleural dose of Ad.IFNβ (91). At 60 days, three patients with MPM had a clinical response and four patients had progressive disease. IFNβ was detectable in the pleural fluid of eight patients, indicating successful gene transfer.

A follow-up phase I trial with Ad.IFNβ evaluated giving a second intrapleural dose (92). Repeated administration was safe, and 2 of 10 MPM patients had a clinical response by CT scan at 2 months. This lack of improvement in response with repeat dosing was likely from rapid development of neutralizing antibodies, as humoral immune responses were consistently detected but not the cellular responses more essential to antitumor immunity. Although these two IFNβ gene therapy trials were encouraging for stimulating an immune response, further dose modifications or combination therapy are needed to have a more significant impact on outcome.

When Ad.IFNβ was no longer commercially available, a subsequent phase I gene therapy study by Sterman and colleagues was completed using replication-incompetent adenovirus expressing IFNα2b (Ad.IFNα2b) (93). Clinical responses were encouraging, with five of nine patients having stable disease or tumor regression at 60 days. This led to a phase II trial combining Ad.IFNα2b with chemotherapy in 40 patients (94). Patients received two intrapleural doses of Ad.IFNα2b on days 1 and 4, followed by chemotherapy on day 15 with standard of care pemetrexed/platinum doublet for chemotherapy naïve patients (first-line treatment, 18 patients). If this was second-line chemotherapy, gemcitabine or pemetrexed was given (22 patients). Partial responses were observed in 25% of patients and stable disease in 62.5%. Although the median overall survival of 13 months was not significantly improved from standard treatments, six patients lived more than 24 months. Based on these results, a randomized phase III trial is planned.

Studies using replication-competent, oncolytic adenoviruses for MPM are scarce. A study by Cerullo and colleagues evaluated an oncolytic adenovirus (Ad5-D24-GM-CSF) modified for tumor selectivity and with insertion of a transgene for GM-CSF to augment immune response (64, 121). The virus was administered once intratumorally to 20 patients with advanced solid cancers, including 2 patients with MPM. Both patients had received prior chemotherapy. Reflecting the clinical benefit rate of 47% among all patients, one case of MPM had stable disease and lived over 1 year; the other case had progressive disease and lived about 100 days. No serious adverse events occurred.

A phase I study published in 2016 used Ad5/3-D24-GM-CSF in patients with advanced solid tumors (95). Twelve patients were enrolled including two with MPM. Multiple IT injections were given along with oral cyclophosphamide. Clinical response rate at 3 months was 40%; one MPM patient had stable disease, and the other had progressive disease. Tumor-infiltrating leukocytes increased following treatment in 11 of 12 patients. Interestingly, both patients with MPM showed increased PD-L1 expression posttreatment, relevant for potential future combination studies with immune checkpoint inhibitors.

HSV Type 1

An enveloped, double-stranded DNA virus, HSV-1 has a large 152 kb genome. About 30 kb of the genome is non-essential, which allows space for insertion of transgenes (33). The human pathogenesis of HSV-1 causes oral and genital lesions, latent infection in peripheral nerves, and less frequently CNS complications. This natural tropism for neuronal tissue led to early studies on brain tumors and also necessitates viral gene deletions to attenuate neurotoxicity in all oncolytic experiments using HSV-1 (58, 59, 122). Recombinant HSV-1 has been studied in a number of malignancies including colorectal, pancreatic carcinoma, and melanoma (31, 123, 124). In fact the only FDA-approved oncolytic virus, T-VEC for melanoma, is a modified HSV-1.

A preclinical study by Kucharczuk and colleagues in 1997 evaluated the replication-competent, neuroattenuated HSV-1716 as oncolytic virotherapy for mesothelioma (125). Neuroattenuation was achieved by deletion of both $\gamma_1$34.5 genes encoding the protein ICP34.5. The virus efficiently replicated in and lysed human mesothelioma cells *in vitro*. The same human mesothelioma cell line was then used to establish intraperitoneal tumors in immunodeficient mice. Fourteen days later, the mice were given HSV-1716 by intraperitoneal injection, resulting in decreased tumor burden and increased survival compared to controls. No viral dissemination was detected in non-tumor tissue.

Another preclinical study evaluated three different replication-competent, oncolytic herpesviruses: G207, NV1020, and NV1066 (126). G207 has both $\gamma_1$34.5 genes deleted along with inactivation of the ICP6 gene for additional attenuation in non-replicating tissues. NV1020, originally designed as an HSV vaccine, has deletions encoding the genes ICP0, ICP4, latency-associated transcripts, one copy of $\gamma_1$34.5, and UL24, all resulting in loss of virulence. NV1066 has single copy deletions of ICP0, ICP4, and $\gamma_1$34.5, plus the addition of GFP for viral imaging. Each virus was tested against 11 different MPM cell lines *in vitro*, including each histologic subtype of MPM—epithelioid, sarcomatoid, biphasic, and mixed. All three viruses were cytotoxic to each cell line, even at low multiplicities of infection (the ratio of viral particles to tumor cells). A murine model of MPM treated with NV1066 decreased tumor burden and increased survival.

Adusumilli and colleagues at Memorial Sloan-Kettering Cancer Center did several additional elegant *in vitro* studies combining NV1066 with other therapies. The first study evaluated viral replication and cytotoxicity in 10 human MPM cell lines infected with NV1066 with or without cisplatin (127). The combination proved synergistic, at least partly attributable to cisplatin-induced DNA damage upregulating the protein GADD34 that in turn potentiates replication and cytotoxicity of the mutant HSV-1.

The second study combined NV1066 and radiation therapy in multiple human MPM cell lines and found synergistic or additive effects depending on the cell line, based on the same mechanism of GADD34 upregulation (128). A murine flank tumor model demonstrated reduced tumor growth with the combination compared to controls or either therapy alone. Importantly, in both of these studies, cytotoxicity was maintained with dose reductions, which may allow for decreased toxicity if such combination therapy advances to further trials.

Human studies of oncolytic herpesviruses for mesothelioma have not been completed. An ongoing phase I/IIa study of HSV-1716 for inoperable MPM began recruiting in 2012. The virus is delivered into the pleural cavity in single or multiple doses, with safety as primary outcome and efficacy as secondary outcome. Study completion is expected in 2016 (97).

Vaccinia Virus

An enveloped, double-stranded DNA virus in the poxvirus family, vaccinia virus has a large ~190 kb genome that facilitates insertion and deletion modifications to improve oncolytic efficacy. Vaccinia replicates in the cytoplasm, posing no risk for host genome integration. An attenuated vaccinia virus was used to eradicate smallpox. Pathogenesis of the wild-type virus in immunocompetent humans is limited to a mild viral syndrome of fever, rash, and myalgias (26, 33).

Vaccinia viruses have been studied in a number of solid tumors in humans including breast, melanoma, and prostate (129–131). Some of these recombinant viruses are described as vaccines, since the objective is stimulation of an antitumor immune response rather than active viral replication causing oncolysis. JX-594, the most clinically advanced oncolytic vaccinia virus, has deletion of the thymidine kinase gene and an inserted transgene to express GM-CSF (132). A phase III trial combining JX-594 with sorafenib for treatment of hepatocellular carcinoma is now recruiting patients (133, 134).

Preclinical and human studies have evaluated treatment of mesothelioma with vaccinia viruses. The replication-competent vaccinia virus GLV-1h68 has deletions of the hemagglutinin and thymidine kinase genes for attenuation and insertion of three transgenes including GFP for viral imaging (135). GLV-1h68 successfully replicated in and lysed multiple MPM cell lines *in vitro* (136). The same study then established a murine model of MPM followed by intrapleural delivery of the virus that resulted in decreased tumor burden and increased survival. The GLV-1h153 virus, a modification of the parent virus GLV-1h68 by insertion of the hNIS gene, proved similarly effective for *in vitro* and murine models with the addition of radioiodine-based imaging for viral infection (137).

A study by Acuna and colleagues in 2014 evaluated vaccinia virus as adjuvant therapy following surgery in a murine model of malignant peritoneal mesothelioma (138). An oncolytic strain with deletions in thymidine kinase and vaccinia growth factor genes for tumor selectivity was used, the double-deleted vaccinia virus (vvDD). A single intraperitoneal dose of vvDD prolonged survival compared to controls. When combined with incomplete cytoreductive surgery, survival was not significantly prolonged compared to either vvDD alone or surgery alone. This led the investigators to propose that the effectiveness of vvDD as an adjuvant therapy following surgery may be limited to microscopic disease after complete surgical resection, although further studies have not yet been completed to confirm this hypothesis.

Human studies with vaccinia virus from MPM are limited. A small pilot study published in 2000 used a vaccinia virus with insertion of the IL-2 gene into the thymidine kinase gene region, a replication-competent virus with tumor cell selectivity (98). Six patients received multiple IT injections. Treatment was well tolerated, and viral gene expression was detected for up to 3 weeks after injection despite the development of antivaccinia antibodies; however, no tumor responses were seen.

An early phase I trial with the oncolytic JX-594 vaccinia virus enrolled 23 patients with metastatic solid tumors, including 1 patient with MPM (99). In contrast to IT or intrapleural administration in nearly every other study, this virus was given intravenously as vaccinia has natural mechanisms to prevent inactivation in blood (139, 140). Following a single intravenous administration, the patient with MPM had a partial response for greater than 10 weeks.

Measles Virus

An enveloped RNA virus with a small ~15 kb genome, measles virus (MV) is a well-known human pathogen that occasionally causes serious illness in non-vaccinated individuals. The attenuated Edmonston strain is used for oncolytic virotherapy given its proven safety profile and also natural tumor specificity due to the upregulation of CD46 on tumor cell surface that the virus uses for cellular uptake (26, 47, 48). Other favorable characteristics of MV are a stable genome and cytoplasmic replication.

Oncolytic measles viruses have been studied in both solid and hematologic malignancies (141, 142). The most visible success thus far is a preliminary report from the Mayo Clinic of two relapsed, refractory myeloma patients given attenuated MV intravenously, with one patient achieving a complete remission lasting 9 months (143). This phase I/II study for myeloma patients is continuing to enroll patients (144).

Several preclinical studies with MV in MPM have been completed. The first *in vitro* experiment used the live attenuated Schwartz strain to evaluate oncolytic activity and immune response against human mesothelioma cells and normal mesothelial cells (145). The mesothelioma cells were more susceptible to infection and viral-induced cell death than the mesothelial cells, attributed to increased CD46 expression on the cancerous cells. Dendritic cells phagocytized the apoptotic MV-infected mesothelioma cells, resulting in dendritic cell maturation and priming of CD8+ T cells. Although *in vitro*, these results were encouraging for MV-stimulating antitumor immunogenicity.

Li and colleagues used a murine model of mesothelioma to study Edmonston strain MV with insertion of the IFNβ and NIS genes (MV–mIFNβ–NIS) (146). After confirming infectivity and replication of the virus *in vitro*, mice were injected subcutaneously in the flank with mesothelioma cells. After tumors grew to 5 mm, different MVs were injected intratumorally. Tumors injected with MV–mIFNβ had increased immune cell infiltration and decreased angiogenesis compared to tumors injected with the parent MV without mIFNβ expression. These pathological findings correlated with median survival, which increased from 20 days for control mice, to 45 days for mice receiving MV without mIFNβ, to 65 days with MV-mIFNβ. A peritoneal mesothelioma mouse model showed similar improvements in survival for each virus. In addition, the NIS gene facilitated non-invasive radioiodine imaging.

A more recent *in vitro* study published in 2015 evaluated the mechanism of MV for tumor cell selectivity. Twenty-two MPM cell lines were tested for infectivity and replication of MV, along with four healthy cell lines. Interestingly, the amount of CD46 expression did not predict for MV infectivity, contrary to previous assumptions. A better correlate for sensitivity to MV was the ability to mount a complete type 1 IFN response. Cell lines unable to generate or respond to IFNα or IFNβ, the case for 70% of the MPM lines, were more susceptible to MV infection (147). These data have implications for predicting response in future studies of MPM to oncolytic MV.

No human studies of MV for mesothelioma have yet to be completed. A phase I trial using the attenuated Edmonston strain with insertion of the NIS gene (MV–NIS) is currently enrolling patients with MPM confined to a single pleural cavity (100). The virus is administered intrapleurally once every 28 days for up to six cycles. Primary and secondary objectives are maximum tolerated dose, safety, and toxicity; tertiary objectives are measurements of viral activity, immune response, and efficacy.

Other Oncolytic Viruses for Mesothelioma

Adenovirus, HSV-1, vaccinia virus, and MV are the most extensively studied virotherapy vectors for mesothelioma. A more limited number of studies have evaluated additional viruses including VSV, NDV, reovirus, and Sendai virus.

An RNA virus in the Rhabdoviridae family, VSV has no known pathogenesis in humans. Exposure is possible in those working with livestock or mice; otherwise the general population is immune naïve. This lack of pre-formed immune response is an advantage when introducing VSV as an oncolytic virus (65). VSV displays natural tumor selectivity *via* induction of the antiviral type 1 IFN pathway. In healthy cells with intact IFN signaling, this prevents viral replication, whereas tumor cells with a defective IFN response allow viral infection to proceed unimpeded (148).

Recombinant VSV engineered to express IFNβ (VSV–IFNβ) augments both the antiviral defense in healthy tissue and the immune response against tumor cells. Several preclinical studies have evaluated VSV–IFNβ against mesothelioma. A murine model of subcutaneous and intraperitoneal tumors injected with VSV–IFNβ showed reduced tumor growth and increased survival compared to controls (149). Safety was also enhanced, with less neurotoxicity with mouse IFNβ.

A second study looked at mesothelioma cell lines *in vitro* and correlated cytotoxicity from VSV–IFNβ with the extent of IFN responsiveness (150). Partial responsiveness, measured by upregulation of PKR and other elements after viral infection, led to resistance to cytolysis. Conversely, downregulation of p48 and PKR caused sensitivity to the virus. The authors proposed that testing tumor cells for IFN responsiveness might provide a predictive marker for this virotherapy.

NDV is an RNA avian paramyxovirus that causes serious disease in fowl but only mild disease in humans. Similar to VSV, the tumor specificity of NDV is dictated through a defective type I IFN pathway in tumor cells (151). A preclinical study in mesothelioma with NDV engineered to express GFP showed effective oncolysis against multiple mesothelioma cell lines *in vitro* (152). An orthotopic model of MPM in mice was then treated with either single or multiple intrapleural doses of NDV. Animals receiving multiple treatments had decreased tumor burden, measured by bioluminescence imaging of GFP. Survival was longest in those receiving multiple treatments and shortest in the control group.

A phase I trial using a replication-competent NDV enrolled 79 patients with advanced solid malignancies, including 2 cases of mesothelioma (101). The virus was administered intravenously at various dose levels and intervals. Of the 9 patients with objective tumor responses, 1 patient with peritoneal mesothelioma received over 30 doses of virus with a 35% tumor reduction, improved performance status, and no cumulative toxicity. A post-treatment tumor biopsy showed active NDV replication. Despite this encouraging result, no further human studies with NDV for mesothelioma have been completed.

Reovirus and Sendai virus are two additional RNA viruses that have been studied in combination with chemotherapy for mesothelioma. In a murine model of MPM, Sendai virus with cisplatin showed synergistic effects (153). A phase I trial evaluated intravenous reovirus plus docetaxel in 25 patients with advanced cancer (102). The one patient with mesothelioma had a minor response.

The Future of Oncolytic Virotherapy for Mesothelioma

The current paradigm for treatment of MPM emphasizes a multimodality approaching with surgery, radiation, or chemotherapy. Most studies of oncolytic therapy for MPM have been as monotherapy, necessary to confirm viral activity, dosing, and safety in preclinical and early-phase human trials. However, a number of studies have successfully combined virotherapy for mesothelioma with chemotherapy (94, 127, 154), radiation (128), and surgery (138, 155). Given the documented safety but overall limited efficacy thus far when administered as monotherapy, future studies will likely use oncolytic viruses as an adjuvant to more established therapy (156).

Combining the immune checkpoint inhibitors with oncolytic viruses is of exceptional interest given the synergistic mechanisms of immune activation. In fact since the recent approval of the oncolytic virus T-VEC for melanoma, a study has already shown improved efficacy with T-VEC when given with the CTLA-4 inhibitor ipilimumab (72). A study by Patel and colleagues finding increased tumor expression of PD-L1 after

treatment with VSV–IFNβ in a murine model of non-small-cell lung cancer indicates the potential of PD-1/PD-L1 agents to increase efficacy (65). Early phase trials with immune checkpoint inhibitors for mesothelioma are in process, with promising preliminary results (157).

The ability to molecularly engineer recombinant viruses to improve safety and efficacy has led to rapid advances in oncolytic virotherapy over the last several decades. Preclinical work for mesothelioma is now starting to move into more significant human studies with several clinical trials currently recruiting patients (97, 100). As the field continues to develop, more studies are needed to determine how oncolytic virotherapy is best utilized alongside current treatment for mesothelioma.

AUTHOR CONTRIBUTIONS

DP wrote the first draft of this article in consultation with RK. RK conceived and edited the article, as well as drafting the format.

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Immune System, Friend or Foe of Oncolytic Virotherapy?

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Oncolytic viruses (OVs) are an emerging class of targeted anticancer therapies designed to selectively infect, replicate in, and lyse malignant cells without causing harm to normal, healthy tissues. In addition to direct oncolytic activity, OVs have shown dual promise as immunotherapeutic agents. The presence of viral infection and subsequently generated immunogenic tumor cell death trigger innate and adaptive immune responses that mediate further tumor destruction. However, antiviral immune responses can intrinsically limit OV infection, spread, and overall therapeutic efficacy. Host immune system can act both as a barrier as well as a facilitator and sometimes both at the same time based on the phase of viral infection. Thus, manipulating the host immune system to minimize antiviral responses and viral clearance while still promoting immune-mediated tumor destruction remains a key challenge facing oncolytic virotherapy. Recent clinical trials have established the safety, tolerability, and efficacy of virotherapies in the treatment of a variety of malignancies. Most notably, talimogene laherparepvec (T-VEC), a genetically engineered oncolytic herpesvirus-expressing granulocyte macrophage colony stimulating factor, was recently approved for the treatment of melanoma, representing the first OV to be approved by the FDA as an anticancer therapy in the US. This review discusses OVs and their antitumor properties, their complex interactions with the immune system, synergy between virotherapy and existing cancer treatments, and emerging strategies to augment the efficacy of OVs as anticancer therapies.

Keywords: oncolytic virus, cancer, immunotherapy, innate immunity, adaptive immunity

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INTRODUCTION

Oncolytic viruses (OVs) are viruses that selectively infect and kill malignant cells, leaving surrounding healthy cells unharmed. In addition to direct cytotoxic activity, OVs engage and amplify host immune responses, leading to the destruction of residual malignant cells and establishment of lasting antitumor immunity. Initial interest in the use of viruses to treat cancer dates back to observations made in the early 1900s of tumor regression in the context of natural viral infection (1). However, the feasibility of this approach was initially limited by viral pathogenicity and associated toxicity in human patients. Recent advances in genetic engineering technology enabling modifications that enhance the safety and efficacy of OVs spurred a renewed interest in oncolytic virotherapy (OVT). Improved tumor selectivity and inherent self-replication kinetics allow for targeted therapy and localized therapeutic amplification, reducing the risk of systemic toxicity.

Oncolytic viruses based on several different vectors including adenovirus, herpes simplex virus (HSV), vaccinia virus, Newcastle disease virus, measles virus, and reovirus have been shown to be tumor-specific, relatively non-toxic, and capable of inducing robust antitumor immune responses in animal models and human patients (2, 3). Talimogene laherparepvec (T-VEC), a genetically

engineered HSV-based OV, was recently approved for the treatment of melanoma, representing the first oncolytic virus to be approved by the FDA as an anticancer therapy. Numerous clinical trials (**Table 1**) have demonstrated synergy between OVT and other standard and emerging anticancer therapies (4–14). Combination treatment, particularly with immune-modulating therapies, continues to be a promising field of research.

Activation of the host immune system is a crucial component of OV-mediated tumor destruction. However, immune responses can also prematurely terminate OV infection, precluding therapeutic efficacy. Optimization of viral replication and propagation as well as the generation of anticancer immunity remains a significant challenge facing OVT. With a better understanding of the complex immunological interactions between OVs, tumor cells, and the host immune system, the next generation of OVs will be poised to realize the full immunotherapeutic potential of OVT.

ONCOLYTIC VIRUSES

At the core of OVT is the natural propensity of viruses to infect malignant cells. This preference stems from an overlap between the cellular changes incurred during oncogenesis and those induced by viral infection. Cancer cells evolve to resist apoptosis and growth suppression, evade immune-mediated destruction, and proliferate indefinitely, characteristics also conducive to viral replication (15). Additionally, many tumors develop defects in cellular antiviral response pathways, like type I interferon (IFN) signaling, rendering them more susceptible to viral infection (16).

While some viruses, such as H1 autonomously replicating parvoviruses, reoviruses, Newcastle disease viruses (NDVs), vesicular stomatitis virus (VSV), mumps virus, etc., have a natural preference for infecting specific types of human tumor cells, others can be genetically modified to enhance tumor cell selectivity, including adenovirus, measles, vaccinia, and HSVs (3, 17). Various approaches have been explored for engineering the ideal oncolytic viral vector that will selectively target, infect, and destroy tumor cells, while sparing normal cells. Viral coat proteins can be altered to recognize specific tumor cell surface markers or utilize tumor-expressed proteases for cellular entry (3, 18). Genes necessary for viral replication can be placed under the control of tumor-specific promoters, or deleted entirely, rendering viral replication conditional upon genes constitutively active in malignant, but not normal, cells (3, 4).

OVs AND TUMOR MICROENVIRONMENT (TME)

Virus-Mediated Tumor Cell Destruction

Oncolytic viruses mediate tumor cell death *via* direct and indirect mechanisms, functioning as both direct cytotoxic agents and therapeutic cancer vaccines (**Figure 1**). These mechanisms are connected by the propensity of many OVs to induce immunogenic forms of tumor cell death, including immunogenic apoptosis, necrosis, pyroptosis, and autophagic cell death, which activate host immune responses (19, 20). Immunogenic cell death (ICD) is characterized by cell surface exposure of calreticulin and heat shock proteins and the release of immune-stimulating molecules like ATP, uric acid, and high-mobility group box 1. Unlike normal apoptosis, which is mostly non-immunogenic and at time tolerogenic, ICD can induce antitumor immune response *via* dendritic cell (DC) activation. ICD of tumor cells also releases tumor-associated antigens (TAAs) that can be used to generate antigen-specific antitumor immunity (21–24).

Native Antigen-Presenting Cells (APCs) and Viruses

Antigen presenting cells, such as DCs, are crucial mediators of innate and adaptive immunity, facilitating the generation of immune responses by releasing cytokines and activating naïve T cells. Recruited to sites of infection and inflammation, such as those induced by immunogenic tumor cell death, DCs capture viral and tumor antigens released during oncolysis and present them to naïve T cells, thereby initiating the generation of antigen-specific adaptive immune responses that mediate targeted destruction of residual and recurrent tumor cells (25).

TABLE 1 | List of oncolytic viruses currently being tested in clinical trial.

Virus	Name	Phase	Tumor	Combination	Reference
Adenovirus	ONYX-015	III	Squamous cell carcinoma head and neck (SCCHN)	Cisplatin	Khuri et al. (4)
		I/II	Pancreatic cancer	Gemcitabine	Hecht et al. (5)
		Pilot	Advancer cancers	Irinotecan + 5-FU or IL-2	Nemunaitis et al. (6)
		I/II	Advanced sarcoma	Mitomycin-C, doxorubicin, cisplatin	Galanis et al. (7)
	Oncorine (H101)	III	SCCHN or esophageal cancer	5-fluorouracil + cisplatin or adriamycin	Xia et al. (8)
	Ad5-CD/Tkrep	I	Prostate cancer	5-fluorocytosine, valganciclovir, radiation	Freytag et al. (9)
	ONCOS-201	I	Solid tumors	Cyclophosphamide	Ranki et al. (10)
Herpes simplex virus	Talimogene laherparepvec	I/II	SCCHN	Radiation, cisplatin	Harrington et al. (11)
		Ib	Melanoma	Ipilimumab (CTLA-4 inhibitor)	Puzanov et al. (12)
	G207	I	Glioma	Radiation	Markert et al. (13)
Reovirus	RT3D	I/II	Advanced cancers	Carboplatin/paclitaxel	Karapangiotou et al. (14)
Vaccinia	GL-ONC1	I	Head and neck carcinoma	Cisplatin, radiotherapy	NCT01584284
	JX-594 (Pexa-Vec)	I/IIa	Colorectal cancer	Irinotecan	NCT01394939

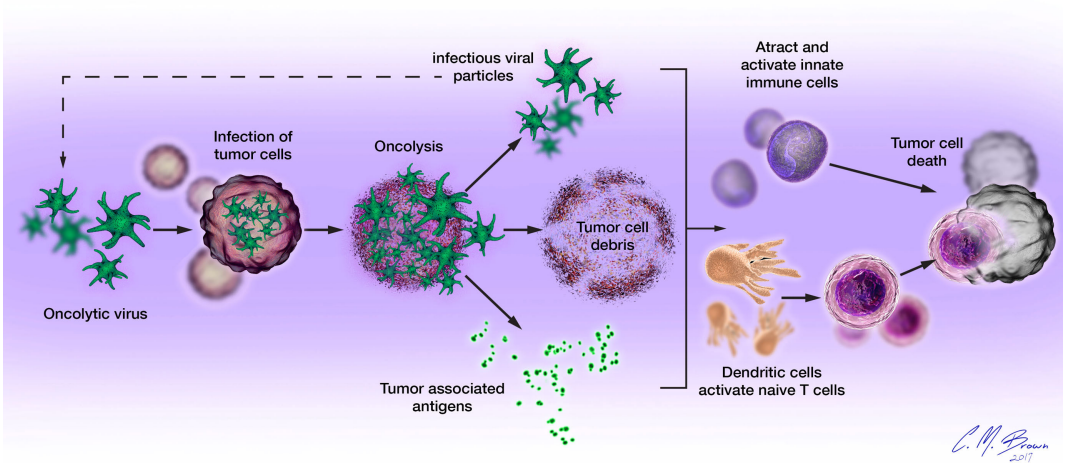

FIGURE 1 | Oncolytic viruses (OVs) mediate tumor cell destruction by two main mechanisms: (1) direct lysis of infected cells, OVs selectively infect malignant cells, hijacking their cellular transcription, and translation mechanisms in order to replicate. Termination of the viral replication cycle induces tumor cell lysis and release of infectious viral progeny. Oncolysis also releases viral particles, tumor-associated antigens, and cellular damage-associated molecular patterns like calreticulin, heat shock proteins, and cellular ATP in a highly inflammatory process, termed "immunogenic cell death" and **(2) induction of host antitumor immune responses**. Cellular detection of viral infection and the products of oncolysis trigger the rapid activation of host antiviral responses and influx of immune cells that mediate the destruction of residual infected and uninfected tumor cells. The direct recognition and killing of tumor cells is primarily mediated by natural killer cells of the innate immune system and tumor antigen-specific CD8+ cytotoxic T lymphocytes of the adaptive immune system.

Tumor/Virus-Induced Cytokine Production

The TME is often characterized by a state of profound immunosuppression. Tumors overexpress cytokines like interleukin-10 and transforming growth factor-β (TGF-β), which inhibit natural antitumor immune responses. Tumor-derived cytokines and chemokines also include those promoting growth and vascularization like tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (25).

Viral infection stimulates the release of cytokines (IL-1, IL-6, IL-12, IL-18, IFN-γ, and TNF-α) and chemokines (RANTES, MIP-1α/β) from infected cells and resident and infiltrating immune cells, altering the balance of pro- and anti-inflammatory factors within the TME (26, 27). In addition to direct antiviral and immunoregulatory activities, these compounds mediate the recruitment of cytokine-releasing immune cells with additional effector functions. Viral infection and resulting localized inflammation enhance the effector functions of infiltrating immune cells, counteract tumor-induced immunosuppression, and facilitate the generation of antitumor immunity (27).

IMMUNOLOGIC BARRIERS TO SUCCESSFUL OVT

Viral infection and oncolysis naturally activate innate and adaptive immune responses that are known to contribute to the killing of malignant cells. However, host immune responses to viral infection have also been shown to be detrimental to the overall efficacy of OVT. Numerous preclinical studies have demonstrated reduced viral replication, earlier clearance, and decreased antitumor efficacy in immunocompetent, compared to immunocompromised, hosts (2, 6, 28). Mechanisms of immunologic barriers to successful OVT are shown in **Figure 2**. The avidity and timing of oncolysis and activation of different components of the host immune response seem to play vital roles in determining the nature and extent of their relative contributions to the overall efficacy of OVT, with vector species and malignancy-specific differences (29–31).

OVs AND INNATE IMMUNITY

Detection of viral infection triggers the production of antiviral proteins, elaboration of cytokines, and recruitment of immune cells to the site of infection. Type I IFNs are antiviral proteins that reprogram gene expression in infected and uninfected cells to directly inhibit viral replication. IFNs also induce cell cycle arrest and apoptosis, upregulate major histocompatibility complex (MHC) expression, stimulate B cell immunoglobulin synthesis, and prompt the development and proliferation of memory T cells (27). Among first responders to viral infection are APCs and other innate immune cells, including neutrophils and NK cells (27, 32). In addition to the release of antiviral cytokines, these cells have unique mechanisms through which they can contribute to the antitumor efficacy of OVT. Neutrophils react to pathogens by secreting reactive oxygen species and proteases, inducing necrotic cell death and localized inflammation (4). In a heterotopic murine model of colon cancer, intratumoral neutrophil accumulation in response to OV infection resulted in tumor vasculature destruction and widespread tumor cell apoptosis (33). NK cells have also been shown to be key effectors of OV-induced antitumor immune responses (20, 23, 29). They specifically target cells lacking MHC molecules or displaying virally induced markers of cellular stress

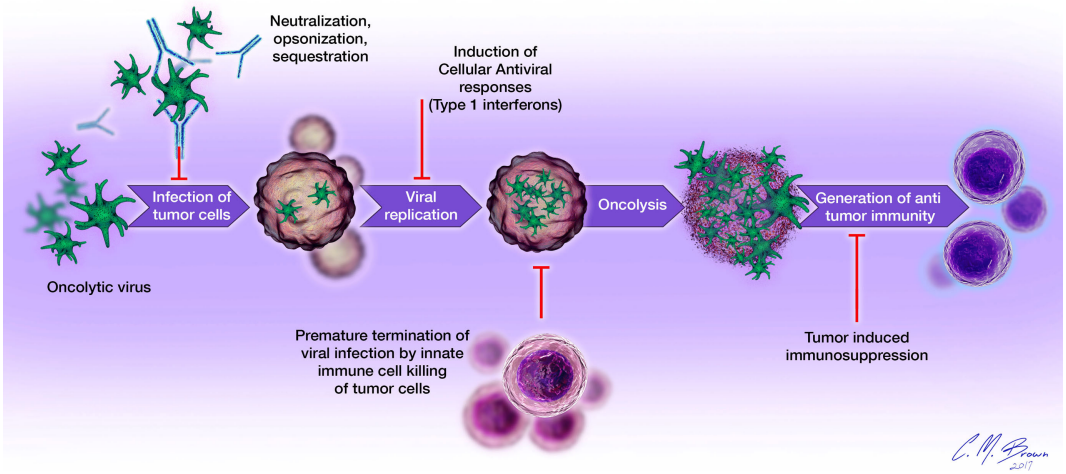

FIGURE 2 | Immunologic barriers to successful oncolytic virotherapy: (1) oncolytic virus delivery to tumor sites is impeded by the presence of neutralizing antibodies, complement proteins, and sequestration in organs such as the liver and spleen; (2) cellular antiviral responses, such as type I interferon signaling limits viral replication within tumor cells; (3) destruction of infected tumor cells by cells of the innate immune system (neutrophils, macrophages, NK cells) prematurely terminates viral infection; (4) tumor-induced immunosuppression (elaboration of immunosuppressive cytokines, accumulation of regulatory T cells, overexpression of negative checkpoint regulators of T cell function) inhibits the generation and effector functions of antigen-specific antitumor immune responses.

like MIC-A/B, inducing cell death by releasing granzyme and perforin enzymes, and activating apoptosis-inducing receptors (27, 28, 34). The agonist/antagonist relationship of the immune system and OV is not static but evolves with the phase of the infection and tumor destruction.

Decreasing Virus Clearance

In order to exert maximal therapeutic effects, OVs must persist long enough and induce sufficient oncolysis to stimulate the generation of long-lasting adaptive antitumor immunity. However, viruses are foreign pathogens and naturally elicit host immune responses mediating their clearance. Upon introduction to the body, viral particles become coated with neutralizing antibodies and are eliminated in a complement-dependent fashion (35). Destruction of infected tumor cells by infiltrating innate immune cells and viral antigen-specific T cells can also terminate OV infection before full therapeutic effects have been achieved (33). Transient suppression of these early immune responses has potential to improve OV delivery to tumor sites, prolong viral infection, and enhance the overall therapeutic efficacy of OVT.

Inhibiting early intratumoral immune cell infiltration with low dose chemotherapy or TGF-β treatment has been shown to enhance viral replication, decrease clearance, and improve antitumor outcomes in several murine models of glioma (32, 34, 36). A recombinant VSV vector expressing a broad-spectrum chemokine-binding protein had similar effects, substantially prolonging the survival of animals with multifocal hepatocellular carcinoma (37).

Pretreatment with immunosuppressive chemotherapeutics like cyclophosphamide has been shown to improve viral delivery, promote replication, and enhance oncolytic activity of HSV-based OVs in murine models of glioma by depleting antiviral antibodies and impairing complement function (32, 38, 39) Viral coat modification through conjugation of polymers like polyethylene glycol and *N*-[2-hydroxypropyl]meth-acrylamide (HPMA) or lipid encapsulation can shield OVs from neutralizing serum factors and prevent the generation of new antiviral antibodies (2, 24). Alternatively, OVs can be hidden within carrier cell vectors and trafficked to tumor sites. In the context of malignant brain tumors, two cell types that have shown promising preclinical potential as OV carriers are mesenchymal stem cells (MSCs) (40) and neural stem cells (NSCs) (41). Both MSCs and NSCs possess a natural tropism for primary tumors and their metastases and are considered immune-privileged. MSCs have been studied extensively in preclinical settings (40, 42). In a small clinical trial of children with metastatic neuroblastomas refractory to frontline therapies, treatment with autologous MSCs carrying ICOVIR-5, an oncolytic adenovirus, was found to be safe and without significant systemic toxicity (43). For malignant glioma, NSC-based carriers not only improve the clinical efficacy of OV by protecting viruses from the host immune system but also through amplification of therapeutic payloads selectively at tumor sites (44, 45). In a comparison of MSCs and NSCs as cellular carriers for OVs, NSCs conferred a superior therapeutic efficacy in the context of malignant glioma (46). Based on these promising preclinical findings, the FDA recently approved the NSC HB1.F3-CD as a cell carrier carrying CRAd-S-pk7 OV for clinical trials in patients with newly diagnosed malignant glioma.

OV Plus Chemotherapy

Preclinical and clinical studies have demonstrated significantly enhanced antitumor immune and clinical responses in patients receiving combination chemotherapy and OVT (4, 7–11, 14). The first such human clinical trial evaluated ONYX-015 (d11520), a genetically modified adenovirus, in combination with cisplatin

and 5-fluorouracil in 37 patients with recurrent squamous cell head and neck cancer. Objective clinical responses were observed in 65% of treated patients, exceeding response rates seen with either agent alone (4). Chemotherapy complements virotherapy through a variety of known and unknown mechanisms, including the direct killing of malignant cells, enhancement of tumor cell immunogenicity, and suppression of antiviral immune responses (15, 47). Several chemotherapeutic agents, including oxaliplatin, doxorubicin, bleomycin, Bortezomib, and cyclophosphamide, have been shown to induce tumor ICD, promoting the generation of antitumor immune responses (19, 26, 48).

OV Plus Immune Checkpoint Inhibitors

Destruction of malignant cells by the host immune system represents a crucial component of virotherapy. However, many tumors develop mechanisms to suppress the antitumor activity of incoming effector cells, for example by inducing overexpression of immune checkpoint regulators like CTLA-4 and PD-L1. T cell surface CTLA-4 competes with CD28 molecules for interaction with APC costimulatory molecules, transmitting inhibitory signals that suppress initial T cell activation. PD-L1, often overexpressed by tumor cells and tumor-infiltrating lymphocytes, binds PD-1 on activated T cells, inducing anergy or apoptosis (49, 50). Blockade of these molecules has been shown to improve T cell function and restore antitumor cellular immune responses. However, the clinical use of immune checkpoint inhibitors, particularly anti-CTLA-4 treatments, is limited by the high risk of associated severe autoimmune events resulting from systemic, uncontrolled T cell activation (50, 51). The unique ability of OVs to locally deliver and amplify therapeutic agents prompted an exploration of their use in combination with immune checkpoint inhibition. In a syngeneic murine model of malignant melanoma, the targeted, localized delivery of anti-CTLA-4 and anti-PD-L1 antibodies to the TME *via* an oncolytic measles virus induced comparably robust antigen-specific antitumor immune responses without evidence of immune-mediated toxicity (50). In another murine model of melanoma, intratumoral injection of combination NDV OV and anti-CTLA-4 antibody treatment resulted in regression of primary injected tumors and contralateral, untreated tumors, prolonged survival, and enhanced protection from tumor rechallenge as compared to treatment with either agent alone (49).

OV Plus Histone Deacetylase (HDAC) Inhibitors

Histone deacetylase inhibitors are an emerging class of antineoplastic agents that enhance the therapeutic efficacy of OVT primarily by suppressing the induction of IFN-stimulated genes (16). HDAC inhibitors have been shown to augment viral replication, reduce early intratumoral immune cell recruitment, and enhance the oncolytic activity of OVs in a variety of tumor types (16). As epigenetic modifiers of transcription, HDAC inhibitors also shift cellular profiles of gene expression to favor the induction of growth arrest and apoptosis in cancer cells, antagonize tumor angiogenesis, and enhance tumor cell immunogenicity through increased expression of MIC-A/B, MHC, and costimulatory molecules (52).

Increasing Antitumor Immune Response

Following initial OV-mediated tumor debulking, it is advantageous to promote host immune system-mediated destruction of any residual or recurrent malignant cells. This can be accomplished by mitigating tumor-induced immunosuppression, enhancing tumor cell immunogenicity, or directly activating host immune responses. Interventions that promote the development of localized inflammation can both counteract the immunosuppressive nature of the TME and recruit and activate effector immune cells. In a murine model of melanoma, OV expression of IL-12 and IL-18 increased intratumoral infiltration of activated NK cells, CD4+, and CD8+ T-cells, resulting in widespread tumor necrosis and prolonged survival (53). Combination treatment with other compounds that induce cellular stress or DNA damage, like chemotherapeutics, can enhance tumor immunogenicity by stimulating expression of NK cell-activating ligands and provoking tumor ICD (48). Increasing the availability of TAAs within the TME *via* induction of ICD or OV expression of specific TAAs can enhance antigen presentation and the generation of adaptive immunity. Incorporation of the ovalbumin protein within a VSV OV augmented the activation of ovalbumin-specific T cells, leading to increased antitumor effects in mice bearing B16ova tumors (54). Antigen-specific antitumor immune responses can be further enhanced by successive vaccination with two different TAA-expressing viruses, in which the second OV heightens the antitumor effects generated by the primary vaccination. This "prime-boost" method has been shown to induce durable adaptive immune responses that primarily target TAAs, rather than viral antigens (31, 55).

OVs AND ADAPTIVE IMMUNITY

Presentation of viral or TAA to cells of the adaptive immune system activates antigen-specific cellular and humoral immune responses. The primary antitumor effector cells of the adaptive immune system are CD8+ CTLs, which have been shown to be crucial mediators of OV-induced antitumor immunity, recognize specific antigens expressed on MHC class I molecules on the surface of infected and malignant cells and induce cell death through the release of perforin and granzymes. In the context of OVT, CTLs specific to viral antigens appear first, followed by development of TAA-specific CTLs (31). APCs also activate CD4+ T-helper cells, which release pro-inflammatory cytokines, promote CTL development, and are crucial in the development of antitumor immunity. Exposure to viral particles initiates humoral immune responses and the production of immunoglobulins from activated B cells. These neutralizing or opsonizing antibodies inhibit viral function and facilitate the clearance of viral infection (2, 28).

NEXT-GENERATION IMMUNE MODULATING OVT

The host immune response to viral infection remains both an untapped resource and significant challenge facing OVT. The antitumor effects of OVs can theoretically be maximized by mitigating early immune responses to allow OV replication, oncolysis,

and spread, followed by stimulation of the host immune system to destroy any residual tumor cells. Therapeutic manipulation of host immune responses represents a powerful strategy for optimizing both the oncolytic and immunotherapeutic potential of OVs. This can be achieved through combination therapy with chemotherapy, radiotherapy, immune checkpoint blockades, HDAC inhibitors, etc., or with single-agent OVs genetically engineered to express immune-modulating compounds.

In a murine model of colorectal cancer, OV expression of the chemokine RANTES (CCL5) prolonged the persistence of an oncolytic vaccinia virus, increased intratumoral lymphocyte infiltration, and enhanced antigen-specific antitumor responses, particularly in combination with DC-based immunotherapy (56). OV delivery of cytotoxic compounds or prodrug-activating enzymes can induce localized tumor damage without systemic side effects (56). AD5-CD/TKrep, an adenovirus expressing a cytosine deaminase/thymidine kinase (CD/TK) fusion protein that locally activates 5-fluorocytosine and ganciclovir pro-drugs, has been evaluated in two phase I clinical trials in patients with prostate cancer (9, 57).

FUTURE DIRECTION

The future of OVT will focus on understanding and optimizing the complex interactions between OVs, tumor cells, and the host immune system. Elucidating relationships between factors such as patient immune status, malignancy type, tumor mutation profiles, and OV vector species, and patient responses to virotherapy will aid in the development of more efficacious, personalized treatments. Exploration of methods to improve OV access and delivery to tumor sites in terms of optimizing cell carrier-based delivery systems that maximize the therapeutic payload to the TME at the same time modulating host immune system are also promising areas of research. Overall, the most effective anticancer treatments will likely utilize a combination of therapies with different, synergistic mechanisms of tumor destruction.

CONCLUSION

Oncolytic virotherapy is a novel approach to cancer treatment, uniquely combining direct cytotoxicity with antitumor immunotherapy. Clinical trials have established the safety and clinical efficacy of OVs, culminating in the recent FDA approval of the OV, T-VEC for treatment of malignant melanoma. An improved understanding of the relationships between OVs, tumors, and the host immune system will be necessary in the development of the next generation of OVs. Future OVs with improved tumor selectivity and cytotoxicity delivered in combination with immune-modulating therapies will significantly enhance the contribution of OVT to the field of oncology.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Oncolytic Immunotherapy: Conceptual Evolution, Current Strategies, and Future Perspectives

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The concept of oncolytic virus (OV)-mediated cancer therapy has been shifted from an operational virotherapy paradigm to an immunotherapy. OVs often induce immunogenic cell death (ICD) of cancer cells, and they may interact directly with immune cells as well to prime antitumor immunity. We and others have developed a number of strategies to further stimulate antitumor immunity and to productively modulate the tumor microenvironment (TME) for potent and sustained antitumor immune cell activity. First, OVs have been engineered or combined with other ICD inducers to promote more effective T cell cross-priming, and in many cases, the breaking of functional immune tolerance. Second, OVs may be armed to express Th1-stimulatory cytokines/chemokines or costimulators to recruit and sustain the potent antitumor immunity into the TME to focus their therapeutic activity within the sites of disease. Third, combinations of OV with immunomodulatory drugs or antibodies that recondition the TME have proven to be highly promising in early studies. Fourth, combinations of OVs with other immunotherapeutic regimens (such as prime-boost cancer vaccines, CAR T cells; armed with bispecific T-cell engagers) have also yielded promising preliminary findings. Finally, OVs have been combined with immune checkpoint blockade, with robust antitumor efficacy being observed in pilot evaluations. Despite some expected hurdles for the rapid translation of OV-based state-of-the-art protocols, we believe that a cohort of these novel approaches will join the repertoire of standard cancer treatment options in the near future.

Keywords: immunogenic cell death, ICD inducer, antigen, cross-presentation, immune checkpoint blockade, antitumor immunity, T cells, combination

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INTRODUCTION

Successful cancer therapy using oncolytic viruses (OV) is predicated on at least three major (and coordinate) mechanisms of action. Among them, the first is the direct infection of cancer cells and endothelial cells and the subsequent oncolysis of these cells in the tumor microenvironment (TME). The second involves indirect effects of necrosis/apoptosis of uninfected cancer cells and associated endothelial cells in the tumor-associated vasculature leading to reduced angiogenesis (1–3). Finally, antitumor (and antiviral) immunity is elicited/expanded by the OV as a consequence of improved antigen cross-priming and recruitment of immune cells into the TME. More

than 10 years ago, most, if not all, investigators thought that the direct oncolysis was the only major mechanism by which OVs inhibited tumor growth, leading to the terminology of "oncolytic virotherapy," coined by Kirn in 2001 (4). Later, investigators discovered that the host immune response was critical to the antitumor efficacy of oncolytic virotherapy. Briefly, this has been shown through multiple approaches including the use of (1) an OV encoding a tumor antigen to potently activate therapeutic T cell responses (5); (2) reovirus infection of tumor cells to prime antitumor immunity capable of reducing metastatic disease burden (6); and (3) $CD8^+$ T cell depletion resulting in the loss of efficacy associated with OV-based treatment (7). Thus, OV represents a novel form of immunotherapy (8), with Rommelaere and associates formally advocating the term "oncolytic immunotherapy" in their article published in 2011 (9). Since then many other investigators, including our group, have adopted this terminology (10–14). As most investigators have discovered, single modality therapies (including OV) may be insufficient to effect cure in the cancer setting, mandating the development of combination protocols implementing antitumor agents capable of yielding additive or synergistic antitumor benefits. Our discussion will focus on combination regimens likely to yield superior antitumor immunity associated with improved treatment outcomes.

THE CONCEPTUAL SHIFT FROM VIROTHERAPY TO ONCOLYTIC IMMUNOTHERAPY

Although the use of viruses as oncolytic agents has a rich history, the application of genetically engineered viruses to selectively target cancer cells is a relatively recent adaptation (15). The first research article reporting the use of a genetically engineered OV was published by Martuza and colleagues in 1991 (16), in which the authors showed that infection with a thymidine kinase (*tk*) gene-deleted herpes simplex virus (HSV) led to the death of multiple human glioma cell lines, as well as, primary cultures of human glioma cells. Furthermore, they demonstrated that intratumoral inoculation of the *tk* gene-deleted HSV led to the slowed growth of human glioma xenografts in SCID mice and to the extended overall survival of these animals. In most early studies, it was thought that the major mechanism associated with OV treatment benefit involved selective viral replication in cancer cells and consequent tumor cell lysis or apoptosis (17). For example, an oncolytic HSV-mediated tumor inhibition showed equivalent effects in immune-competent and immune-incompetent mice, suggesting that viral oncolysis and not the host immune response was the primary mechanism linked to tumor destruction (18). Thus, investigators at that time paid significant attention to remove viral genes that would limit tumor cell lysis or apoptosis, such as the adenovirus gene encoding E1B-19 kDa protein (19) or vaccinia virus (VACV) genes for SPI-1 and SPI-2 (20). In addition, to accentuate such pathways, OVs commonly incorporated suicide genes or genes promoting apoptosis such as a suicide gene encoding purine nucleotide phosphorylase (21), apoptosis-inducing gene TRAIL (22), or tumor suppressor gene TP53 (23, 24).

Yet, investigators repeatedly noticed the critical role of antitumor T cells in OV-mediated therapeutic efficacy in their studies. In 1999, Martuza and associates found that infection of established CT26 tumors in mice using an HSV-1 OV G207 led to the generation of highly specific, systemic antitumor immunity (25). Later, Vile and associates demonstrated that tumor infection by oncolytic reovirus primes adaptive antitumor immunity (6). They also showed that $CD8^+$ T cells played a critical role in the therapeutic efficacy of intratumorally delivered vesicular stomatitis virus (VSV), with these T cells specific for immunodominant epitopes derived from both viral- and tumor-associated target proteins (5). The authors utilized two approaches to show the important roles of $CD8^+$ T cells in this therapy. First, by increasing the circulating levels of tumor antigen-specific T cells using adoptive T cell transfer, in combination with intratumoral virotherapy, the investigators observed significantly enhanced therapeutic efficacy over either monotherapy. Second, the integration of a tumor-associated antigen (TAA) within the oncolytic VSV was found to increase the level of activation of naive T cells recognizing that antigen, in association with enhanced antitumor activity. As a consequence, they termed their approach an "oncolytic immunovirotherapy" (5). Zhang and associates showed that tumor destruction after delivery of an HSV2-based OV (FusOn-H2) *in vivo* induced potent antitumor immune responses in a syngeneic neuroblastoma model (26, 27). Even UV-inactivated Sendai virus (particle) was shown to eradicate tumors by promoting antitumor immunity as a consequence of blocking the immunosuppressive action of regulatory T cells (Tregs), believed to be mediated *via* the viral particle-induced secretion of IL-6 from activated dendritic cell (DC), independent of cancer cell infection (28). In addition, investigators have developed OV armed with genes to stimulate immune responses, as showcased by T-VEC, originally constructed and tested in 2003 (29). On the basis of an increasing body of evidence, we and others concluded that OVs are promising novel immunotherapeutic strategies (8, 30). More recently, Bhat et al. have coined the term "oncolytic immunotherapy" in reference of their study of oncolytic parvovirus to activate NK cells capable of killing cancer cells in 2011 (9). Hemminki and associates have also applied this term in their clinical study using an oncolytic AdV expressing CD40L, where they observed induction of potent tumor antigen (surviving)-specific $CD8^+$ T cells associated with robust antitumor activity (10). Many in the field have now adopted this nomenclature as it is believed to best reflect the intrinsic immunologic mechanisms of action associated with this class of novel antitumor agents (8, 10–14, 30, 31).

CURRENT STRATEGIES IN ONCOLYTIC IMMUNOTHERAPY

In this section, we will introduce the concept of tumor immunogenic cell death (ICD), how OVs induce ICD, and how this may lead to the development of potent, durable antitumor immune responses in treated individuals. We will then discuss current concepts for preclinical studies and the clinical implementation of OVs as monotherapies or combination protocols integrating a range of chemotherapeutic agents or immunomodulatory compounds.

Immunogenic Cell Death (ICD)

In a previous review, we summarized the developmental concept of ICD and key features of this type of cell death that leads to robust antitumor immune responses (12). Here, we will update this important and evolving paradigm and discuss new findings related to the role of OV-associated ICD with the development of therapeutic antitumor immunity.

Intrinsic to this discussion is the question of how the immune system senses danger associated with pathogenic infection or the development of a pathologic state (such as cancer). As Janeway summarized, the immune system distinguishes self from non-self "events" based on the surveillance of differences and danger signals predicated on so-called immune signals 1, 2, 3, and 0 (32). Signal 0 derives from pathogens and is now called pathogen-associated molecular patterns (PAMPs). In 1994, Matzinger proposed that danger signals are also communicated from the inside of dying cells, i.e., damage-associated molecular patterns (DAMPs) (33, 34). In recent years, ICD in tumor cells has been viewed as critical to the development and sustainability of protective adaptive immune responses. To qualify as ICD, dying tumor cells must possess characteristics associated with immune signal 0 (danger) and signal 3 (inflammatory cytokines) that are required to instruct host DCs to take up tumor cell bodies, to mature and process these antigens into MHC-presented peptides, and to cross-prime antitumor T cells in a manner that results in the activation and expansion of cytotoxic T cells capable of emigration back to sites of disease.

In 2014, a group of key investigators from around the world working on ICD reached a consensus that there were at least three key feature molecules (DAMPs) required for the process of ICD. These include cell surface-exposed calreticulin, extracellular ATP and high mobility group box 1 (HMGB1), and/or the pathways allowing for their emission from dying cells, such as endoplasmic reticulum stress, autophagy, and necrotic plasma membrane permeabilization (35). When Zitvogel, Kroemer, and others originally proposed the concept, ICD included only the consideration of immunogenic apoptosis (36, 37). However, in 2013, our group, and that of Inoue and Tani, independently proposed that ICD includes not only immunogenic apoptosis but also necroptosis, autophagic cell death, and pyroptosis of cancer cells (30, 38). This extension has been validated by a number of recent studies. For example, necroptotic cancer cells induce ICD, and vaccination with such dying cancer cells induces efficient antitumor immunity (39). With a greater understanding of various mechanisms of cell death, the concept of ICD has continued to evolve. This year, Galluzzi and colleagues have further revised the ICD concept to now include additional types of cell death (such as necroptosis, pyroptosis) as we and other groups had originally proposed in 2013 (30, 38, 40).

A variety of therapeutic regimens and factors induce ICD in cancer cells (41). They include physical (radiotherapy and photodynamic therapy) (42), chemical (such as anthracyclines, oxaliplatin) (41), and biological ones. These biological agents include some OVs, immunogenic peptide (43), and other microorganisms and their products as they are potent PAMPs and more. We may arbitrarily think that infection with OVs automatically makes tumor cells highly immunogenic; however, this is not a guarantee as many viruses have evolved molecular mechanisms that subvert the exposure of DAMPs (such as ecto-CRT), thereby limiting the magnitude of ICD and thus consequent immune detection of such infected cells (12, 44). Indeed, such viruses induce cell death *via* non-immunogenic (sterile) apoptosis.

OVs Induce *Bona Fide* ICD in Cancer Cells and May Interact Directly with Immune Cells, Leading to the Activation of Innate and Adaptive Immune Cells

Even though a variety of OVs have been shown to induce some features of ICD, few have been conclusively shown to represent *bona fide* inducers of tumor ICD. Based on the consensus-recognized ICD signature molecules (i.e., ecto-CRT, extracellular ATP, and HMGB1), only one OV thus far appears to meet the criteria for designation as an ICD-promoter: coxsackievirus B3 (45). However, a number of other OVs may also induce *bona fide* ICD, as they indeed serve to prime/induce adaptive antitumor immunity *in vivo*. The list is quite long and includes oncolytic adenovirus (46), influenza virus (47), HSV (25, 48, 49), measles virus (MeV) (50), NDV (51), VSV (5), and Sendai virus (52). However, we wish to emphasize that significantly more investigations will be required to validate such conjecture.

Some unarmed OVs possess the potential to activate innate and adaptive immunity. For example, an HSV-2 mutant, called ΔPK (due to the deletion of ICP10 that has protein kinase activity), has strong oncolytic activity for melanoma, induced mainly by a mechanism other than replication-induced cell lysis. It was found that it induced multiple non-redundant programmed cell death pathways (53). ΔPK inhibited the secretion of IL-10 from melanoma cells through virus replication and *c*-Jun *N*-terminal kinase/*c*-Jun activation. The virus-induced IL-10 inhibition led to enhanced cell surface expression of MHC class I chain-related protein A, the ligand for NKG2D receptor expressed on NK and $CD8^+$ T-cells. Concomitantly, ΔPK also enhanced the secretion of TNF-α, GM-CSF, and IL-1β through autophagy-mediated activation pathways of Toll-like receptor 2 and pyroptosis and inhibited the expression of CTLA-4, one of the key negative immune checkpoint molecules (54).

Interestingly, ICD is not the only pathway by which OVs may modulate the host antitumor immune response. OVs may interact directly with immune cells to prime antitumor as well as antiviral immune responses. Reovirus may function as a PAMP interacting directly with DC, thus promoting DC maturation and stimulating the production of pro-inflammatory cytokines that may activate innate antitumor immunity (55). In contrast, reovirus may also infect tumor cells, leading to the (cross) priming of adaptive antitumor immunity (6). VSV can infect DC, leading to the improved capacity of these antigen-presenting cells to prime innate and adaptive antitumor immunity (56). The interaction of VACV with DC is a complex story. *In vivo*, both $CD8^+$ and $CD8^-$ DC are infected with VACV, resulting in the generalized upregulation in the expression of costimulatory molecules. However, IL-12 production is restricted to a subset of non-infected DCs (57). Interestingly, VACV may modulate the biological activity of another important immune cell type in the TME. Tumor-associated

$CD11b^+Ly6G^+$ myeloid-derived suppressor cells (MDSCs) are normally immunosuppressive. Oncolytic VACV recruits MDSC with enhanced iNOS expression, which leads to beneficial anti-tumor activity. Depletion of iNOS-producing cells leads to very rapid tumor growth postvirus injection. These results suggest that the virus-induced $iNOS^+$ MDSCs could represent an important antitumor effector cell population in the TME (58).

Many studies have shown that OVs elicited antitumor immunity, and this significantly contributed to the overall efficacy of the virus-mediated cancer therapy. As early as 1999, Toda et al. have shown that an oncolytic HSV (G207) could function as an *in situ* vaccine to induce specific antitumor immunity (25). An OV (e.g., MeV, Parvovirus H-1, or reovirus) would induce cancer cell oncolysis and allows DC to cross-prime tumor-specific $CD8^+$ T cell response (6, 59, 60). As we will discuss later, arming the OVs with immune-stimulatory molecules would further promote eliciting potent antitumor immunity. A number of groups have shown that when the OVs encode a TAA, these OVs worked effectively as cancer vaccines (61–64). The antitumor immunity mounted by OVs have also been demonstrated in human cancer patients treated with oncolytic MeV (62, 65), HSV (66), AdV (67), and VACV(68).

OVs Expressing Th1-Stimulatory Molecules

To enhance the efficacy of antitumor immunity (**Figure 1**), many investigators have armed OVs with immune stimulatory genes. These may include costimulatory molecules, cytokines, and chemokines, such as IL-2, IL-12, IL-18, IFN-α/β, TNF-α, or GM-CSF, that are capable of promoting the development of cytotoxic immune effector cells.

All of these viruses are designed to further stimulate systemic antitumor immunity and to promote the trafficking of immune cells into the TME. Arguably, the best-studied OVs have been those armed with GM-CSF. The first such agent in the class approved by FDA is T-VEC, a HSV armed with GM-CSF for the treatment of patients with advanced-stage melanoma. Pexa-Vec, a VACV armed with GM-CSF, is currently being evaluated in a PHOCUS (phase III) global clinical trial in the setting of advanced-stage hepatocellular carcinoma (HCC). An additional oncolytic AdV armed with GM-CSF, designated CG0070, is being assessed for efficacy in a phase III clinical trial for the treatment of high-grade non-muscle invasive bladder cancer after failure to treatment with Bacillus Calmette–Guérin (BCG).

T-VEC represents a rationally designed OV to stimulate anti-tumor immunity based on engineering the viral vector to encode an immune-stimulatory gene. First, the virus was modified through deletion of two non-essential viral genes for replication in cancer cells (ICP34.5 and ICP47). ICP34.5 is a neurovirulence factor gene and its deletion attenuates viral pathogenicity and enhances tumor-selective replication (69). The second viral gene is ICP47, and its encoded protein enhances viral neurovirulence by limiting $CD8^+$ T cell responses (70). Deletion of the ICP47 gene reduces viral-mediated suppression of antigen presentation and increases the expression of the HSV Us^{11} gene. The virus

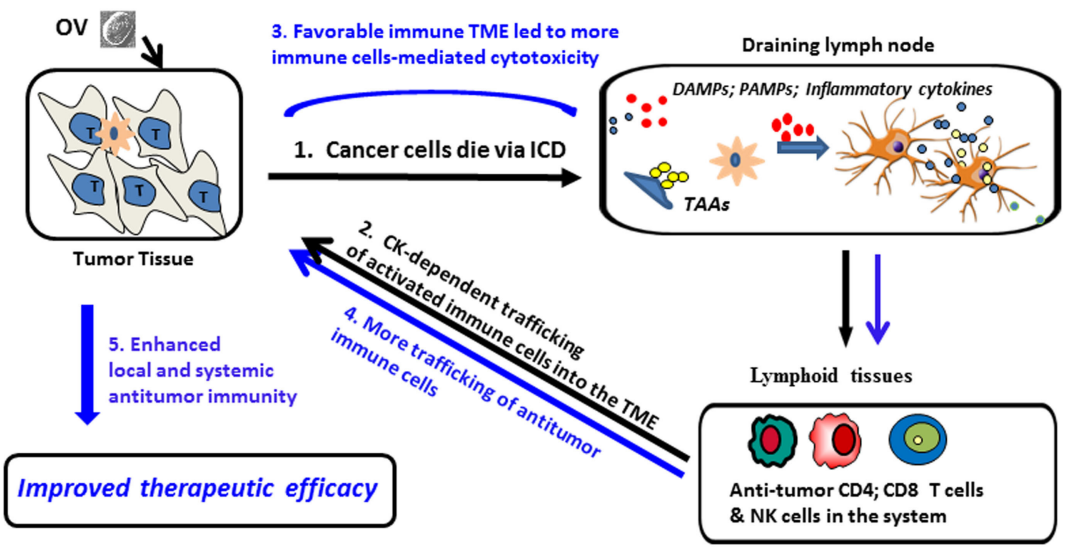

FIGURE 1 | Proposed model for ICD and pro-inflammatory cytokines/chemokines (Th1) promotion of oncolytic virus (OV)-mediated antitumor-immunity. (1) OV infects tumor cells and induces ICD, leading to the release/presentation of signal 0 [damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs)], along with tumor-associated antigens (TAAs) to dendritic cells (DCs), resulting in DC activation and Ag cross-presentation to antiviral and antitumor immune cells (activated NK cells, $CD4^+$ and $CD8^+$ T cells), followed by clonal expansion and maturation of antitumor T effector cells. (2) Cytokines/chemokines released during the acute inflammation in the tumor microenvironment (TME) promote trafficking of therapy-induced immune cells into the TME; (3) inflammation in the TME is sponsored by both viral- and tumor-reactive T cells, with immune-mediated eradication of tumor cells and tumor-associated stromal cells. Additional danger signals (signal 0), inflammatory cytokines, and chemokines (signal 3) and TAAs (signal 1) further activate tumor-associated DCs, overcoming local immunosuppression and prolonging the survival and functionality of antitumor immune cell populations; (4) reiterative rounds of DC-mediated cross-priming continue to allow for delivery of new (reinforcement) T immune effector cells into the TME (5) allowing for sustained antitumor efficacy within disseminated sites of disease.

was then modified by insertion of cDNA encoding the cytokine GM-CSF. The infection of cancer cells by T-VEC induces ICD and local expression of GM-CSF, resulting in the recruitment, activation, and maturation of antigen-presenting cells, which are competent to promote tumor-specific T-cell responses (29).

Other OVs have been armed with chemokine genes. Expression of CCL5 (RANTES) from an OV has been shown to recruit DC, macrophages, NK, and $CD8^+$ T cells into tumor sites, in association with the development of enhanced tumor antigen-specific $CD8^+$ T cell and NK cell-mediated immune responses (71, 72). Recently, we have developed an OV encoding the chemokine CXCL11, designed to recruit $CXCR3^+$ antitumor T effector cells and NK cells into the TME to mediate improve therapeutic efficacy (73). Although infection with this OV indeed led to these expected outcomes, we unexpectedly observed that vvDD-CXCL11 (but not parental vvDD) induced a systemic increase in tumor-specific IFN-γ-producing $CD8^+$ T cells in treated animals. In an immunogenic tumor model, this therapy led to tumor regression and extended survival benefit, which was strictly dependent on $CD8^+$ T cells and IFN-γ, but not $CD4^+$ T cells (73). However, in a non-immunogenic tumor model, treatment with vvDD-CXCL11 monotherapy was not effective, necessitating its combination with a drug cocktail chosen for its ability to (re) condition the TME, which led to improved therapeutic efficacy in the MC38 colon tumor model (74).

Oncolytic virus expressing costimulatory molecules have also been explored. An oncolytic VACV expressing the 4-1BBL T-cell costimulatory molecule (rV-4-1BBL) was shown to be moderately effective in treating poorly immunogenic B16 melanomas in mice. Interestingly, when rV-4-1BBL treatment was combined with a lymphodepletion regimen, the authors observed enhanced tumor MHC class I expression, the promotion of viral persistence, and the rescue of effector-memory $CD8^+$ T cells in association with improved therapeutic efficacy (75). When an oncolytic VACV was combined with an agonist antibody (Ab) specific for the costimulatory molecule 4-1BB (CD137), the dual treatment led to enhanced antitumor immunity and robust suppression of tumor growth in murine models (76). Enhanced immunity was associated with increased numbers of ($CD11b^+$ and $CD11c^+$) myeloid cells in tumor draining lymph nodes and enhanced infiltration of both NK cells and $CD8^+$ T effector cells into the TME (76). Allison and associates have recently constructed an oncolytic NDV expressing the inducible costimulator and shown that when applied as an intratumoral therapy in combination with systemic CTLA-4 blockade, which treated mice exhibit enhanced infiltration of activated T cells in both virus-injected and uninjected, distal tumors that is curative in the B16-F10 tumor model (77).

We and other investigators in the field continue to search for new and exciting factors for inclusion in cutting-edge OV-based immunotherapies. In this regard, one of our groups has recently discovered the potent antitumor action of the IL-1 family member IL-36γ, which coordinately activates $CD8^+$ T cells, NK cells, and Tγ/δ cells and synergizes with TCR activation and the type-1 polarizing cytokine IL-12 (78). When present within the TME, IL-36γ exerts profound antitumor activity *in vivo*, suggesting the great potential of this pro-inflammatory cytokine in OV-based cancer therapeutics.

Combination of OV with Other Therapeutic Regimens/Drugs to Favorably Correct and Optimize the Immunologic TME

The cellular cross-talk between tumor cells and stromal cells within the TME, which is often mediated through soluble factors, creates an immunosuppressive environment that allows for enhanced viral replication and oncolytic activity in immune-deficient mice (79). The expression of VEGFR, which promotes tumor angiogenesis and progression, sensitizes the tumor vasculature to infection by oncolytic VACV (80). However, the TME coordinately inhibits protective antitumor immune responses that are crucial to the overall therapeutic efficacy of OVs applied to the immunocompetent (tumor-bearing) host. As a consequence, investigators have developed a variety of strategies including arming viruses with therapeutic genes or coapplying pharmaceutical interventions that promote ICD and/or that facilitate antigen cross-presentation in support of developing therapeutic antitumor T cell responses (81). We will discuss six strategies in this section.

Combination of OV with Conventional Chemotherapeutic Agents That Induce ICD

Many traditional chemotherapeutic agents possess the capacity to enhance host immunity (82). It is therefore logical to combine OV with this type of conventional drug to effect greater clinical benefit in the cancer setting. Combination treatments utilizing OVs and other pharmaceutical drugs have been reviewed extensively by Forbes et al. (83). We will discuss two recent studies to illustrate the most critical points. In the first study, the authors used autophagy stimulating or inhibitory drugs to determine if autophagy meaningfully impacts the outcome of oncolytic virotherapy. They showed that chloroquine or rapamycin significantly potentiate NDV-mediated oncolytic activity in mice bearing drug-resistant lung cancer (84). In this case, the exact mechanisms underlying treatment benefit remain to be elucidated. In another study, treatment with HSV-1 ICP0 null OV KM100 alone was determined insufficient to break immune tolerance in a breast tumor model; however, Workenhe et al. showed that by combining the virus with the ICD-inducing chemotherapy agent mitoxantrone, a significant survival benefit was gained for mice bearing Her2/neu TUBO-derived tumors. The take-home lesson was that such combination OV-based regimens coordinately enhances tumor immunogenicity, breaks immunologic tolerance established toward TAAs, and elicits superior therapeutic benefit (85).

Combination with Other Immunotherapies to Recruit and Sustain Protective Antitumor Immunity in the TME

By using tumor explant models, we investigated the impact of 3 in-clinic drugs for their ability to productively modulate the inflammatory characteristics of the TME: IFN-α, poly-I:C (a TLR3 ligand), and a COX-2 inhibitor (86–88). Tumor tissues reacted to individual drugs heterogeneously. A combination of IFN-α and poly-I:C uniformly enhanced the production of preferred (type-1 T cell recruiting) chemokines CXCL10 and CCL5, while reducing local production of CCL22, known to recruit

suppressor cell populations. The addition of a COX inhibitor to this combination further enhanced these effects (86). We then applied this cocktail of agents to a colon tumor model in conjunction with the delivery of an oncolytic VACV. Sequential treatment with the virus vvCXCL11 and then the drug cocktail resulted in the upregulated expression of Th1-attracting CKs and a reduction in expression of the Treg-attracting CKs (CCL22 and CXCL12), in concert with enhanced trafficking of tumor-specific $CD8^+$ T cells and NK cells into the TME. Notably, this combination regimen led to the greatest degree of therapeutic antitumor activity and to the long-term survival of the treated mice (74).

Another strategy is to engineer OV with a gene that serves as an antagonist to a dominant suppressor cell type or suppressor soluble mediator in the TME. MDSCs are one of the major regulatory cell subpopulations in the TME, where they promote tumor growth and progression (89). The inhibition of tumor-derived prostaglandin-E_2 (PGE_2) would be expected to block the induction of MDSCs and the recovery of NK cell activity (90). 15-Prostaglandin dehydrogenase (15-PGDH) is a tumor suppressor protein that is responsible for the degradation of PGE_2. Walker et al. have constructed an oncolytic HSV expressing 15-PGDH and demonstrated that the delivery of this virus mitigates immune suppression and inhibits the growth of primary and metastatic breast cancer in a murine model (91). Recently, Hou et al. have also shown that an oncolytic VACV expressing this enzyme overcomes local immunosuppression, leading to profound changes in protective immune function within the TME. Such engineered OVs promote robust adaptive antitumor immunity and sensitize established and previously resistant tumors to regulation by immunotherapies (92).

Use a Vaccine Monotherapies or Combination Therapies

That OVs may function as effective cancer vaccines and impediments to their biologic activity have been discussed extensively in several recent reviews (30, 93–95). Here, we will focus on a discussion of prime-boost strategies as these relate to the use of OVs as cancer vaccines.

Heterologous prime-boost vaccination, a well-documented regimen to elicit robust $CD8^+$ T cell responses, has been applied within the context of oncolytic immunotherapy. The first such study was carried out by Wan and colleagues, employing an antigen-expressing VSV and AdV. Intranasal delivery of the OV VSV-hDCT resulted in the activation of both $CD4^+$ and $CD8^+$ DCT-specific T-cells. These responses were significantly increased by subsequent booster vaccination using recombinant Ad (Ad)-hDCT. This regimen resulted in enhanced therapeutic efficacy against established B16-F10 melanomas in mice (96). In another study, the authors used recombinant VSV as a booster vaccine and demonstrated a massive increase in the secondary expansion of $CD8^+$ antigen-specific T cells after priming with recombinant AdV (97). Vile et al. have also recently showed that a prime-boost vaccine regimen using distinct OVs (reovirus and VSV), when applied in combination with immune checkpoint blockade results in improved antitumor immunity/efficacy in the B16 melanoma model (98). Song, Kim, and others have developed a hybrid regimen using a complex of DNA and oncolytic AdV to treat malignant melanoma in a syngeneic mouse model (99). In this protocol, MART1 plasmid was used as a DNA-based vaccine to induce specific immunity, while the gene encoding murine GM-CSF and shRNA against mouse TGF-β2 were codelivered with MART-1 cDNA *via* an oncolytic AdV. This heterologous prime-boost vaccine strategy resulted in delayed tumor growth, likely resulting from (i) the induction of anti-MART1 T effector cells, (ii) enhanced antigen-presentation driven by GM-CSF and TGF-β2 shRNA, (iii) tumor growth inhibition by TGF-β2 shRNA, and (iv) tumor cell-specific OV-induced oncolysis (99).

Combination with CAR T Cell-Based Adoptive Immunotherapy

CAR T cells represent one of the most promising new approaches in cancer immunotherapy (100), with only a single study thus far integrating OV (101). In this report, an oncolytic Ad (Ad5Δ24) was armed with chemokine genes CCL5 and IL-15 and applied as a recruiter (*via* CCL5) and sustainer (*via* IL-15) of CAR-T cells (reactive against the tumor-associated ganglioside GD2) into/within the TME. Application of the OV was observed to enhance the function of CAR T cells *in vivo*, with the combination immunotherapy extending overall survival in mice bearing neuroblastomas.

Combination with Bispecific T-Cell Engagers (BiTEs)

So far two studies have explored this novel approach. Song and associates constructed an oncolytic VACV encoding a secretory BiTE composed of two single-chain variable fragments specific for CD3 and the tumor cell surface antigen EphA2 (EphA2-TEA-VV) (102). This virus, when combined with human T cells, exhibited potent antitumor activity in a lung cancer xenograft model. Earlier this year, Alemany and associates generated an oncolytic AdV encoding a BiTE (cBiTE) coordinately targeting EGFR and CD3 (ICOVIR-15K-cBiTE). Intratumoral injection of this recombinant AdV increased the persistence and accumulation of tumor-infiltrating T cells *in vivo*. This OV, when combined with peripheral blood mononuclear cells or T cells exhibited enhanced antitumor efficacy (103). The results from these two studies suggest that BiTE-armed OVs may overcome some key limitations associated with current oncolytic virotherapy-based strategies.

Combination with Complement Inhibition

Natural barriers in the blood, including neutralizing antibodies and complement, likely limit our ability to repeatedly administer the same OVs intravenously. As a consequence, it makes sense to consider means by which to coordinately inhibit complement activation to improve the utility and antitumor efficacy of OV-based immunotherapies. We showed that inhibitors of C5 complement enhanced the infection of cancer cells by VACV *in vitro*, even in the absence of antivaccinia antibodies (104). In a recent study, Evgin et al. demonstrated that in immunized rats, complement depletion stabilized VACV in the blood, resulting in the improved delivery of virus into the TME (105).

Combination with Immune Checkpoint Blockade

Immune checkpoint blockade-based immunotherapy has made major advances over the past several years, to now become standard of care in the setting of many forms of cancer. Since the anti-CTLA4 Ab (ipilimumab) was FDA approved for use in patients with advanced-stage melanoma in 2011, immune checkpoint antagonists (including anti-CTLA4 and anti-PD-1/PD-L1 antibodies) have now been approved for use against six forms of cancer. Immune checkpoint molecules are a natural means used by the immune system to maintain homeostasis, ensuring self-tolerance and the prevention of pathologic autoimmunity. In tumor tissues, however, these signals are often upregulated, allowing for progressively growing tumors to evade local protective immune responses (106).

Despite enthusiasm for the continued clinical use of immune checkpoint blockade as a general strategy to combat cancer, this approach works best in patients who exhibit existing evidence of ongoing antitumor immune responses, and it fails in cases where the TME is devoid of a protective immune signature. Furthermore, even in the setting of advanced-stage melanoma, only 15–25% patients exhibit durable objective clinical responses. Thus, there exists obvious potential for synergy between therapeutic regimens using OVs and immune checkpoint blockade. Mechanistically, OVs offer the possibility of priming, boosting, and recruiting effector T cells into the TME, where immune checkpoint blockade may serve to enhance/sustain the potency of antitumor TIL *via* the removal of inhibitory signals (94, 107).

In such combination immunotherapies, the immune checkpoint antagonist Ab could be physically delivered as a protein or encoded by a recombinant OV used to infected cancer cells. The first study for such a combination approach was published by Hemminki and his team in 2012, demonstrating that targeted cancer immunotherapy could be achieved using an oncolytic AdV encoding a fully humanized monoclonal Ab reactive against CTLA-4 (108). Since then, several original research papers on this exciting combination strategy have been published (109–118).

Zamarin and others demonstrated in mouse models that localized immunotherapy with oncolytic NDV combined with anti-CTLA4 Ab could cure the majority of treated tumor-bearing mice, while treatment with NDV alone was effective in only 10% of cases. Importantly, this combinatorial strategy was observed to induce an immune response against both virally infected and control, uninfected tumors, with minimal reactivity noted against unrelated, third-party tumors. Interestingly, the antitumor efficacy of this approach was dependent on $CD8^+$ T-cells, NK cells, and type I IFN, but not on oncolysis. Treatment with this combination of oncolytic NDV and anti-CTLA4 Ab led to systemic tumor rejection and subsequent protection of the host against tumor rechallenge in poorly immunogenic tumor models (111).

Vile and colleagues have used a prime-boost vaccine regimen with separate OVs in concert with immune checkpoint blockade to further improve antitumor efficacy in combination approaches (98). They hypothesized that reovirus-induced $CD8^+$ antitumor T cell responses, when combined with the VSV-ASMEL-induced $CD4^+$ Th17 responses, would result in potent antitumor immunity/efficacy. In their study, tumor-bearing mice were first treated with intratumoral injection of reovirus, followed by intravenous delivery of VSV-ASMEL. This regimen significantly improved the overall survival of mice bearing subcutaneous B16 melanoma. Finally, the triple combination immunotherapy significantly enhanced survival of mice, with improved frequencies of durable cures (versus mono- or dual-component treatment cohorts), in association with robust Th1 and Th17 immune responses against tumor antigens (98).

In our recent study, we explored the efficacy of combined therapy using oncolytic VACV and anti-PD-L1 Ab in murine colon and ovarian cancer models (118). We hypothesized that an oncolytic VACV would elicit antitumor adaptive immune response and attract T cells into the tumor, with the resulting inflammation promoting PD-L1 expression in both cancer and immune cells, making the TME susceptible to subsequent treatment with the anti-PD-L1 antagonist Ab. We determined that the combination immunotherapy facilitated tumor infiltration of effector $CD8^+$ and $CD4^+$ T cells (expressing IFN-γ, ICOS, granzyme B, and perforin), while reducing the prevalence of PD-$L1^+$ cells and exhausted PD-1^+CD8^+ TIL in the TME. The combination protocol also resulted in superior antitumor efficacy (versus the component monotherapies) and extended overall survival. We predict that these combination OV/immune checkpoint blockade-based immunotherapies will expand the use of checkpoint inhibition to a much wider population of cancer patients (118).

CLINICAL STUDIES WITH OVs

Starting in the year 2000, a variety of OVs have been tested in clinical trials. Many phase I studies with a variety of OV have been conducted, mostly dealing with safety and feasibility issues. Some OVs have been tested in phase II or beyond. Since 2010, nine phase II/III clinical trials employing four types of OVs have been reported (**Table 1**). In this section, we will focus on those OVs in phase II trials and then briefly discuss the OVs with completed phase III trials, and two phase Ib clinical studies combining T-VEC with immune checkpoint blockade in patients with advanced melanoma.

Two oncolytic HSVs have now been tested in four phase II trials treating patients with three different types of cancer. In the first trial, NV1020 was evaluated in patients with pretreated refractory metastatic colorectal cancer, where treatment was observed to stabilize liver metastases with minimal toxicity (119). In a second trial, Kaufman et al. assessed local and systemic immune responses after T-VEC was injected directly into melanoma lesions. They determined that (i) established tumors contained elevated levels of Treg, suppressor T cells (Ts), and MDSC at baseline and (ii) T-VEC treatment enhanced local and systemic antigen-specific T cell responses in association with decreased levels of Treg, Ts, and MDSC in those patients who exhibited objective clinical responses to therapy (66). In a third study, T-VEC was combined with radiotherapy and cisplatin for the treatment of patients with untreated stage III/IV squamous cell carcinoma of the head and neck (SCCHN) (120). Finally, Kaufman and others compared the efficacy of intratumorally delivered T-VEC versus non-injected non-visceral or visceral

TABLE 1 | Phase II clinical trials in cancer patients with oncolytic viruses (OVs) (from year 2010 to current).

OV	Combination or others	Cancer type (patient number)	Primary endpoints	Clinical responses	Reference
Herpes simplex virus (HSV) (NV1020)		Refractory metastatic colorectal cancer (19 in phase II)	Toxicity and efficacy	50% patients with stable disease	Geevarghese et al. (119)
HSV (T-VEC)		Metastatic melanoma patients (50)	Local and distant antitumor immunity	(1) Elevated levels of regulatory T cells (Tregs), suppressor T cells (Ts), and myeloid-derived suppressor cell (MDSC) within established tumors (2) Direct injection of T-VEC induces local and systemic antigen-specific T cell responses and decreases Treg, Ts, and MDSC in patients exhibiting therapeutic responses	Kaufman et al. (66)
HSV (T-VEC)	Radiotherapy + cisplatin	Untreated stage III/IV squamous cell carcinoma of the head and neck (17)	Safety and efficacy	(1) 82% patients showed tumor responses by RECIST (2) 93% pathologic complete regression (3) DFS 82% at 29 months	Harrigton et al. (120)
HSV (T-VEC)	Systemic versus local responses	Stage IIIc or IV melanoma (50)	Comparison of efficacy in directly injected lesions, and uninjected non-visceral and visceral lesions	(1) Lesions directly injected: 67% decreased in size; 46% completely resolved (2) Uninjected non-visceral lesions: 41% decreased in size; 30% completely resolved	Kaufman et al. (121)
Reovirus (RT3D; same as Reolysin®)	Carboplatin + paclitaxel	Advanced malignancies (31)	Safety and efficacy	(1) No dose-limiting toxicity (2) One complete response, 6 partial responses, 9 stable disease, and 8 disease progression	Karapanagiotou et al. (122)
Reovirus (Reolysin®)		Advanced melanoma (21)	Safety and efficacy	(1) Viral replication (2/21) (2) No objective response (3) Median time to progression and survival were 45 and 165 days	Galanis et al. (123)
Reovirus (Reolysin; Pelareorep)	Paclitaxel/ carboplatin	Metastatic pancreatic adenocarcinoma (arm A, $n = 36$)		(1) The majority of PFS time was without toxicity or progression (4.3 months) (2) Patient immunophenotype appeared important (3) Overall, pelareorep was safe but does not improve PFS	Noonan et al. (124)
AdV	Radiation	Intermediate-risk prostate cancer -(21 in the arm)	Acute (≤90 days) toxicity	When used combined, a clinically meaningful reduction in positive biopsy results at 2 years	Freytag et al. (125)
Vaccinia virus (JX-594; Pexa-Vec)		Advanced hepatocellular carcinoma ($n = 30$)	To determine the optimal dose	(1) JX-594 replication and granulocyte-macrophage colony-stimulating factor expression preceded the induction of anticancer immunity (2) Median survival of 14.1 months compared to 6.7 months on the high and low dose, respectively	Heo et al. (68)

lesions. They found that the therapeutic efficacy was greatest in the injected lesions, intermediate in non-injected non-visceral lesions, and lowest in visceral lesions (121).

Reovirus has also been evaluated in three phase II trials treating various advanced forms of cancer. Reolysin (RT3D) administered with carboplatin and paclitaxel has been evaluated for safety and efficacy in patients with SCCHN (122). The authors report no dose-limiting toxicity, with a large fraction of patients exhibiting stable disease, as well as, several PR or CR (4%). Reolysin has also been used to treat patients with advanced-stage melanoma *via* intravenous delivery, where again the treatment was observed to be well tolerated, with evidence for virus replication in tumor biopsies (123). Furthermore, Reolysin has been applied alone or in combination with paclitaxel/carboplatin for the treatment of patients with metastatic pancreatic adenocarcinoma in a randomized phase II trial (124). The approach was found to be safe, although the combination therapy was not superior to carboplatin/paclitaxel alone in improving patient progression free survival.

Other OVs have also been tested in phase II clinical trials. Oncolytic AdV applied in combination with radiation has been used to treat intermediate-risk prostate cancer in a prospective randomized phase II trial (125), clinically meaningful reductions in positive biopsies noted at 2 years posttreatment. To date, the most encouraging results have been obtained in a trial using Pexa-Vec (JX-954) to treat patients with liver cancer (68), where

coordinate viral replication and GM-CSF expression in tumors was observed, with therapy-induced antitumor immunity also being detected. In this trial, the duration of patient survival was directly related to viral dose, with a median survival of 14.1 months in the high-dose cohort versus 6.7 months in the low-dose group.

Four phase III trials involving administration of OVs have been completed or remain open to patient accrual at this time. H101 is a recombinant human AdV type 5 with E1B deletion (presumably) conferring conditional replication in p53-deficient cancer cells. China approved the clinical use of H101 in 2005 (Oncorine®). In a multicenter, open, randomized, and parallel controlled clinical study, H101 combined with chemotherapy was reported to be superior to chemotherapy alone with a good safety profile in patients with squamous cell carcinoma of head and neck and the esophagus (126). In the United States, T-VEC was used in a successfully completed phase III OPTiM study and was FDA approved for the treatment of patients with advanced-stage melanoma in 2015 (127). A third OV, Pexa-Vec (JX-594), is currently being investigated in a worldwide phase III PHOCUS trial in patients with hepatocellular carcinoma. Finally, CG0070 (AdV expressing GM-CSF) is currently being evaluated in BOND2, a phase III pivotal study, examining treatment efficacy against high-grade, non-muscle invasive bladder cancer after failure to BCG therapy.

As we have discussed previously, positive clinical results were obtained from HCC patients in South Korea receiving Pexa-Vec (68). In contrast, its TRAVERSE Phase 2 study of Pexa-Vec in second-line advanced liver cancer in the United States (in 2013) did not meet its primary endpoint. It will be interesting to analyze the contradictory results in Asia and North America in greater detail. We would argue that the success of OV, as a form of immunotherapy, critically depends on intrinsic or therapeutically inducible cancer immunogenicity. In Asia, infection with hepatitis B virus is the more common cause of HCC, while in the United States, hepatitis C virus (HCV) is a more common etiologic agent. HCV may also possess a higher capacity to evade the immune system (128). As of today, we still do not have an effective vaccine against HCV, and we would hypothesize that liver cancers (mostly HCC) resulting from chronic HCV infection may be generally less immunogenic than those tumors caused by HBV infection.

We have been developing the WR strain VACV as an OV (129). Phase I clinical trials with vvDD, a double viral genes-deleted tumor-selective OV, have now been completed. The first-in-human dose-escalation trial of vvDD was performed in 16 patients with advanced-stage solid tumors, predominantly colorectal cancer (130). Viral dose escalation, delivered intratumorally, proceeded without dose-limiting toxicities, up to a maximum feasible dose at 3.0e9 pfu. Viral replication in tumors was reproducibly observed, with virus recovered from both injected and non-injected tumors. In summary, vvDD delivered intratumorally was well tolerated in patients, with viral administration leading to selective infection of injected and non-injected tumors, with coordinate antitumor activity noted. In a second trial, we delivered the virus intravenously into cancer patients (131). Again, we observed no dose-limiting toxicities or treatment-related severe adverse events. Viral genome DNA was detectable in patient blood shortly after virus administration, with prolonged viral replication detected in tumor tissues isolated from two patients. Viral replication was not found in non-tumor tissues, with the exception of sites of injury. It is worth noting that the best clinical responses were observed in the two patients with melanoma in these two trials. This could reflect the consensus that melanoma is a particularly immunogenic type of cancer (and possibly preferred target for immunotherapy (132)) and/or that skin is the normal target tissue for infection by VACV (possibly making it easier for VACV to induce ICD in cutaneous forms of cancer).

Szalay, Fong, and others have also been developing LIVP strain-derived oncolytic VACV GLV-1h68 (commercial name: GL-ONC1) (133). Multisite clinical studies have demonstrated a favorable safety profile and hinted at the potential use of GL-ONC1 as an effective therapeutic agent (e.g., ASCO Annual Meeting 2015). Two ongoing phase I clinical trials are currently evaluating i.v.-administered GL-ONC1 along with concurrent chemoradiotherapy for patients with locoregionally advanced head and neck carcinoma and intrapleural administration of GL-ONC1 for patients with malignant pleural effusion.

At this time, the most exciting clinical studies appear to be those combining OV with immune checkpoint blockade. A phase Ib study using T-VEC with ipilimumab, an anti-CTLA-4 antagonist Ab, in patients with unresectable stage IIIb/IV melanoma has been recently reported (134). Nineteen patients were included in the safety analysis. No dose-limiting toxicities were observed. The objective response rate reached 50%, with 44% of patients exhibiting durable responses lasting ≥6 months. The conclusion of the study was that the combined treatment had a tolerable safety profile and appeared to have greater efficacy than either monotherapy.

Pembrolizumab is an anti-PD-1 antagonist Ab. Previous clinical studies have shown that clinical administration of this Ab leads to greater progression-free survival and overall survival than ipilimumab in melanoma patients, suggesting that a combination of T-VEC with pembrolizumab might be more effective than the combination with ipilimumab. An ongoing phase I–III study was designed to explore this combination for patients with unresected melanoma (NCT02263508) (135). In the phase Ib study of 21 patients, the reported ORR was 57%, with 24% of patients with confirmed complete response. The disease control rate was 71%. A phase III randomized, double-blind, placebo-controlled trial (MASTERKEY-265) is now planned for 660 patients with unresectable state IIIb/IV melanoma.

CONCLUSION AND PERSPECTIVES

The TME in the setting of advanced-stage cancers is highly immunosuppressive (136). As we and others have previously suggested, this immunosuppressive property poses a double-edge sword in consideration of OV-based immunotherapy. Such suppression limits immune regulation of viral replication in support of direct oncolysis, but it represents a major impediment to the development, targeting and operational integrity of protective antitumor immunity that appears crucial to the

durable clinical success of OV-based interventional strategies. How we manipulate this delicate balance may likely determine the optimal benefits that can be achieved using such treatment modalities in the clinic. Notably, administration of OVs often leads to ICD of cancer cells, a process in which dying tumor cells expose/release multiple potent danger signals (signal 0) and pro-inflammatory cytokines (signal 3), while in some cases, coordinately upregulating their expression of MHC class I and II antigens. ICD in the TME begets efficient tumor antigen-cross presentation (signals 1 and 2) by tumor-associated DC that serve as the instigators of robust type-1 T cell responses capable of limiting tumor growth/metastasis. Combinatorial OV-based approaches allow for the fine tuning of the immune microenvironment within tumors, leading to removal of suppressive cells/factors and the recruitment and maintenance of therapeutically induced antitumor immune cells. Such combinational approaches, incorporating chemotherapeutic drugs, vaccines, or adoptive immune cell therapies, hold great clinical promise in optimizing the therapeutic potential of OV-based interventional approaches.

There also remains great need to further investigate mechanisms underlying patient resistance to oncolytic immunotherapy and any OV-associated toxicities. There are primary, adaptive, and acquired resistance to OV-mediated and other cancer immunotherapy (137). As our understanding for mechanisms of resistance continues to improve, we will be in position to rationally design combinatorial strategies to safely overcome such resistance. Our recent study combining OV and anti-PD-L1 represents one such study (118). There is also need to define biomarkers associated with clinical response (or resistance, toxicity) to treatment with oncolytic immunotherapy. Only a few studies have been published in this area of research to date. In this regard, serum HMGB1 has been shown to be a predictive and prognostic marker for successful oncolytic immunotherapy with AdV (138). In another study, immunoglobulin-like transcript 2 has been identified as a biomarker of therapeutic response to oncolytic VACV (13). These types of studies may enable us to better predict OV-based treatment outcomes in future clinical trials.

A number of hurdles remain that limit wide-spread use of OV-based therapies in the cancer setting. The first hurdle is the inability of OV to efficiently deliver and propagate throughout the entire tumor and to infect cancer cells that are at extended distances from the site of virus injection or from the original site of infection site after systemic delivery, which limits the ability of this approach to achieve consistent therapeutic responses in patients with disseminated disease. The tumor matrix also hinders virus diffusion throughout a given lesion. Some suggested means to circumvent this limitation have been offered. For example, the engineered overexpression of matrix metalloproteinases-1 and -8 significantly depletes tumor-associated sulfated glycosaminoglycans, resulting in increased tumor perfusion and greater distribution of injected virus in association with improved therapeutic efficacy (139). Similarly, enforced expression of hyaluronidase by OV led to improved virus spread throughout the tumor and to greater therapeutic benefit (140). Losartan, an angiotensin II receptor antagonist, appears capable of enhancing the distribution and efficacy of nanomedicines, including OVs (141). Another reason for the low efficiency of virus distribution throughout the tumor reflects the relatively high interstitial fluid pressure of the TME (142). In this regard, blood flow may affect the intratumoral extravasation of systemically delivered OVs. Indeed, one recent study demonstrated that perfusion pressure greatly affects the intratumoral extravasation of OVs (143). Antiangiogenic therapies, through their induction of collagen degradation, can also enhance intratumoral distribution of oncolytic AdV (144). Clearly, additional investigations will be required to further improve upon tumor uptake and intralesional distribution of OVs to yield more effective cancer therapies.

A second hurdle involves the need to develop a broad repertoire of therapeutic immune cells that circulate systemically to impact disseminated disease, which typically evolves over time (145). Such timing can be adversely affected by antiviral immunity that may clear the OV prematurely, thus reducing therapeutic efficacy. For example, HSV-mediated oncolytic virotherapy for glioblastoma is often improved with the suppression of innate immune responses, leading to increased viral replication and subsequent oncolysis (146, 147). However, the boosting of antiviral immunity has also been shown to be required for efficient OV-mediated therapy benefits in some tumor models (7, 148, 149) and can play a "helper" role in the evolution of adaptive antitumor immunity elicited by OV, with the ultimate therapeutic efficacy requiring a delicate balance of the avidity, potency, and timing of the immune response directed against the virus versus the tumor (150).

A third hurdle reflects toxicities associated with OVs. In patients receiving Imlygic (T-VEC), adverse reactions, including fatigue (50%), chills, pyrexia, nausea, influenza-like illness, injection-site pain, and vomiting, occurred in over 20% of treated patients, with the most common grade 3/4 adverse reaction being cellulitis (127). Given the use of a live virus, Imlygic can cause life-threatening dissemination of herpetic infections in immunocompromised patients. As a result, the use of T-VEC is contraindicated in immunocompromised patients and in pregnant women. We have recently evaluated OV derived from the WR strain of VACV (vvDD), and based on our findings, patients with actively healing wounds, or those with acute inflammatory conditions involving the skin or oral mucosa, should be excluded from using this OV (131). It also should not be used by immunocompromised patients.

Finally, accumulating evidence suggests that microbiota play an important role in the initiation, progression, and dissemination of cancer, not only at epithelial barriers but also in sterile tissues. Perhaps more importantly, barrier tissue microbiota can modulate cancer patient response to interventional therapy (including immunotherapy), as well as, patient adverse events to therapy (151). In this regard, it will be critical to further study the relationship between OV and microbiota in the host to better predict the likelihood of therapeutic efficacy versus treatment-associated toxicity.

In summary, it is indeed an exciting time to work in field of cancer immunotherapy. By combining with other forms of cancer immunotherapy, especially modulation of immune checkpoint pathways (impacting signal 2) and adoptive cell therapies,

the future appears bright for the effective use of OV-based immunotherapy in the cancer setting.

AUTHOR CONTRIBUTIONS

ZG collected and read relevant papers and designed and drafted the manuscript. WS revised and polished the whole manuscript. All other authors have made suggestions to the manuscript. All authors have read and approved the final manuscript.

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MINI REVIEW
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Sharpening the Edge for Precision Cancer Immunotherapy: Targeting Tumor Antigens through Oncolytic Vaccines

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Cancer immunotherapy represents a promising, modern-age option for treatment of cancers. Among the many immunotherapies being developed, oncolytic viruses (OVs) are slowly moving to the forefront of potential clinical therapeutic agents, especially considering the fact that the first oncolytic virus was recently approved by the Food and Drug Administration for the treatment of melanoma. OVs were originally discovered for their ability to kill cancer cells, but they have emerged as unconventional cancer immunotherapeutics due to their ability to activate a long-term antitumor immune response. This immune response not only eliminates cancer cells but also offers potential for preventing cancer recurrence. A fundamental requirement for the generation of such a strong antitumor T cell response is the recognition of an immunogenic tumor antigen by the antitumor T cell. Several tumor antigens capable of activating these antitumor T cells have been identified and are now being expressed through genetically engineered OVs to potentiate antitumor immunity. With the emergence of novel technologies for identifying tumor antigens and immunogenic epitopes in a myriad of cancers, design of "oncolytic vaccines" expressing highly specific tumor antigens provides a great strategy for targeting tumors. Here, we highlight the various OVs engineered to target tumor antigens and discuss multiple studies and strategies used to develop oncolytic vaccine regimens. We also contend how, going forward, a combination of technologies for identifying novel immunogenic tumor antigens and rational design of oncolytic vaccines will pave the way for the next generation of clinically efficacious cancer immunotherapies.

Keywords: cancer immunotherapy, oncolytic vaccines, tumor antigens, antitumor immunity, T cells, tumor major histocompatibility complex ligandome

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INTRODUCTION

Immunotherapies have steadily emerged as a powerful treatment option for patients with various types of cancers. While employing the immune system to fight cancer was first proposed in the late nineteenth century, it was only recently that the improved understanding and novel discoveries in classical and tumor immunology have led to the design of more targeted and efficacious

immunotherapeutics (1–4). One such immunotherapy utilizes oncolytic viruses (OVs), which were originally discovered for their direct cancer-killing properties (5). Historically, case reports of cancer regression following infection with unrelated viruses started appearing around the early twentieth century (6). But it was not until the 1990s that concrete evidence demonstrated the ability of certain viruses to preferentially target cancer cells (7–9). In recent times, the advent of new technologies allowing for customization of viruses, combined with the urgent need for novel and effective therapies for cancer treatment, has led to a new impetus for OV research (10). OVs have a dual mechanism of action against tumors. First, they can preferentially replicate in and directly kill cancer cells, in a mechanism known as oncolysis (11). Second, the immunological events induced following the administration of OVs awaken the previously suppressed immune system to become activated and target tumor cells more effectively (12). This activation of the immune system is the most promising aspect of oncolytic virotherapy. One of the major players of the immune system responsible for targeting cancer cells are T cells and the effective activation of tumor-specific T cells can lead to long-term antitumor immunity and protection against cancer recurrence (13).

For the activation of antitumor T cells, the primary requirement is the presentation of a tumor antigen *via* major histocompatibility complex (MHC) molecules of antigen-presenting cells (APCs) (14). Antigens, usually identified as small peptide molecules of approximately 8–18 amino acids in length, are expressed *via* MHC class I and II molecules, and lead to the activation of antigen-specific CD8 and CD4 T cells, respectively (15). Tumor antigens can be derived from peptide fragments of mutated oncoproteins and tumor suppressors, aberrantly expressed cellular proteins, modified glycoproteins, oncofetal proteins, tissue-specific differentiation proteins, and proteins derived from oncogenic viruses (16, 17). Identification of such tumor antigens to activate antigen-specific T cell responses in tumors represent a highly attractive target for cancer immunotherapies today (16, 18). In addition to the antigenic peptide presented through the MHC molecule, the complete activation of T cells requires two other signals: costimulatory molecules on APCs and the presence of the appropriate cytokines in the immune milieu (19, 20). Thus, ongoing research to improve cancer immunotherapies aim to target one or more of these signals to effectively stimulate clinically relevant antitumor T cells.

In this mini-review, we highlight the studies that have incorporated tumor antigens in OVs to enhance antitumor immune responses and consequent therapeutic benefits in the context of cancer. We discuss recent studies completed using a variety of viral systems, as well as combinations of multiple strategies used to elicit the most efficacious immune response. We also throw light on some of the challenges in this area of research and emphasize the need for combining recent, cutting-edge technologies for tumor antigen discovery with oncolytic virus research for generating more efficacious cancer treatments.

ONCOLYTIC VACCINE THERAPY

The first generation of OVs primarily focused on direct killing of tumor cells. OVs can replicate preferentially in tumor cells due to deregulated signaling pathways (8, 9, 21, 22) resulting in increased susceptibility of tumor cells to viral infections (22–24). When it was observed that the direct killing of tumor cells led to the release of novel tumor antigens in the tumor microenvironment and the subsequent activation of immune responses (25–29), strategies began to be focused on the modulation and optimization of these immune responses to achieve maximum clinical benefit. The overexpression of tumor antigens *via* OVs represents one such strategy that makes OV-based cancer therapies more potent by driving immune responses to be directed specifically toward the tumor. OVs that are genetically modified to express tumor antigens are commonly known as "oncolytic vaccines."

Vesicular Stomatitis Virus (VSV)

In recent times, many OVs have been found to be amenable for therapeutically desired genetic modifications. Among these, VSV has been the subject of extensive genetic manipulation and consequent investigation on antitumor immunity in the context of cancer treatment. For example, studies have shown that VSV-expressing tumor antigens human papilloma virus oncogene E7 (VSV-E7) and human dopachrome tautomerase (VSV-hDCT) can induce tumor antigen-specific CD8 cytotoxic T cell responses (30, 31). Therapeutic vaccination with VSV-E7 led to reduced TC-1 tumor volumes, and VSV-hDCT generated antigen-specific CD4 T cell responses in addition to CD8 T cells in murine melanoma (30, 31). Another study employed the popularly used ovalbumin (ova) as a surrogate tumor antigen expressed in murine melanoma cells to demonstrate that the administration of VSV engineered to express ova (VSV-ova) led to increased activation of naïve T cells, as well as increased number of ova-specific, antitumor T cells (32).

Furthermore, VSV-based oncolytic vaccines have also been indicated as promising candidates to be used in combination therapies. A recent study demonstrated that in combination with stereotactic ablative radiation therapy, VSV-ova helped control local and systemic disease in a murine oligometastatic melanoma model (33). In addition, oncolytic vaccines can be administered in combination with adoptive transfer of antigen-specific T cells for enhanced therapeutic benefits compared to either treatment alone. For instance, a melanoma-derived tumor antigen gp100-expressing VSV (VSV-gp100) combined with adoptive transfer of gp100-specific T cells resulted in increased survival of mice with established melanoma that was accompanied by the development of antitumor T cell responses (34, 35). Similar results were also observed by combining VSV-ova and adoptive transfer of ova-specific T cells (32). Taken together, these studies in preclinical models demonstrated that oncolytic vaccines may be combined with current clinical treatment options to achieve improved therapeutic and immune responses.

Another interesting approach to develop efficacious VSV-based oncolytic vaccines was to employ tumor-derived cDNA libraries. Specifically, cDNA libraries derived from cancer cell lines are expressed in VSV, followed by screening and administration of these library-based oncolytic vaccines in tumor-bearing mice. VSV expressing a cDNA library created from normal prostate cells has been shown to lead to the rejection of mouse prostate

cancers with little autoimmunity as measured by whitening of whiskers and tail, hair depigmentation, abnormal immune cell infiltration, and tissue destruction (36). A subsequent study employed VSV to express a melanoma cDNA library, which was screened *in vitro* for immunogenic tumor antigens, and demonstrated that a combination of three specific VSV-cDNA viruses infected established melanoma tumors and induced tumor rejection *via* T_H-17 responses (37).

These studies using cDNA library-expressing VSV also highlighted the importance of treating cancers according to their origins. First, it was observed that primary and recurring tumors must be targeted in a different manner and thus, "recurrence" libraries were developed. In one study employing a murine melanoma model, 14 out of 16 recurrences were found to have mutated BRAF, so VSV-BRAF was used to successfully target the recurring tumors (38). Second, the anatomical site of cancer development is another important consideration for oncolytic vaccine design. For example, a combination of VSV viruses derived from the melanoma cDNA library (VSV-N-RAS, VSV-CYTC-C, and VSV-TYRP-1) that was successful in treating subcutaneous melanoma could not treat intracranial melanoma tumors. Instead, a therapeutic combination targeting new tumor antigens in the context of intracranial tumors (VSV-HIF-2α, VSV-SOX-10, VSV-C-MYC, and VSV-TYRP-1) was shown to promote long-term survival of mice with intracranial melanoma (39). Building on this, another study showed that tumors of different histological origin shared immunological signatures based on their location and could be targeted specifically (40). The study used a glioma model in comparison to the intracranial melanoma model to establish that different tumors growing in the same location shared location-specific immunological signatures that could be targeted with the right combination of oncolytic vaccines. Of note, this study was among a few that combined oncolytic vaccines with immunological checkpoint inhibitors (ICIs) to reveal that ICIs enhanced the impact of oncolytic vaccines by reactivating T_H-1 and T_H-17 responses. Using ICIs like anti-PD1 and anti-CTLA4 antibodies in combination with OVs represents the next frontier in cancer immunotherapy as these complementary therapies are emerging as synergistic therapeutic partners of each other (41–44). Going forward, identification of novel tumor antigens (discussed in Section "Future directions and concluding remarks") for expression in viral vectors combined with effective combination therapy *via* ICIs represents an emerging paradigm of cancer immunotherapy.

Vaccinia Virus (VV)

Another virus that has allowed for ease of genetic manipulation and, thus, lent itself to oncolytic vaccine research is VV. VV has been employed in multiple studies for prophylactic vaccine development. Studies using oncofetal tumor antigen animal models like carcinoembryonic antigen (CEA) and glycoprotein oncofetal tumor antigen (5T4) transgenic mice have been widely used to study the prophylactic capacity of VV vectors. One such study demonstrated that VV-expressing CEA administered in CEA transgenic mice led to the development of CEA-specific T_H-1 responses and peptide-specific cytotoxicity. The authors noted that neither CEA antibodies nor CEA-specific T cell responses were elicited in CEA transgenic mice in response to endogenous or administered CEA in the absence of virus, indicating the usability of this animal model for more aggressive vaccinations (45). Upon virus infection, protection against CEA-specific cancer cells was observed without any effect on normal tissue-expressing CEA (45). Another study employing VV-expressing human and mouse 5T4 (VV-h5T4, VV-m5T4) demonstrated that mice vaccinated with these vectors showed retarded tumor growth upon challenge with syngeneic melanoma and colorectal cancer cells (46). No autoimmune toxicity in the form of wasting, respiratory problems, affected mobility or weight loss was seen in this study. Put together, these studies reaffirmed the potential of oncolytic vaccines as safe therapeutic options for treatment of cancers with low off-site toxicity.

Engineering genetic elements other than tumor antigens for immunomodulation is a common strategy for developing viral vectors. VV has provided an excellent platform for engineering costimulatory elements in conjunction with tumor antigens to incorporate other important factors for T cell activation. One such study demonstrated the expression of a T cell engager (TCE) element along with tumor antigen EphA2 in a VV vector (47). The TCE is a special secretory element used to specifically bind and activate T cells *via* CD3. The study noted that the virus killed tumor cells and induced a bystander killing of non-infected tumor cells *in vitro*. The modified VV also had potent antitumor activity *in vivo* in a lung cancer xenograft model (47). Another study employed VV to express a triad of costimulatory molecules (B7-1, ICAM-1, and LFA-3) along with oncofetal tumor antigen CEA, and the administration of the modified virus increased survival of colon adenocarcinoma tumor-bearing mice due to induced CD8 and CD4 T cell responses. Clinical serum and urine assays combined with histopathology showed no classical indicators of autoimmune responses, a typical complication when targeting oncofetal antigens like CEA (48).

The final factor that determines the quality of the generated T cell responses is the presence of cytokines in the immune environment. Expressing cytokines *via* viral vectors represents a promising strategy for the development of a robust antitumor immune response, and thus far, many cytokines including IL-2, TNF, IFN, and GM-CSF have been expressed through OVs (49–52). In 2015, the U.S. Food and Drug Administration approved the first oncolytic virus T-VEC for use in clinics. T-VEC employs modified herpes simplex virus (HSV)-expressing GM-CSF to enhance the generation of APCs (53). In the context of oncolytic vaccines and cytokines, a study using MB49 cancer cells expressing male tumor antigen HY was used to evaluate the efficacy of VV-overexpressing HY (VV-HY) and GM-CSF (VV-GM-CSF) to overcome tumor-associated immune tolerance. The study noted that the administration of both viruses together led to the generation of splenic HY-specific CD8 T cells, indicating development of systemic immunity (54). Overall, consideration of all the signals required for an effective T cell response is an important parameter for future studies employing oncolytic vaccines to activate holistic antitumor responses that are qualitatively superior to current therapeutic regimens.

Other Viruses

Several other viruses have been employed as oncolytic vaccines to target tumor antigens. A unique study worth highlighting employed Sindbis virus engineered with β-galactosidase (SV-β-gal) to demonstrate memory T cell responses that conferred protection against tumor re-challenge with antigen-specific and non-specific colon cancer cells in mice (55). The authors demonstrated that the influx of NKG2D-expressing antigen-specific CD8 T cells in the tumor was important for the development of long-lasting memory responses (55). Another study compared homologous vaccination strategies with Semliki Forest virus, adenovirus, and pox virus, and found that Semliki Forest virus provided potent protection in P185 tumors and showed increased levels of systemic antitumor-specific central memory T cells (56). However, the role of memory T cell development upon oncolytic vaccine administration remains largely unknown and poorly characterized. Considering the importance of antitumor T cell memory responses in long-term protection, the focus of future studies aimed at dissecting the immunological implications of oncolytic vaccines should consider memory T cells.

Other viruses that have been employed to target tumor antigens include Newcastle disease virus (NDV) and HSV. A study using NDV-expressing β-galactosidase-derived antigenic peptide (NDV-β-gal) found enhanced antitumor immune responses in a murine colon cancer model (57). The importance of cytokines in enhancing antitumor T cell responses has been discussed earlier. This study also demonstrated that coadministration of NDV-β-gal with NDV-expressing IL-2 led to increased frequency of tumor-infiltrating antigen-specific T cells and enhanced tumor regression (57). In another study, HSV was employed to express xenoantigen prostatic acid phosphatase (HSV-hPAP). This study demonstrated that HSV-hPAP caused reduced tumor growth and increased survival in mice bearing prostate tumors (58). A complete list of studies employing OVs to target tumor antigens has been summarized in **Table 1**.

HETEROLOGOUS VIRUS PRIME-BOOST STRATEGIES

One of the major challenges that oncolytic vaccines face is the induction of an undesired antiviral immune response against the viral vector. This antiviral immunity reduces the efficacy of the OV treatment by clearing the virus prematurely. In the context of oncolytic vaccines, immune responses against the viral vector may dominate the response against the tumor antigen, generating a much more subdued antitumor response. To overcome this obstacle, strategies that effectively redirect the immune responses against the tumor must be employed. One such strategy that we shall discuss is the heterologous virus prime-boost strategy.

The heterologous virus prime-boost strategy exploits the quick and effective immunological recall responses to redirect the maximal potential of immunity toward the tumor antigen. In the first priming step, a tumor antigen is expressed using one oncolytic virus vector, whereby the immune system "sees" and responds to the tumor and viral antigen for the first time. In the second boost step, a different viral vector is chosen to express the same tumor antigen, resulting in a primary immune response against the second viral vector while a stronger memory immune response is induced against the tumor antigen. Thus, this clever manipulation allows for the skewing of immunity toward antitumor responses over antiviral responses.

In preclinical studies, adenoviruses (Ad), VSV, and VV have emerged as common viral vectors that can be adapted for evaluation of priming and boosting strategies. Using the previously discussed tumor antigen hDCT, studies have shown that priming with Ad-hDCT and boosting with Maraba virus-expressing hDCT led to increased hDCT-specific T cell responses and survival of melanoma tumor-bearing mice (59). Another study using the same tumor antigen in a murine melanoma model demonstrated that priming with VSV-hDCT followed by a booster with Ad-hDCT also greatly enhanced antigen-specific T cell responses and led to enhanced efficacy in a prophylactic as well as a therapeutic setting (31). This study also demonstrated that VSV could boost hDCT-specific T cell responses generated by Ad-hDCT priming, indicating that VSV could successfully be used to either prime or boost tumor T cell responses (31). Another study demonstrating that the prime-boost strategy is effective irrespective of the order in which certain versatile viral vectors are administered used VV and Semliki Forest virus both overexpressing ova, resulting in enhanced antitumor activity and increased levels of ova-specific CD8 T cells in a murine ovarian surface epithelial carcinoma animal model (60). VV has also been used in combination with Sindbis virus in TC-1 tumor models for the prime-boost strategy, where priming with Sindbis-E7 and boosting with VV-E7 resulted in effective antitumor immune responses and the generation of increased numbers of E7-specific CD8 T cells (61).

Unfortunately, one of the major challenges with the heterologous virus prime-boost strategy involves the rapid killing of APCs by effector T cells before the memory T cells are engaged to produce a strong antitumor T cell response. A unique study with oncolytic vaccines addressed this issue by delivering oncolytic vaccines with B cells. Here, the immunodominant gp33 peptide derived from lymphocytic choriomeningitis virus was expressed in three different viruses VSV, Ad, and VV to demonstrate that B cells loaded with the booster virus can elicit better antigen-specific secondary T cell responses, which were dependent on antigen presentation by dendritic cells (62).

Altogether, the heterologous virus prime-boost strategy represents an innovative approach to redirecting immune responses in oncolytic vaccine therapy and warrants further research in the areas of viral vector selection and delivery of vaccines. These strategies combined with effective oncolytic vaccine design and vector development can help maximize antitumor immune responses and the development of efficacious clinical regimens.

CHALLENGES FOR ONCOLYTIC VACCINE THERAPY

Oncolytic vaccine development has come a long way from its initial stages but still has ways to go. As alluded to earlier, implementation of strategies like the heterologous virus prime-boost

TABLE 1 | Summary of oncolytic vaccine studies using a variety of viral vectors targeting respective tumor antigens.

Antigen	Implementation strategy	Route of delivery	Physiological effect	Reference
Vesicular stomatitis virus				
E7	Monotherapy	Intramuscular	Antigen-specific CD8 T cell responses Tumor volume reduction	(30)
DCT	Monotherapy	Intranasal	Antigen-specific CD8 and CD4 T cell responses	(31)
DCT	Heterologous prime-boost	Intranasal	Increased antigen-specific T cells Enhanced prophylactic and therapeutic efficacy	(31)
Ova	Monotherapy	Intratumoral	Increased T cell activation Increased antigen-specific T cells	(32)
Ova	Combination therapy	Intravenous	Local and systemic disease control	(33)
gp100	Combination therapy with adoptive transfer	Intratumoral	Increased antigen-specific T cells Elimination of established tumors	(34, 35)
Various	Viral expression of cDNA libraries	Intravenous	Tumor rejection *via* CD4 T_H-17 responses Anatomy-specific immune signatures of tumors	(36–40)
gp33	Novel delivery approach	Multiple	Oncolytic vaccine delivery using B cells	(62)
Vaccinia virus				
CEA	Monotherapy	Subcutaneous	Antigen-specific CD4 T cell responses Peptide-specific cytotoxicity No autoimmune responses	(45)
CEA	Engineered with costimulatory elements	Intravenous	Activation of CD4 and CD8 T cells Increased survival	(48)
5T4	Monotherapy	Intravenous/intramuscular	Retarded tumor growth No autoimmune responses	(46)
Ova	Heterologous prime-boost	Intraperitoneal	Increased antitumor activity Antigen-specific CD8 T cell responses	(60)
E7	Heterologous prime-boost	Intraperitoneal	Antigen-specific T cell responses	(61)
HY	Combination therapy	Intratumoral	Systemic antigen-specific CD8 T cell responses	(54)
EphA2	Engineered with T cell engager element	Intraperitoneal	Direct killing of cancer cells Bystander killing of cancer cells	(47)
gp33	Novel delivery approach	Multiple	Oncolytic vaccine delivery using B cells	(62)
Adenovirus				
DCT	Heterologous prime-boost	Intravenous	Antigen-specific T cell responses Increased survival	(59)
DCT	Heterologous prime-boost	Intramuscular	Increased antigen-specific T cells Enhanced prophylactic and therapeutic efficacy	(31)
Trap1a	Heterologous prime-boost	Intradermal	Effective tumor protection Increased CD8 T cell responses	(56)
gp33	Novel delivery approach	Multiple	Oncolytic vaccine delivery using B cells	(62)
Newcastle disease virus				
β-gal	Combination therapy with NDV-IL-2	Intratumoral	Increased tumor regression Increased antigen-specific TILs frequency	(57)
Herpes simplex virus				
PAP	Monotherapy	Intravenous	Reduced tumor growth Increased survival	(58)
Sindbis virus				
β-gal	Monotherapy	Intraperitoneal	Memory T cell responses Antigen-specific and non-specific immunity	(55)
E7	Heterologous prime-boost	Intramuscular	Antigen-specific T cell responses	(61)
Semliki Forest virus				
Trap1a	Homologous injections	Intradermal	Increased tumor-specific central memory	(56)
Trap1a	Heterologous prime-boost	Intradermal	Effective tumor protection Increased CD8 T cell responses	(56)
Ova	Heterologous prime-boost	Intraperitoneal	Increased antitumor activity Antigen-specific CD8 T cell responses	(60)
Maraba virus				
DCT	Heterologous prime-boost	Intravenous	Antigen-specific T cell responses Enhanced survival of mice	(59)
Pox virus				
Trap1a	Heterologous prime-boost	Intradermal	Effective tumor protection Increased CD8 T cell responses	(56)

DCT, dopachrome tautomerase; Ova, ovalbumin; gp100, glycoprotein 100; gp-33, lymphocytic choriomeningitis virus-derived peptide; CEA, carcinoembryonic antigen; 5T4, glycoprotein oncofetal tumor antigen; EphA2, Ephrin type-A receptor 2; Trap1a, tumor rejection antigen P1A; β-gal, β-galactosidase; PAP, prostatic acid phosphatase.

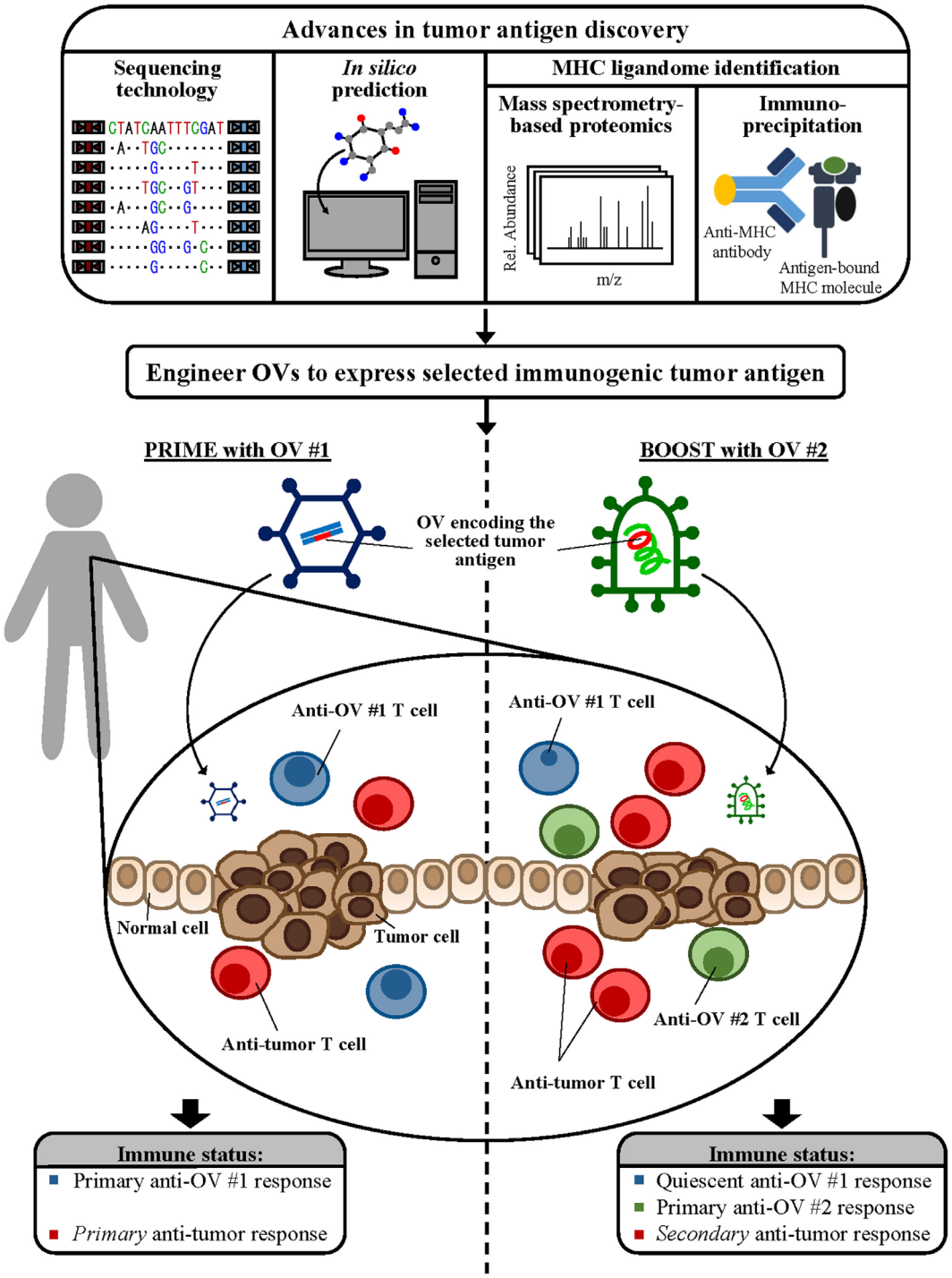

FIGURE 1 | Combining novel antigen discovery platforms to design oncolytic vaccines. Advances in sequencing technology, *in silico* prediction methods, and mass spectrometry-based proteomics and immunoprecipitation for major histocompatibility complex (MHC) ligandome elucidation allow identification of novel tumor antigen targets. Engineering these novel antigens into powerful viral vectors will provide a platform for the development of the next generation of oncolytic vaccines. Incorporating immunomodulatory strategies, such as the heterologous virus prime-boost as shown, during oncolytic vaccine administration can maximize antitumor immune responses, leading to the development of complete and clinically efficacious antitumor treatment options.

warrants more research. An important factor when extending such strategies to clinics is a consideration of pre-existing immunity to common viral vectors, which may lead to reduced efficacy in humans. Understanding pre-existing immunity combined with choosing the most effective routes of delivery, which depends on the type of tumor being targeted, will help

achieve maximum efficacy of oncolytic vaccines in clinics. Routes of delivery employed in the reviewed studies, along with other parameters and findings, have been summarized in **Table 1**. More research on developing the criteria for matching tumor type with routes of delivery is much needed for effective translation of preclinical models to bedside treatments. Moreover as discussed above, development of autoimmunity upon implementation of oncolytic vaccines has been a long-standing scientific concern. Minimizing autoimmune responses and maximizing tumor-specific T cell responses needs to be a major focus of future studies aimed at developing oncolytic vaccines. In this context, choice of tumor antigen can play an important role in not only maximizing antitumor immunity but also reducing off-site toxicity if carefully selected. In light of ongoing burgeoning developments in the areas of cancer genomics and proteomics, it is believed that novel antigens that are highly tumor-specific, and thus are non-autoreactive, will be available for such targeting (63, 64). In the following section, we discuss how, going forward, novel tumor antigens discovered *via* a combination of computational and high throughput approaches provide the best chance for selecting antigens to be targeted in tumors and represent an emerging frontier in cancer immunotherapy.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Novel tumor antigen discovery is a fundamental cornerstone in cancer immunotherapy and provides an enormous knowledge base for oncolytic vaccine research to draw from. Cutting-edge technological advances in tumor antigen discovery have identified a wide range of potential targets that can be incorporated in the next generation of oncolytic vaccines for designing highly targeted and efficacious therapies. One of these major advances is in the field of sequencing; new and improved sequencing technologies are now available to identify and categorize mutations at the level of genes, exons, and RNA from many different cancer types (65–67). In addition, recent developments in computational techniques allow better *in silico* prediction and identification of immunogenic tumor antigens (68–74). Finally, advances in peptide isolation and identification by immunoprecipitation and mass spectrometry-based proteomics, respectively, have led to the development of novel approaches to unearth new tumor antigens with greater precision (75–77). One such result of modern research combining these advances in different fields has led to the discovery of the high-confidence tumor MHC ligandome, which represents an array of tumor-derived peptide antigens that are bound to MHC molecules. These tumor antigens are identified using immunoprecipitation of peptide-MHC molecules followed by mass spectrometry analysis of the peptides eluted from the MHC molecules. Such antigens have been collectively called as ligandome-derived tumor-associated antigens (LiTAAs) (78–81). A recent breakthrough study used a combination of exome sequencing, proteomics, and computational modeling to identify novel tumor antigens that could be targeted *via* peptide-based vaccines (82). Going forward, the tumor MHC ligandome will serve as a unique and effective library for identifying novel tumor antigens to be targeted using oncolytic vaccines. More importantly, as opposed to the traditional peptide-based vaccines to deliver LiTAAs, oncolytic vaccines provide the advantage of administering LiTAAs in the presence of all the necessary immune signals for the proper activation of long-term antitumor immunity.

Combining this unique capability of oncolytic vaccines with novel tumor antigen discovery paradigms and effective, timely immunological intervention *via* strategies like the heterologous virus prime-boost will aid the design of the most clinically efficacious cancer immunotherapeutics (summarized in **Figure 1**). With the ever-increasing knowledge bases that have not yet been effectively translated to the bedside, concerted efforts to adapt these uniquely exciting strategies to develop therapeutic models of high clinical relevance are more important today than ever before and mark the next frontier in precision cancer immunotherapy research.

AUTHOR CONTRIBUTIONS

NH, YK, and SG developed the concept. NH prepared the figures and wrote the manuscript. YK participated in writing and technical editing of the manuscript. PL and SG supervised and edited the manuscript.

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REVIEW
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Oncolytic Viruses—Natural and Genetically Engineered Cancer Immunotherapies

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There has long been interest in innovating an approach by which tumor cells can be selectively and specifically targeted and destroyed. The discovery of viruses that lyse tumor cells, termed oncolytic viruses (OVs), has led to a revolution in the treatment of cancer. The potential of OVs to improve the therapeutic ratio is derived from their ability to preferentially infect and replicate in cancer cells while avoiding destruction of normal cells surrounding the tumor. Two main mechanisms exist through which these viruses are reported to improve outcomes: direct lysis of tumor cells and indirect augmentation of host anti-tumor immunity. With these factors in mind, viruses are chosen or modified to selectively target tumor cells, decrease pathogenicity to normal cells, decrease the antiviral immune response (to prevent viral clearance), and increase the antitumor immune response. While only one OV has been approved for the treatment of cancer in the United States, and only two other OVs have been approved worldwide, a wide spectrum of OVs are in various stages of preclinical development and in clinical trials. These viruses are being studied as alternatives and adjuncts to more traditional cancer therapies including surgical resection, chemotherapy, radiation, hormonal therapies, targeted therapies, and other immunotherapies. Here, we review the natural characteristics and genetically engineered modifications that enhance the effectiveness of OVs for the treatment of cancer.

Keywords: oncolytic viruses, cancer immunotherapy, oncoimmunology, pathogens, viruses

INTRODUCTION

The implementation of immunotherapeutic strategies for the treatment of cancer has gained prominence over the past decade in preclinical development and clinical practice. Traditional oncological approaches, including surgery, radiation, and chemotherapy, aim to directly remove or kill cancer cells. In contrast, immunotherapy seeks to enhance the host immune system's ability to eliminate cancer cells resulting in tumor regression, antitumor immune memory formation, and ultimately in durable responses (1, 2). Induction of the host immune system *via* increases in innate and adaptive immune surveillance of and response against the tumor can provide lasting positive outcomes in

cancer patients (3). Initiating the human body's ability to recognize and destroy malignancies is often better tolerated by patients long term in comparison to traditional therapies. However, the use of immunotherapies in conjunction with traditional therapies may further increase treatment efficacy and lead to prolonged survival (2, 4–6). Immunotherapies have also been shown to be effective against recalcitrant disease and are, therefore, being tested and utilized frequently in difficult clinical situations for patients with advanced-stage disease. Delivering immunotherapy to patients with earlier stage cancers may lead to an increase in the proportion of patients who exhibit clinical benefit.

A novel addition to the anticancer treatment armamentarium is use of oncolytic viruses (OVs). Observations of spontaneous tumor regression after naturally occurring viral infections gave rise to the notion that OVs can be incorporated as treatments (7). Although numerous naturally occurring OVs exist, recently immense interest has revolved around genetically modifying viruses to create new cancer therapeutics (8, 9). OVs function by preferentially targeting and killing tumor cells while simultaneously stimulating the immune system and creating antitumor immunity (4–6). This dual mechanism of action allows for direct local antitumor response (leading to tumor regression) as well as the induction of the innate and adaptive components of the immune system (leading to the recognition and removal of systemic disease and prevention of recurrence) (7). The predilection of these viruses to preferentially infect tumor cells while sparing normal surrounding cells allows for an excellent therapeutic ratio. The ability of OVs to cause immune infiltration into tumors bridges the gap to immunotherapy (1, 4–6), and immunologic outcomes are being reported from ongoing and completed clinical trials (**Table 1**).

As our understanding of cancer biology and virus–host cell interactions improves in concert with genetic engineering, the ability to manipulate the viral genome gains importance. For

TABLE 1 | Select clinical trials of oncolytic viruses with clinical and immune outcomes data (9–17).

Virus strain	Study	Trial design	Number of patients	Clinical outcomes	Immunological outcomes
Herpes simplex virus (HSV) type 1 (HSV-1)	Talimogene Laherparepvec Improves Durable Response Rate in Patients with Advanced Melanoma (9)	Phase III	436	Improved durable response rate (16.3 vs. 2.1%), overall response rate (26.4 vs. 5.7%), and longer median survival (23.3 vs. 18.9 months) in patients with non-surgically resectable melanoma receiving T-VEC vs. GM-CSF (9)	Regression of 34% of uninjected non-visceral and 15% of visceral tumors (11). Earlier Phase II study reported increased MART-1 specific T cells in regressing tumors and decreases in intratumoral regulatory T cells, suppressor T cells, and myeloid-derived suppressor cells in responding patients (12)
HSV-1	Talimogene Laherparepvec in Combination with Ipilimumab in Previously Untreated, Unresectable Stage IIIB-IV Melanoma (10)	Phase Ib/II	19	50% objective response rate and 44% of patients had a durable response lasting ≥6 months. 18-month progression-free survival was 50%, and overall survival was 67%	Significant increase in total CD8+ T cells and activated CD8+ T cells (CD3+, CD4−, HLA-DR+). Significant upregulation in activation marker ICOS on CD4+ T cells
Reovirus	Randomized Phase II Trial of Oncolytic Virus Pelareorep (Reolysin) in Upfront Treatment of Metastatic Pancreatic Adenocarcinoma (13)	Phase II	73	Addition of pelareorep to carboplatin and paclitaxel did not improve progression-free survival compared to carboplatin and paclitaxel alone	Increased natural killer cells or B cells in patients with improved disease control rate
Reovirus	Phase II trial Intravenous Administration of Reolysin (Reovirus Serotype-3-Dearing Strain) in Patients with Metastatic Melanoma (14)	Phase II	21	No objective responses seen and 6 patients with stable disease for >8 weeks	Extensive necrosis in metastases of one patient and demonstrated viral replication in melanoma metastases in 2 of 13 tumors. Significant increase in neutralizing anti-reovirus titers in 13 patients
Vaccinia virus	Use of a Targeted Oncolytic Poxvirus, JX-594, in Patients with Refractory Primary or Metastatic Liver Cancer: A Phase I Trial (15)	Phase I	14	JX-594 injection was generally well tolerated. Neutralizing antibodies do not prevent efficacy	Interleukin 6, Interleukin 10, and TNF-α peaks at 3 h and at later time points (days 3–22)
Coxsackievirus	Phase II Calm Extension Study: A Study of Intratumoral CAVATAK™ in Patients with Stage IIIc and Stage IV Malignant Melanoma (16)	Phase II	57	38.6% of evaluable patients demonstrated durable responses in both injected and uninjected melanoma metastases	Increased immune-cell infiltration (in particular CD8+ cells) and increased PD-L1 expression on immune cells. Gene expression analysis 4 days pre and post biopsy samples indicated Th-1 gene shift
Coxsackievirus	Phase Ib Study of Intratumoral Oncolytic Coxsackievirus A21 (CVA21) and Systemic Pembrolizumab in Subjects with Advanced Melanoma: Interim results of the CAPRA clinical trial (17)	Phase Ib	22	Best overall response rate of 60% and stable disease in 26.7% of patients	Increase in number of PD-L1-expressing immune cells and increase in CD8+ and CD4+ T cells observed 8 days post treatment

example, talimogene laherparepvec (T-VEC) is a herpes simplex virus (HSV) type 1 (HSV-1) that is the first OV to be approved by the FDA for the treatment of advanced melanoma (9, 18). Although derived from a naturally occurring HSV strain, it has been genetically engineered to preferentially target cancer cells and to result in the production of an immune factor, granulocyte macrophage colony-stimulating factor (GM-CSF), to improve the antitumor immune response. T-VEC is now being tested in other cancers and in rational combinations with standard and immune-targeted therapies (9, 18).

This review will describe a series of OVs used in clinical trials and care settings, as well as, in testing and development. It will especially focus on mechanisms underlying the oncolysis of naturally occurring OVs and the genetic modifications that have been made to improve the therapeutic ratio provided by OVs. A number of important considerations exist in terms of choosing a virus for potential use as a therapeutic oncolytic agent. These include targeting the OV to the appropriate tissue or cell type and mechanisms of action (including specific lysis of tumor cells and the activation of an effective immune response) (**Figure 1**). These are described in this review, while other considerations, including bioavailability and safety have been described in detail in our earlier review (19). Ultimately, this review will address the quickly progressing field of OVs and their role in the fields of oncology and immuno-oncology.

TARGETING

Targeting: Natural

In the development of cancer therapeutics, targeting may be the most difficult obstacle to overcome. Targeting can be achieved by choosing viruses with natural tropisms to specific tissues or cell types or by engineering these tropisms (**Figure 2**). While research studies tend to highlight differences between tumor cells and normal cells, in reality their similarities are much more numerous. This presents the problem of specific drug targeting that allows for destruction of the tumor without effecting normal (i.e., non-cancerous) tissue. OVs present two distinct, although not mutually exclusive, advantages by which they can specifically target cells. The first is the basic tenet of viral infection, that viruses naturally exploit permissive cells for infection through expression of the necessary surface receptors that allow viral entry and through the modulation of host defense pathways that allow viruses to avoid detection. The second is the permissiveness of viruses to accept modifications engineered to increase their specificity against cancer cells, while at the same time being limited in their effect on normal cells.

Cell permissivity for viral infection begins with the ability of the virus to identify and enter its cellular target. This is mediated primarily through the expression of cell surface receptors. Several OVs in use have been chosen based on their inherent ability to use cell surface molecules for entry that are abnormally upregulated in cancer cells. An example of this is the use of HVEM, nectin-1, and nectin-2 by T-VEC (20). This augmented expression has been noted in multiple tumor types and renders these cancer cells more susceptible to herpesvirus infection than normal cells. Another example of cancer cell surface molecule-specific tropism includes CD46. This molecule is aberrantly expressed by tumor cells to subvert the complement pathway and is frequently overexpressed by malignant cells to avoid recognition and elimination. This can be taken advantage of by use of the measles virus (Edmonston strain) which utilizes CD46 for cell entry, making tumor cells overexpressing this receptor susceptible to oncolysis (21). Similarly, overexpression of intracellular adhesion molecule 1 (ICAM-1) and decay accelerating factor in malignancies such as breast cancer, multiple myeloma, and melanoma can be taken advantage of by coxsackievirus, including coxsackievirus A21 (CAVATAK) (22–24). A series of other OVs are being developed for clinical use based on their natural tropism for cancer cells. For example, poliovirus has a natural tropism for the cell surface marker CD155. This receptor is frequently upregulated by cancers because it affords protection from innate immunity for the pathogen by downregulating antitumor natural killer (NK) cell responses (25). Echovirus, another enterovirus, has tropism for ovarian cancer because of higher expression of integrin α2β1

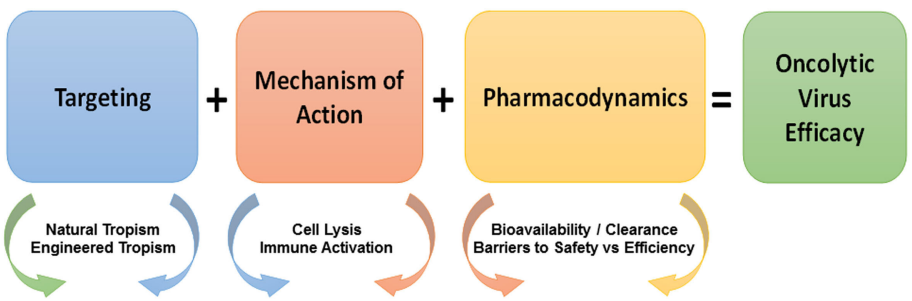

FIGURE 1 | Considerations in the development of oncolytic viruses (OVs). Considerations in the development of efficacious OV immunotherapy include targeting, mechanism of action, and pharmacodynamics. Targeting (blue box) is dependent on the natural and engineered tropism of viruses for tumor vs. normal cells. The mechanism of action (red box) of OVs is dependent on the immune mechanisms and the non-immune mechanisms of OVs, which are further enhanced by the combination of OVs with traditional and emerging antitumor therapeutics. OVs share pharmacodynamic considerations (orange box) with other small molecule drugs as well as raise new fundamental issues in terms of bioavailability vs. clearance and barriers to safety vs. efficiency. Overlapping arrow colors signify the existent overlap between the listed considerations.

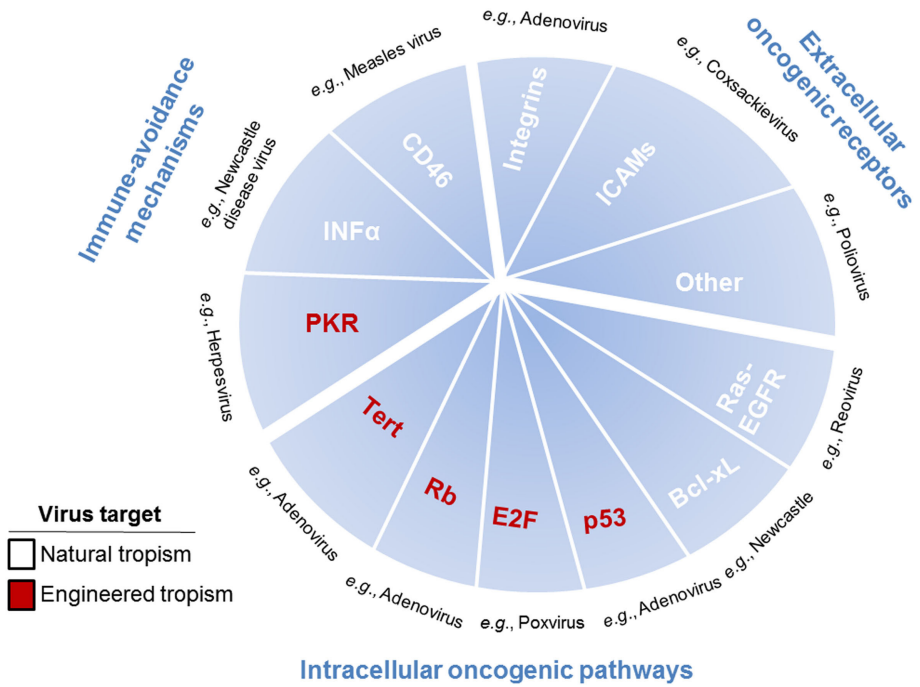

FIGURE 2 | Pathways, receptors, and mechanisms used by oncolytic viruses (OVs) to target cancer cells. OVs target cancer cells through pathways, receptors, and mechanisms used to promote tumor growth, including immune-avoidance mechanisms, extracellular oncogenic receptors, and intracellular oncogenic pathways. Immune-avoidance, extracellular receptor, and intracellular pathway targets that are overexpressed or repressed to inherently allow tumors to avoid immune responses can simultaneously can be used for targeting OVs to cancer cells. Extracellular receptors include surface molecules [such as integrins, ICAMs, and others (CD155, laminin receptors, etc.)] inherently expressed by some tumor cells, which are utilized by viruses for specific targeting to cancer cells rather than normal cells. Intracellular pathways are utilized in tumor cells to promote proliferation and survival, which are required for viral propagation, thus enhancing cancer cell susceptibility to oncolytic viral infection. Single examples of viruses utilizing each of the described targets are listed in black text. Mechanisms, pathways, and receptors that enhance cancer cell targeting as part of the natural tropisms of OVs are listed in white text and as part of the engineered tropisms of OVs are listed in red text.

(26). Besides making use of cell surface receptors designed to subvert the immune system, some OVs use receptors utilized by tumor cells to enhance metastasis. Cancer cells may upregulate the laminin receptor to allow for invasion and increased motility. The Sindbis virus targets this receptor, allowing for specific targeting of cells with increased metastatic potential (27).

Cancer cells use more than aberrant cell surface receptor expression to fuel carcinogenesis. Specifically, they manipulate the transcriptional and signaling networks of cells to increase survival, proliferation, immune evasion, and metastasis. Viruses use many of these same pathways to propagate and infect cells. These parallels lead to cancer cells being more permissible to viral infections based on the overlaps between carcinogenesis and viral infection. An example is cancer's resistance to cell death, often achieved by increased expression of antiapoptotic molecules such as those of the Bcl family. This increase in antiapoptotic molecules increases the targeting of cancer cells by certain OVs. Newcastle disease virus, for example, specifically targets cancer cells overexpressing Bcl-xL, a protein that inhibits apoptosis. This gives the virus time to incubate, multiply, and form syncytia, which is imperative to the survival and spread of Newcastle disease virus (28). The Ras signaling pathway represents another pathway altered in cancer cells. Ras is involved in the regulation of cell death and proliferation. Reovirus and vaccinia have been identified as OVs due to their ability to specifically target cancer cells driven by the Ras pathway. Reovirus preferentially proliferates in Ras-transformed cells (29). Reovirus infects healthy cells and begins viral RNA production. This viral RNA causes activation of natural cellular defenses including the PKR pathway, which leads to translation inhibition, thus stopping viral production and spread. In cancer cells in which this pathway has been manipulated; however, the PKR pathway is not activated, which allows increased viral production and lysis (29). Vaccinia virus, a pox family virus, targets malignancies that overexpress EGFR. It requires EGFR-RAS signaling to replicate. These viruses encode a ligand that can bind to the EGFR receptor, resulting in RAS activation. This leads to the increased production of virus and propagation of the infection (30).

Further, a number of viruses have been identified and taken to clinical trial that are oncolytic through preferentially targeting transformed cells by mechanisms that have not yet been fully elucidated. For example, Seneca valley virus is being clinically evaluated for its therapeutic efficacy against neuroendocrine tumors based on its natural tropism for cancer cells with neuroendocrine features (31, 32). One possibility is that it utilizes targets (including CD56, chromogranin A, and potentially synaptophysin) that

are associated with neuroendocrine tumors. Another example is parvovirus H-1PV, which preferentially targets glioblastoma, pancreatic carcinoma, and other tumors through complex mechanisms that are not yet fully understood. One possibility is that parvoviruses may target transformed cells through increased cellular proliferation allowing for production of viral DNA and proteins, including those viral components that are needed for lysis of the cell (33). For these viruses and others, it may also be that they require cellular proliferation and that cancer, a disease of abnormally elevated proliferation, creates a natural tropism for OVs, thus providing a means for therapeutic targeting of cancer.

Targeting: Engineered

Advances in molecular biology have afforded the OV field an opportunity to alter the DNA sequences of viruses and thus engineer viruses that are more specific for cancer cells than their normal counterparts. Currently, in clinical trials and in preclinical development, such viruses employ multiple mechanisms, including expression of modified receptors for cellular entry, restriction of critical viral protein expression *via* cancer-specific promoters, and deletion of viral proteins that prevent apoptosis in healthy cells.

To target ovarian cancer, an oncolytic adenovirus was engineered so that its capsid incorporated a specific arginine–glycine–aspartic acid (RGD) protein motif so that it could bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (34) since these cell surface receptors are overexpressed in ovarian cancer (35). Similarly, to allow for targeting of melanoma by a lentivirus, the E2 glycoprotein of the Sindbis virus, which has a natural affinity to these cells, has been expressed (36). This establishes the concept that OVs may be able to be engineered to directly target cancer cells based on unique cell surface receptors.

Cancer cells inherently have altered signaling pathways that allow for uncontrolled proliferation. Molecular biology is allowing viruses to be modified and provided tropisms that take advantage of protein regulation and signaling in cancer cells. One technique used is the manipulation of viral genes so they are under the control of modified promoters. Adenoviruses are commonly engineered in this manner based on their large viral genome, which allows for the incorporation of long DNA sequences thus permitting multiple modifications to be made to the native virus. E1A is an adenoviral protein that inhibits retinoblastoma (Rb)-mediated cell cycle arrest, allowing for sustained viral replication. It has been manipulated in various ways to allow for selective viral tropism in tumor cells. In an engineered virus specific to prostate cancer, for example, this gene has been modified to be under a prostate-specific antigen (PSA) promoter. PSA is a protein that is created specifically in normal and malignant prostate cells. This modification leads to E1A expression, and therefore, viral proliferation and oncolysis, that only occurs in prostate cells (37). In other cells, however the adenovirus will not produce E1A and therefore Rb-induced apoptosis will occur normally, thus halting infection. The KH901 virus is also a modified adenovirus that expresses E1A in actively dividing cells. This is achieved by having E1A transcription coupled to human telomerase reverse transcriptase (hTERT) and further being restricted by the presence of E2F-1 (cell cycle regulator) on the hTERT promoter (38). Here, using the human telomerase promoter increases the number of different tumors susceptible to this adenovirus. E1A manipulation has also been used in the development of CG0070. This virus has been modified to selectively replicate in cells that have an Rb deficiency or defect. Normally, Rb binds to E2F, inhibiting this factor's activity and its ability to conjugate with other molecules (39). In the context of Rb depletion, E2F is free to bind. Therefore, the E1A protein has been placed under the control of the E2F-1 promoter in this virus (40). Again, multiple cancers are susceptible because E2F-1 regulation of E1A expression is restricted to cells with defective Rb, which is a common mutation in cancers. The tumor microenvironment is often hypoxic, protecting the tumor cells from traditional therapies, such as radiation, that require oxygenation to work optimally. However, OVs with A1E manipulation can be engineered to take advantage of such an environment. Specifically, HYPR-Ad is an adenovirus whose E1A expression is transcriptionally regulated by HIF-1α, a protein induced by the hypoxic environment (41).

In addition to restricting viral replication to tumor cells, adenoviruses have been engineered to use targeted delivery of suicide genes with promoters that have increased activity. One example is the placement of the HSV-1 thymidine kinase (TK) suicide gene under the control of an osteocalcin promoter. Osteocalcin is overexpressed in patients with bone metastases. This modification restricts the toxicity of the suicide gene to cells with an active osteocalcin promoter and increases susceptibility of cancer cells with overactive osteocalcin promoters (42). This strategy of targeting cancer cells by using promoters that are tissue-specific or enriched in the tumor limits the effects of the viruses to these areas, which can improve the therapeutic ratio by limiting side effects spatially.

To promote tumorigenesis, cancer cells disrupt natural immune system antiviral defenses and thus do not function like normal cells. This allows for the engineering of viruses to induce proliferation and lysis of cancer cells while inducing apoptosis in normal cells. Adenoviruses, for example, normally act to prevent abortive apoptosis in normal cells through the E1B protein, which binds to and inactivates the p53 protein. OVs ONYX-15 and H101 have been engineered to prevent expression of E1B (43). While these viruses retain the ability to infect normal cells, they will not actively replicate in them because the cells will undergo apoptosis, thus stopping the spread of the virus to other nearby normal cells. Cancer cells, on the other hand, that downregulate or inactivate p53, will remain susceptible to viral replication and lysis, and allow the spread of the virus to other cancer cells.

Other viruses, including oncolytic herpesvirus have been likewise engineered to take advantage of the disruption of standard antiviral responses that are inherent to cancer cells. Cancer cells commonly have overactive Ras-MAPK signaling which blocks phosphorylation of PKR. Such PKR blockade permits cellular proliferation. The HSV JS1 strain (the backbone of T-VEC) has been engineered with deletions of ICP 34.5 and US11. ICP34.5 acts as a neurovirulence factor required for viral replication that works by interacting with PCNA, and the US11 gene product downregulates expression of PKR. These virulence factors are required in normal infection, but since tumor cells undergo aberrant division and frequently downregulate PKR, this attenuated virus is able to preferentially infect cancer cells (44).

DIRECT CELL LYSIS

Cell Lysis: Natural

The first-line mechanism of action of OVs is their direct lysis of cancer cells (**Figure 3**). Some OVs take advantage of dysregulate apoptotic pathways in cancer cells to shunt cells toward other forms of death. One example of this is parvovirus H-1PV, which is currently being evaluated in a phase I/IIa clinical trial for metastatic inoperable pancreatic ductal adenocarcinoma (PDAC), while the first parvovirus clinical trial in patients with progressive recurrent glioblastoma has been recently completed. Toward prolonged survival, many tumors, including glioma and PDAC, actively dysregulate apoptotic pathways, which are inhibited even in the context of viral infection. Thus, H-1PV utilizes the cathepsin-mediated pathway to cause tumor cell death (45, 46). This type of non-apoptotic death is immunogenic and through a bystander effect, leads to an increase of interferon (IFN)-γ, inflammatory cytokines, and tumor neoantigen exposure from oncolytic cell death, which together, can lead to antitumor immune responses (46, 47).

Cell Lysis: Engineered

Other OVs have been engineered to induce cell death through mechanisms involving both virolysis and the introduction of suicide genes. Some adenoviruses have been modified to express HSV-1 TK. Once the virus targets the cancer cell using its specific natural or engineered tropism, it allows for a unique mechanism of cell killing when combined with thymidine analogs (e.g., acyclovir or ganciclovir). Unlike normal human TK, the HSV-1 TK can activate these thymidine analogs by converting them into monophosphates (48). These monophosphates are subsequently incorporated into the DNA of replicating cells which leads to chain termination and cell death. To further increase specificity, tumor-specific promoters are used to regulate the expression of HSV-1 TK. Clinical trials in which the osteocalcin promoter regulates HSV-1 TK expression have been used to target bone metastases. Similar to HSV-1 TK, another viral suicide gene, adenovirus death protein has been incorporated into OVs in preclinical studies to increase cell death (49). "Arming" OVs with suicide genes enhances their overall efficacy through increasing the ability of these viruses to directly kill cancer cells. Other OVs have been engineered to express pro-apoptotic molecules, such

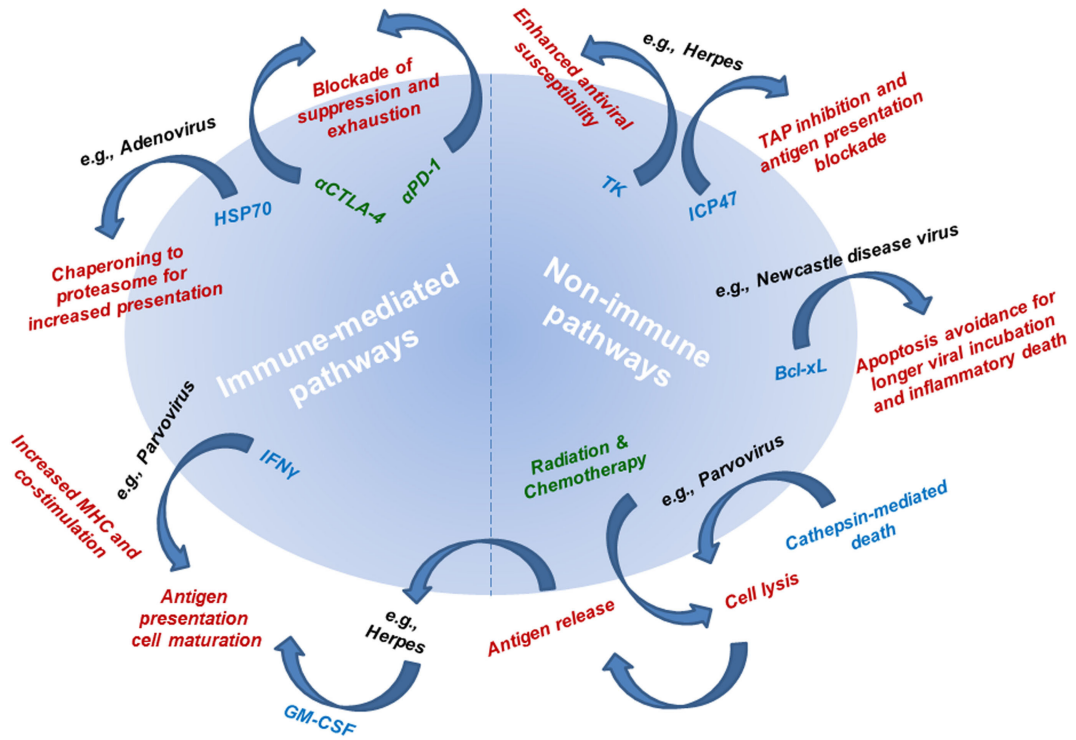

FIGURE 3 | Mechanisms of action underlying the efficacy of oncolytic viruses (OVs). Mechanistic pathways are categorized as immune-mediated and non-immune pathways. Non-immune pathways are utilized by OVs to avoid apoptotic (non-immunogenic) death to allow sufficient viral infectivity and spread, while at the same time resulting in cell lysis. Connecting non-immune and immune pathways is antigen release as a result of viral infection-induced cell lysis. The mechanism underlying immune-mediated pathways includes delivery or utilization of immune mediators that allow for improved tumor antigen presentation and subsequent tumor-targeted immune responses. Radiation therapy and chemotherapy (leading to cell lysis and antigen release) and immunotherapy (leading to blockade of antitumor response suppression and exhaustion) are being combined with OVs to simultaneously target multiple mechanistic pathways for improved antitumor therapeutic responses. Mechanisms of action are listed in red text. Single examples of viruses utilizing each of the described mechanisms are listed in black text next to their respective mechanism. Mediators responsible for each mechanism are listed in blue text next to their respective mechanism. Therapies used in combination regimens with OVs are listed in green text.

as TNF-related apoptosis-inducing ligand (TRAIL), which classically have been associated with apoptosis, but recently also shown to be involved in necroptosis (i.e., programmed necrosis) (50–53).

IMMUNE ACTIVATION

Immune Responses: Natural

Beyond using the inherent lytic potential of OVs to provide direct killing of the cancer cell, viral infection of cancer cells elicits the standard antiviral immune response to clear the viral infection (**Figure 3**). In this process, it is proposed that antitumor responses can be revitalized or initiated by converting the environment from a suppressive into an inflamed tumor microenvironment. Briefly, viral infection results in the increase of pro-inflammatory cytokines, which recruit and activate both innate and adaptive immune cells. Further, viral infection results in the release of potent immune stimulators, toll-like receptor (TLR) ligands, which are critical for activating antigen-presenting cells (APCs), NK cells, and T cells. The combination of the release of cytokines and of TLR ligands as a result of infection of cancer cells with OVs is proposed to alleviate tumor-induced immune suppression. In addition, viral lysis leads to the release of intracellular tumor antigens that have not been presented and thus otherwise remain hidden to the immune system. Thus, oncolytic viral infection may release cancer antigens, including neoantigens (to which the immune system has not yet been tolerized) in the context of an inflammatory immune response, thus generating an effective antitumor response.

Mechanisms that some malignancies use to subvert the host immune system, in turn make them more susceptible to OVs. An example of this is interference with the type I IFN signaling pathway, which acts systemically to heighten antiviral and antitumor immune responses and locally to decrease cellular proliferation and increase p53, thereby activating the host apoptotic pathways (54). A number of cancers manipulate this pathway by decreasing type I IFN expression, decreasing receptor expression, or altering signaling downstream. While this leads to an optimal environment for cancer replication, it is also ideal for viral infection since in healthy tissues viruses are frequently cleared by the type I IFN responses. This, therefore, allows OVs, including vesicular stomatitis virus (VSV), vaccinia, Newcastle disease virus, mumps virus, alphaviruses, rabbit myxoma virus, and others to have specificity for these cells and the resulting microenvironment.

Additional factors have to be considered for oncolytic viral treatments. These include understanding the ability of a virus to hide from the immune system as well as possible antibodies generated against a virus since immunity may already exist to certain viruses. Some OVs are ones that humans are commonly exposed to, including adenovirus and poxviruses. Therefore, neutralizing antibodies may be present in the serum prior to treatment and further antibodies produced more rapidly based on immunological memory (55). However, exposure to and neutralizing antibodies against some OVs are less common (e.g., Seneca Valley virus). Such OVs provide an advantageous therapeutic window for treatment prior to immune neutralization of the virus (32).

Immune Responses: Engineered

In addition to the immune-mediated mechanism of action for OVs that is a result of standard antiviral responses, OVs have been specifically engineered to further potentiate the immune response (**Figure 3**). To improve immune-mediated tumor destruction, OVs induce cancer cells to express pro-inflammatory cytokines, increase antigen presentation by cancer cells, and promote a more immunogenic form of cancer cell death. Cancer cells prevent immune destruction through altering the tumor microenvironment by recruiting immune suppressive cells and producing cytokines that limit antitumor responses. OVs specifically target cancer cells and are engineered to modify the suppressive tumor microenvironment. T-VEC was engineered with two copies of the human GM-CSF gene based on this idea. This immune stimulatory molecule can recruit professional APCs including dendritic cells, promote presentation of cancer antigens, lead to an influx and maturation of immune cells, and activate NK cells and tumor antigen-specific T cells (20, 56–58). Similar modifications have been made in adenoviruses and vaccinia virus (59, 60). In addition, adenoviruses have been engineered to enhance intrinsic antigen presentation by the cancer cell. Specifically, they have been modified to overexpress heat shock protein 70 (HSP70) in their target cells. This leads to increased intracellular protein trafficking to proteasomes, which directly leads to the increased availability of protein fragments for antigen presentation. HSP70 also has the unique characteristics of being directly associated with antigen presentation and allows for more peptides to be seen by APCs because these cells have an affinity for HSP70-linked peptides (61).

Natural serotype switching provides an advantageous method of treatment for viruses that possess multiple serotypes such as VSV and adenoviruses. However, serotype switching can also be mimicked through engineering of a virus to aid in immune system evasion and has been successfully done in measles virus (62). Other methods developed to avoid antibody neutralization include the encapsulation of OVs in polymer coatings to ensure viral replication and circulation (63, 64).

CONCLUSION

Despite the immense progress evident in the development of OVs, it is important to note that substantial work remains in regards to understanding the mechanics and the ultimate potential of OVs. Genetic engineering to augment therapeutic efficacy still needs to be studied. Further, the proper administration and doses of different viruses should be investigated. As more information arises describing the use of new OVs, increases in biosafety procedures and protocols will also be a factor to consider. Clinical implications regarding the correct cancers to target and appropriate patients for whom to use oncolytic therapies will also be important to understand.

Oncolytic viruses used in therapy have had modest clinical success as stand-alone treatments for cancer. While efforts are being made to improve the overall efficacy of these viruses as individual therapies (including by engineering better OVs), combining OVs with currently approved cancer treatments may

drastically improve therapy. Because of their unique mechanism of action, specific ability to target cancer cells, and good safety profile, OVs have been combined with many standard cancer therapies including surgery (NCT02714374), chemotherapy (NCT02779855), radiation therapy (NCT02453191), hormone modulators (NCT01867333), targeted therapies (NCT03088176), and even other immunotherapies (NCT02978625) (10, 65–71).

In addition, OVs have been combined with other OVs to enhance cancer cell lysis, improve targeting, and overcome immunity developed against multiple administrations of the same virus. Herpes virus combined with adenovirus in a pancreatic tumor model has been shown to improve the lytic release of adenovirus (72). Echoviral infection, which results in the upregulation of ICAM-1 (the cell entry receptor for Coxsackievirus) has been shown to improve efficacy of coxsackievirus (26). Further, the combination of vaccinia expressing the tumor antigens CEA and Muc1 (Panvac-v) and fowlpox virus expressing the same tumor antigens (Panvac-f) has been shown to improve direct lysis and immune-mediated tumor destruction (73–75).

Further, OVs have been combined with recently FDA-approved immunotherapies to enhance the immune-mediated mechanisms associated with tumor clearance. Specifically, talimogene laherparepvec (T-VEC), a herpes virus encoding GM-CSF, is being assessed for its ability to be combined with the immune checkpoint blockers ipilimumab (an antibody against CTLA-4) (NCT01740297) and pembrolizumab (an antibody against PD-1) (NCT02263508). Similarly, adenoviruses have been combined with checkpoint inhibitors in several ongoing studies in various malignancies including melanoma (NCT03003676), lung cancer (NCT02879760), and breast cancer (NCT03004183). The potential synergy from initiating immune responses with OVs and then blocking tumor immune suppression with immune checkpoint blockade represents an attractive therapy to generate effective antitumor responses. Such responses may be achieved in greater proportions of patients than either therapy has delivered alone. The emergence and imminent regulatory approval of chimeric antigen receptor (CAR)-modified T cells will lead to yet another potential combination therapy. CAR-T cells have been limited in their ability to treat solid tumors, likely secondary to poor trafficking to the immunosuppressive microenvironment (76). Early preclinical work, however, has established the potential benefits of this combination in solid tumors through viral modifications allowing for better tumor infiltration by and survival of CAR-T cells (77). Given the near limitless viral modifications that can be made to locally deliver specific genes and gene products to the tumor microenvironment, OVs have the potential to have an ever expanding role as an adjuvant to current and upcoming systemic immune and non-immune-mediated therapies.

Clinical trials have demonstrated that OVs are tolerable and their common side effects include those present in natural viral infections (e.g., fever, fatigue, and other flu-like symptoms) (78, 79). Improvement of OV specificity for cancer cells can be utilized to achieve the highest possible therapeutic efficacy while limiting side effects. Specifically, the antiviral immune response can at the same time limit the bioavailability and produce dose-limiting side effects, while both the antitumor and antiviral immune response can mediate therapeutic antitumor responses. Another safety consideration of using live virus for treatment is the possibility of transmission to others. However, transmissibility to others is limited because the majority of OVs are attenuated through extensive passaging, neutralized by healthy humans with preexisting antibodies and have reduced ability to spread through saliva and urine. However, precautions should always be taken, as with any live virus use (similar to those currently in place for live virus vaccinations).

Overall, OVs have shown the potential to be a very effective method of immunotherapy that efficiently and preferentially kills cancer cells. OVs have shown great successes as a treatment modality that both directly lyses cancer cells and elicits strong antitumor immune responses. With T-VEC producing significant results in terms of tumor regression in the United States, and with H101 approval in China, the use of OVs as an immunotherapeutic strategy has the potential to change the way we treat our patients. Numerous viruses exist as future therapeutic candidates allowing various cancers to be targeted for treatment. OVs have thus emerged as a fascinating approach to combat cancer and will only continue to improve with increased funding, preclinical studies, and clinical trials.

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Current Immunotherapeutic Strategies to Enhance Oncolytic Virotherapy

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Oncolytic viruses (OV) represent a promising strategy to augment the spectrum of cancer therapeutics. For efficacy, they rely on two general mechanisms: tumor-specific infection/cell-killing, followed by subsequent activation of the host's adaptive immune response. Numerous OV genera have been utilized in clinical trials, ultimately culminating in the 2015 Food and Drug Administration approval of a genetically engineered herpes virus, Talminogene laherparepvec (T-VEC). It is generally accepted that OV as monotherapy have only modest clinical efficacy. However, due to their ability to elicit specific antitumor immune responses, they are prime candidates to be paired with other immune-modulating strategies in order to optimize therapeutic efficacy. Synergistic strategies to enhance the efficacy of OV include augmenting the host antitumor response through the insertion of therapeutic transgenes such as GM-CSF, utilization of the prime-boost strategy, and combining OV with immune-modulatory drugs such as cyclophosphamide, sunitinib, and immune checkpoint inhibitors. This review provides an overview of these immune-based strategies to improve the clinical efficacy of oncolytic virotherapy.

Keywords: oncolytic virus, immune therapy, GM-CSF, prime boost, cyclophosphamide, immune checkpoint, sunitinib

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INTRODUCTION

Despite the introduction of molecular interrogation and personalized medicine strategies for both the diagnosis and treatment of cancer over the past decade, the burden of this disease is still large. In 2016, an estimated 600,000 individuals died from cancer in the USA alone (1). Thus, while there is more efficacy in cancer treatment than ever before, there is still a significant potential for improvement.

Until recently, the myriad of genetic and epigenetic alterations that exist among cancer cells provided a seemingly insurmountable therapeutic challenge. How could one specific drug target all the machinery that the cancer cell uses to grow? Additionally, tumor heterogeneity and resistance mechanisms allow growth of cancer cells under the selective pressures of both the tumor microenvironment and attempted treatments (2). Thus, the answer to these treatment barriers may be in the ability to harness the potential of an equally diverse entity—the human immune system. One unique class of cancer therapeutics that utilizes the immune system is oncolytic viruses (OV).

The recognition that viral infection could play a role in the treatment of cancer first came to light over one hundred years ago (3). Only recently, though, has there been an increasing interest in the field, culminating in the US Food and Drug Administration (FDA) approval of a modified herpes simplex virus (HSV) for use in metastatic melanoma (4). There are numerous other clinical trials of OV currently ongoing (**Table 1**).

TABLE 1 | Selected ongoing clinical trials using oncolytic viruses.

Virus	Name	Mods/effect	Tumor	Phase	Route	Combination	Trial ID
Adenovirus	DNX-2401	*Enhance viral tumor entry*: Δ24-RGD insertion	Glioma, gliosarcoma	I	IT	IFN-γ	NCT02197169
				II	IT	Pembrolizumab	NCT02798406
			Glioma	I	IT	Temozolomide	NCT01956734
	VCN-01	*Enhance intratumoral distribution*: PH20 hyaluronidase insertion	Pancreas	I	IT	Gemcitabine + Abraxane	NCT02045589
			Solid tumors	I	IV	Gemcitabine + Abraxane	NCT02045602
	Colo-Ad1	*Increase tumor specificity*: Chimeric Ad11/3 group B	Ovarian	I/II	IP	–	NCT02028117
			Solid tumors	I	IV	Nivolumab	NCT02636036
				I/II	IV	–	NCT02028442
	AdV-tk	*Increased tumor sensitivity to drug*: TK insertion	MPE	I	IPI	–	NCT01997190
			Pediatric (brain)	I	IT	RT + Valcyclovir	NCT00634231
			Pancreas	I/II	IT	Gemcitabine + RT + mFOLFIRINOX	NCT02446093
			Prostate	II/III	IT	Valcyclovir	NCT02768363
				III	IT	RT + Valcyclovir	NCT01436968
	Oncos-102	*Enhance viral tumor entry and immune activation*: Δ24-RGD-GM-CSF insertion	Melanoma	I	IT	CPA + Pembrolizumab	NCT03003676
			Mesothelioma	II	IPI	Carboplatin/Paclitaxel + CPA	NCT02879669
			Solid tumors	I	IP	Durvalumab	NCT02963831
	CG0070	*Immune activation*: GM-CSF insertion and E3 deletion	Bladder	III	Intravesicular	–	NCT02365818
Coxsackie	CVA21	None	Lung (NSLC)	I	IV	Pembrolizumab	NCT02824965
			Melanoma	I	IT	Ipilimumab	NCT02307149
					IT	Pembrolizumab	NCT02043665
			Solid tumors	I	IV	Pembrolizumab	NCT02043665
Herpes simplex	Talminogene laherparepvec	*Decreased virulence and prolong viral replication*: ICP34.5 deletion, US11 deletion, GM-CSF insertion	Breast	I/II	IT	Paclitaxel	NCT02779855
				II	IT	–	NCT02658812
			H/N	I	IT	Pembrolizumab	NCT02626000
			HCC, Liver Mets	I	IT	–	NCT02509507
			Lymphoma	II	IT	Nivolumab	NCT02978625
			Melanoma	I/II	IT	Ipilimumab	NCT01740297
				II	IT	RT	NCT02819843
						–	NCT02366195
						–	NCT02211131
						Pembrolizumab	NCT02965716
				III	IT	–	NCT02297529
						Pembrolizumab	NCT02263508
			Pediatric	I	IT	–	NCT02756845
			Sarcoma	I/II	IT	RT	NCT02453191
				II	IT	RT	NCT02923778
	HF-10	*Decreased virulence*: UL56 deletion, single partial UL52	Melanoma	II	IT	Ipilimumab	NCT02272855
			Solid tumors	I	IT	–	NCT02428036
	HSV1716	*Decreased virulence*: ICP34.5 deletion	Mesothelioma	I/II	IPI	–	NCT01721018
			Pediatric	I	IT/IV	–	NCT00931931
	G207	*Decreased virulence*: ICP34.5 deletion, UL39 disruption	Pediatric (brain)	I	IT	RT	NCT02457845

(*Continued*)

TABLE 1 | **Continued**

Virus	Name	Mods/effect	Tumor	Phase	Route	Combination	Trial ID
Maraba	MG1	*Tumor antigen to enhance antitumor immune activity*: MAGE-A3	Lung (NSCLC)	I/II	IM	AdMA3 Vaccine + Pembrolizumab	NCT02879760
			Solid tumors	I/II	IM	AdMA3 vaccine	NCT02285816
Reovirus	Reolysin	None	Bladder	I	IT	Gemcitabine + Cisplatin	NCT02723838
			Breast	II	IV	Paclitaxel	NCT01656538
			Colorectal	I	IV	FOLFIRI + Bevacizumab	NCT01274624
				II	IV	FOLFOX + Bevacizumab	NCT01622543
			Myeloma	I	IV	Bortezomib + Dexamethasone	NCT02514382
						Lenalidomide or Pomalidomide	NCT03015922
			Pancreas	I	IV	Pembrolizumab + Chemo	NCT02620423
				II	IV	Carboplatin + Paclitaxel	NCT01280058
			Pediatric (brain)	I	IV	GM-CSF	NCT02444546
			Solid tumors	II	IV	Paclitaxel	NCT01199263
Vaccinia	GL-ONC1	*Increased tumor sensitivity to drug and reduced virulence*: TK disruption, hemagglutinin disruption, F14.5L disruption	MPE	I	IPl	–	NCT01766739
			Ovarian	I	IP	–	NCT02759588
			Solid tumors	I	IV	Eculizumab	NCT02714374
	JX-594	*Immune activation and increased tumor sensitivity to drug*: GM-CSF insertion, TK disruption	Breast, sarcoma	I/II	IV	CPA	NCT02630368
			HCC	III	IT	Sorafenib	NCT02562755
			Solid tumors	I	IT	Ipilimumab	NCT02977156
	PROSTVAC	*Tumor antigen to enhance antitumor immune activity*: PSA, LFA-3, ICAM-1, B7.1 additions	Prostate	I/II	SC	Nivolumab and/or Ipilimumab	NCT02933255
				II	SC	–	NCT02326805
						–	NCT02649439
						–	NCT02772562
						Ipilimumab	NCT02506114
						Docetaxel + Prednisone	NCT01145508
						Docetaxel	NCT02649855
						Flutamide	NCT00450463
						–	NCT02153918
						Enzalutamide	NCT01867333
							NCT01875250
				III	SC	GM-CSF	NCT01322490
Vesicular stomatitis	*VSV-IFNβ-NIS*	*Increased tumor specificity and enhanced sensitivity to radiotherapy*: IFN-β + NIS	Hematologic malignancy	I	IV	–	NCT03017820
			Solid tumors	I	IV	–	NCT02923466

CPA, cyclophosphamide; IM, intramuscular; IP, intraperitoneal; IPl, intrapleural; IT, intratumoral; IV, intravenous; MPE, malignant pleural effusion; SC, subcutaneous; RT, radiotherapy.

OV therapy is based on the finding that certain viruses selectively replicate within cancer cells. Initially, OV therapy was thought to exert its primary anticancer effect through direct tumor oncolysis (apoptosis/autophagy). However, almost 20 years ago, findings by Mastrangelo and colleagues (5) demonstrated that, in fact, another mechanism may be at play with oncolytic virotherapy. Not only did primary tumors decrease in size when injected with an oncolytic vaccinia virus (VV), but non-injected tumors did as well (5). Their findings suggested that OV have the potential to induce systemic antitumor immunity. It is now commonly accepted that exposure of tumor neoantigens after OV-induced oncolysis (**Figure 1A**) can activate both the innate and adaptive arms of the host immune system and direct them specifically toward areas of tumor burden. It is currently unclear to what extent each of these mechanisms contributes to the overall success of clinical efficacy in an individual OV.

Interestingly, there has been only modest success in the introduction of OV to the clinical arena as monotherapies (6, 7). The explanation for these modest results is likely multifactorial, including host antiviral mechanisms limiting effective viral dissemination, development of tumor resistance to key oncogenic signaling pathways typically exploited by OV, and a host of immunosuppressive regulatory factors within the tumor microenvironment. Current clinical approaches utilizing OV seek to enhance their efficacy with complimentary immunotherapeutic strategies (**Figure 1B**).

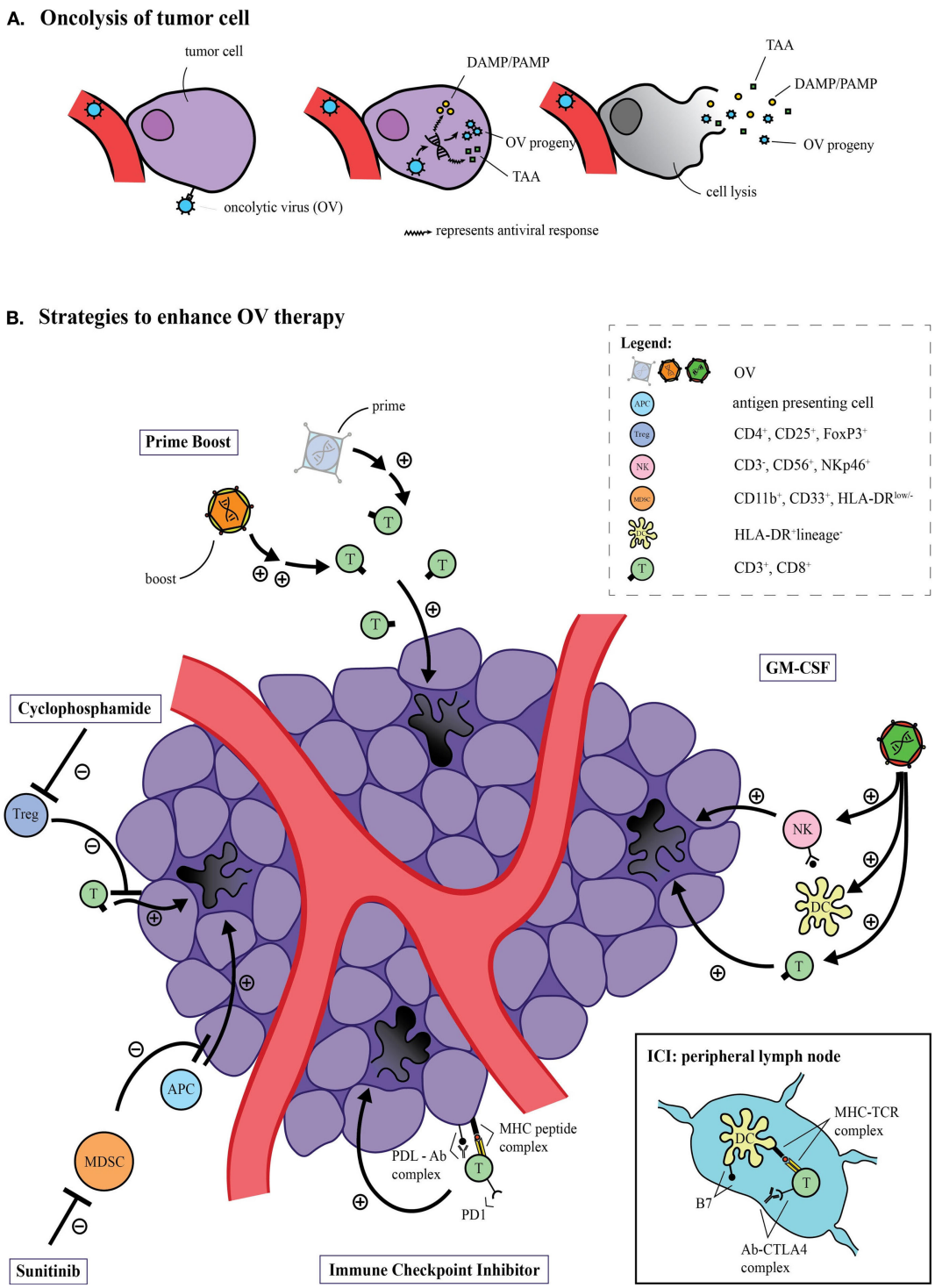

FIGURE 1 | Oncolytic virus (OV)-mediated tumor cell lysis. **(A)** OV can specifically infect cancer cells, and subsequent replication can induce oncolysis. The release of tumor antigens has the potential to activate a systemic antitumor immune response. **(B)** The immune response induced by OV can be improved through several strategies. The prime-boost approach utilizes one priming viral platform carrying tumor-specific antigens, while a second platform—usually an OV—carrying the same antigens boosts the resultant antitumor immune response. The insertion of transgenes, such as GM-CSF, can facilitate antigen presentation on the surface of dendritic cells, and thus augment an antitumor response by recruiting natural killer (NK) cells and inducing tumor-specific cytotoxic T-cells. Immune checkpoint inhibitors can function both at the level of the tumor, targeting the programmed cell death protein 1 (PD-1) axis or peripherally at the level of the lymph nodes by targeting the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) axis. Both approaches ultimately improve the antitumor response. Immunomodulatory drugs such as sunitinib and cyclophosphamide can augment the antitumor immune response of OV by inhibiting immunosuppressive populations, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), respectively.

As the field of OV is in the midst of renewed excitement and optimism, we seek herein to provide an overview of the most frequently utilized immune-based strategies to improve the clinical efficacy of oncolytic virotherapy and review the available evidence for doing so.

Manipulating OV for Clinical Benefit

The Hallmark Transgene: GM-CSF

Early in the process of bringing OV into the clinical setting, it was realized that certain viral candidates could be genetically modified to reduce virulence and/or be armed with therapeutic transgenes to augment oncolytic activity with local gene delivery. Transgenes to enhance therapeutic benefit of OV are quite varied and include inflammatory cytokines, proteases that degrade the tumor microenvironment, antiangiogenic proteins, prodrug-converting enzymes, and proapoptotic genes (8). In general, the trend in the OV field is to enhance candidate viruses in such a way that their ability to induce antitumor immunity is optimized. No transgene has been utilized as frequently or with as much success as GM-CSF.

Ever since the antitumor effects of GM-CSF were first appreciated by Dranoff and colleagues (9), it has held particular interest as a therapeutic adjuvant in immune-based cancer treatments. Based on its effects in cytokine-transduced cancer cell vaccines such as Sipuleucel-T for prostate cancer, it has become an attractive OV therapeutic transgene. By promoting monocyte-to-dendritic cell (DC) differentiation, GM-CSF facilitates antigen presentation on the surface of DCs following viral-induced oncolysis (10). This ultimately leads to a more robust antitumor immune response by recruiting natural killer (NK) cells and inducing tumor-specific cytotoxic T-cells (11).

To date, GM-CSF has been used with success in OV platforms such as HSV (4, 12), VV (13, 14), and adenovirus (AdV) (15, 16). Of these, HSV and VV have arguably served as the most efficacious platforms. A phase III randomized clinical trial comparing HSV-1 with a GM-CSF Transgene Talminogene laherparepvec (T-VEC) vs. GM-CSF alone in advanced melanoma led to the first FDA approval of an OV. Of 436 patients randomized, 295 were in the T-VEC group and 141 in the GM-CSF arm. The objective response rate (ORR) was 26.4% for T-VEC, including 10.8% with a complete clinical response, vs. 5.7% for GM-CSF alone. Despite not quite reaching statistical significance, those in the T-VEC arm achieved an overall survival of 23.3 vs. 18.9 months in the GM-CSF group, thus demonstrating a meaningful trend toward improved survival (4).

The utility and efficacy of T-VEC are currently being explored across a variety of cancer types with phase II clinical trials open in breast (NCT02658812), lymphoma (NCT02978625), and sarcoma (NCT02923778). Additionally, another randomized phase III trial in melanoma is open exploring the value of adding T-VEC to the programmed cell death protein 1 (PD-1) inhibitor, pembrolizumab, for treatment of unresected melanoma (NCT02263508).

Furthermore, an oncolytic VV has been programmed with a GM-CSF insertion (JX-594) and has been the subject of much clinical investigation. Early-phase I/II trials have been completed with JX-594 in colorectal cancer (17), melanoma (18), pediatric malignancy (19), and non-specific solid tumors (14). The greatest clinical promise, however, has been seen with JX-594 in hepatocellular carcinoma (HCC). A phase II dose-finding trial demonstrated significant survival benefit with high doses (14.1 months) compared to low doses (6.7 months) of JX-594 (20). Furthermore, it was found that objective tumor responses were present in both injected- and non-injected tumors, indicating a possible element of systemic antitumor immunity. Studies of this OV in a preclinical setting have demonstrated that tumor oncolysis is mediated by antibodies in a complement-dependent nature (21), likely related to its ability to increase the release of specific tumor neoantigens/epitopes to the systemic circulation. Further exploration of its efficacy in HCC is currently ongoing, with a phase III trial open for recruitment (NCT02562755) with or without with the VEGFR tyrosine kinase inhibitor Sorafenib.

It is important to consider that despite the clinical promise of OV expressing a GM-CSF transgene, the underlying mechanisms mediating antitumor activity are both poorly understood and subject to controversy. There are little data surrounding the specific mechanistic contributions of GM-CSF to the success of the OV previously mentioned. Moreover, despite the recognition that GM-CSF has a certain level of antitumor potency, it is also intricately linked to the modulation (increase) of immunosuppressive myeloid-derived suppressor cells (MDSCs) (22). Specifically, not only has GM-CSF been shown to increase MDSC numbers in transplantable tumor models (23) but it has also been implicated as the main factor driving MDSC generation in these models (24). Thus, further study is needed to determine the best use of GM-CSF with OV in order to maximize its antitumor effects, while minimizing its recruitment and proliferation of immunosuppressive MDSCs.

"Boosting" OV Efficacy: The Prime-Boost Strategy

Based on the success of traditional vaccinations to combat virally induced disease, vaccinating patients with tumor antigens has been a therapeutic approach of interest in cancer, although has only demonstrated modest success to date. Eliciting a successful systemic immune response against tumor antigens requires the breaking of tolerance that typically prevents host antitumor immunity. One answer may be to utilize viral delivery platforms. One problem with this approach lies in that the use of viral vectors may induce a competitive immune response against the viral antigens, rather than the tumor antigens of interest (25). A solution is to utilize the emerging heterologous "prime-boost" approach. For example, tumor-specific antigens can be encoded into the backbone of one viral platform to prime the immune system before being introduced to a second viral platform carrying the same antigens that upregulates, or boosts, the resultant antitumor immune response.

Classic viral vaccine vectors are non-replicating and therefore do not have oncolytic properties. However, the prime-boost strategy with non-OV has still seen demonstrable clinical applicability. PROSTVAC, which is utilized in prostate cancer, is the prototypical example. Despite not utilizing an OV platform, ongoing clinical trials of PROSTVAC are highlighted in **Table 1**, as success of this platform to date demonstrates the power of the prime-boost strategy in viral-based cancer vaccination.

There are two members of the *Rhabdoviridae* family that have been investigated for use as OV, both belonging to the *Vesiculovirus* genus—vesicular stomatitis virus (VSV) and Maraba virus. These enveloped ssRNA viruses were first noted to have oncolytic potential in 2000 when VSV was demonstrated to induce tumor regression in a mouse xenograft model of melanoma (26). VSV is a promising oncolytic agent due to its reasonable safety profile and lack of preexisting neutralizing antibodies in humans—problems that have been encountered with other OV platforms. It has been demonstrated that VSV can be utilized effectively as a cancer vaccine, with increased capacity as part of a heterologous prime-boost strategy (27, 28). In a murine model of melanoma, VSV vaccine not only induced upregulation of tumor-specific immunity but also decreased adaptive antiviral immunity leading to an increase in the overall survival of treated animals (27). Following the early preclinical success of VSV, other mammalian cell-trophic rhabdovirus family members were screened for oncolytic capacity (29). From this study, Maraba virus was identified as having the broadest oncotropism, which could be further enhanced with the induction of two-point mutations (L123W in M and Q242R in G). In a direct comparison to a similarly mutated VSV in a murine model of metastatic colorectal cancer, this Maraba virus (MG1) induced total tumor clearance in 100% of treated animals, as compared to 30% in VSV (29). Later studies specifically investigating a Maraba MG1 expressing a melanoma antigen demonstrated its inability to prime an adaptive immune response but significant capacity as a boosting vector. In a syngeneic murine model of melanoma, utilizing Maraba MG1 had dramatic effects leading to significantly extended median survival and complete remission of 20% of animals treated (30). Preclinical promise has allowed Maraba MG1 to move into early-phase clinical trials, with two currently ongoing (NCT02879760, NCT02285816). Both trials utilize a non-replicating AdV vector for priming with MG1 as the boost. Results are not yet available.

Synergistic Strategies with OV and Immune-Modulatory Drugs

Cyclophosphamide (CPA)

Cyclophosphamide is a commonly used anticancer agent that non-specifically causes DNA alkylation and induces apoptotic cell death. Additionally, CPA can modulate the immune system through its ability to kill proliferating NK cells, T cells, and B cells with relatively low clinical doses (31). Thus, CPA has been investigated for a synergistic effect along with OV and has demonstrated improved tumor destruction in preclinical models of reovirus (RV) (32, 33), VV (34), measles (35), and AdV (36). Specifically, in a murine model of melanoma, preconditioning with CPA led to an increased intratumoral viral level of oncolytic RV and led to enhanced antitumor efficacy (32). Additionally, one study demonstrated that CPA treatment in conjunction with OV therapy leads to control of the host antiviral response, a problem that can dampen effective OV proliferation, especially in viral platforms that are ubiquitous in humans (37). Furthermore, CPA can potentiate OV replication by suppressing local innate immune cells (38) and depleting regulatory T cells (Tregs), thus enhancing antitumor activity of cytotoxic T-cells (11). Recently, a number of early-phase clinical trials investigating OV synergy with CPA have been completed in oncolytic AdV (solid tumors) (15), oncolytic RV (pediatric tumors, solid tumors) (39, 40), and oncolytic Seneca Valley Virus (neuroendocrine tumors) (41). These trials, however, did not examine the role of CPA specifically in advancing the efficacy of the OV platforms. Furthermore, two current early-phase clinical trials utilizing CPA and an AdV platform are being conducted (NCT00634231, NCT02879669) as well as one trial utilizing CPA and an oncolytic VV (NCT02630368). The general landscape of cancer immune therapies, however, is gravitating toward more tumor-specific therapies. As such, other immune-modulatory agents are being explored, and CPA's role as a synergistic treatment strategy to compliment OV therapy is diminishing.

Immune Checkpoint Inhibitors (ICIs)

Immune checkpoint inhibitors function as immune suppression antagonists. Normally crucial for the maintenance of self-tolerance, immune checkpoint proteins can be overexpressed by tumors as a way to evade detection by the host immune system (42). The first immune checkpoint to be targeted for therapeutic benefit was cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), but superior clinical outcomes, broader clinical applications, and more favorable safety profiles have led PD-1 and its cognate ligand (PD-L1) inhibition to be the new vogue. Importantly, PD-1/PD-L1 inhibition can be combined with CTLA-4 antagonists. PD-L1 expression specifically is induced on activated T cells following a stimulatory signal from IFN-γ (43). CTLA-4 acts at the level of the draining lymph node for T cell priming. Conversely, the PD-1/PD-L1 pathway only inhibits activated T cells, which attenuates the potential for loss of self-tolerance. Since many tumors overexpress PD-L1 (44), they can escape recognition by tumor-infiltrating lymphocytes. Inhibiting this pathway effectively "removes the brakes" on the normal immune response. The impressive success of PD-1/PD-L1 inhibition as monotherapy in phase III clinical trials of melanoma (45), non-small cell lung cancer (46), renal cell carcinoma (RCC) (47), and urothelial carcinoma (48) has led to FDA approval for clinical use. One crucial problem with ICI is that despite their profound efficacy in responding patients, the majority of patients are non-responders (49, 50). This can possibly be explained by the lack of active tumor-specific T cells in the tumor microenvironment. As OV therapy can induce antitumor adaptive immunity, it seems as though ICI and OV could be a perfect therapeutic match.

Preclinical success marrying ICI with OV therapy has been encouraging. Specifically, a study conducted by Zamarin and colleagues (51) demonstrated the potential for combining CTLA-4 inhibition with an oncolytic Newcastle disease virus in a murine model of melanoma. They found that OV therapy alone triggered a systemic antitumor immune response, but accumulated T cells overexpressed CTLA-4, leading to an immunosuppressive tumor microenvironment and diminished treatment efficacy. Adding in CTLA-4 inhibition, however, improved the antitumor response, leading to increased long-term survival of dually treated animals. This response was dependent on NK cells, $CD8^+$ T cells, and type I interferon (51). Although still ongoing, one clinical trial (NCT01740297) utilizing T-VEC and CTLA-4 blockade has promising interim results; ORR has

been found in 41% of treated patients and complete responses in 24%. Given that T-VEC monotherapy has a reported ORR of 26% and a complete response rate of 10.8% (4), the combination therapy with CTLA-4 blockade seems to be an improvement. Additionally, a preclinical study in a murine model of melanoma utilizing an oncolytic RV in combination with PD-1 inhibition demonstrated promising results (52). This group found that combination treatment significantly enhances survival compared to either monotherapy. The enhanced survival was tied to increased activity of NK cells, reduced Tregs, and increased $CD8^+$ antitumor responses (52). Between PD-1 inhibitors nivolumab and pembrolizumab, PD-Ll inhibitor durvalumab, and CTLA-4 inhibitor ipilimumab, there are currently 19 clinical trials ongoing that combine ICI and OV (**Table 1**). Results from these trials are eagerly anticipated in order to assess the value of combining these two immune-based treatment modalities.

Sunitinib

Sunitinib is a multi-tyrosine kinase inhibitor (VEGFR, PDGFR, c-kit, flt3, RET, CSF-1R) that has FDA approval for use in RCC and gastrointestinal stromal tumors. Its primary antitumor effect is through inhibition of VEGFR, leading to a reduced capacity for tumor angiogenesis (53). It is also now understood that sunitinib also has a role in indirectly inhibiting tumor growth through the promotion of antitumor immune responses (54–56). For example, immunosuppressive immune cell populations such as Tregs and MDSC are decreased with sunitinib treatment (54, 55). Its role as an immunotherapeutic adjuvant makes it a suitable candidate for combination with OV. Interestingly, it has been demonstrated that sunitinib can lead to the enhancement of viral replication through targeting innate immune pathways of viral resistance such as double-stranded RNA protein Kinase R (PKR) and RNase (57). The timing of sunitinib administration seems to be of importance, as administering it prior to and during oncolytic RV therapy allowed for the preconditioning of the tumor microenvironment to facilitate a maximal OV-induced antitumor response (58). Although no clinical trials have been initiated utilizing sunitinib and OV, one preclinical study seems to suggest potential for this combination in the treatment of RCC. Sunitinib and an oncolytic RV were found to significantly decrease tumor burden and significantly increase lifespan in a murine model of RCC (59). This therapeutic effect could be explained by their finding that this treatment combination increased the presence of tumor-specific $CD8^+$ T cells and decreased accumulation of both MDSCs and Tregs. Additionally, dually treated mice had protective immunity upon tumor rechallenge. In the same study, Lawson and colleagues (59) also demonstrated similar results in a murine model of squamous cell lung carcinoma, thus highlighting the possible broad application of this treatment strategy. Furthermore, sunitinib combination with an oncolytic VSV led to the elimination of prostate, breast, and kidney malignant tumors in mice (60). Additionally, the antiangiogenic effects of sunitinib can be augmented by the utilization of an oncolytic VV, leading to reduction of tumor growth in murine models of cancer (61). Hopefully, the preclinical success of sunitinib and OV will be replicated in clinical trials once they are initiated.

OTHER STRATEGIES TO ENHANCE OV

Although the focus of this review has been necessarily limited to a handful of combinatorial immunotherapeutic strategies to enhance OV therapy, there are a number of other exciting approaches under preclinical investigation. For example, the combination of adoptive T cell therapy with OV has shown preclinical promise and efforts are underway to bring this strategy to clinical investigation (62, 63). Additionally, a number of different OV platforms are being utilized in combination with inhibitors of histone deacetylases (HDACIs) [reviewed in Ref. (64)]. Although the mechanisms underpinning their tumor tropism are not fully understood (65), HDACIs led to immunogenic cell death of cancer cells thus potentially enhancing antitumor immune responses in synergy with OV (66, 67).

Finally, a transgene-modified oncolytic AdV, NG-348 (PsiOxus Therapeutics), has been recently designed in hopes that it will drive T-cell immune responses within the tumor microenvironment independent of tumor-specific antigens. When two transgenes, a membrane anchored full-length human CD80 and a membrane anchored antibody fragment for the T-cell receptor, are expressed together on the surface of NG-348-infected tumor cells they provide both the T-cell receptor and costimulatory signal required to activate tumor-infiltrating T-cells (68). This strategy mimics that of CAR-T therapies but does not require autologous cell processing or tumor-specific antigens. Furthermore, since the expression of the encoded transgenes is encoded by the endogenous viral major late promoter, their expression is limited to the surface of cells permissive to viral infection—i.e. tumor cells. It is hoped that preclinical testing of NG-348 will ultimately support clinical application.

CONCLUDING REMARKS

Oncolytic viruses represent a promising immunotherapeutic approach to the treatment of cancer. Although clinical trials have demonstrated that their use as a monotherapy is likely insufficient for meaningful efficacy in the clinical arena, it has become clear that the ability for OV to induce a systemic antitumor immune response is intricately linked to their potential for success. Therefore, combining OV with other immunotherapies seems to represent the approach with the most promise. As numerous clinical trials are underway across multiple OV platforms utilizing different immunotherapies for treatment synergy, time will ultimately unveil the potential for OV as a future standard treatment option for our patients with cancer.

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Implications of MicroRNAs in Oncolytic Virotherapy

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MicroRNAs (miRNAs) are an abundant class of small non-coding RNA molecules (~22 nt) that can repress gene expression. Deregulation of certain miRNAs is widely recognized as a robust biomarker for many neoplasms, as well as an important player in tumorigenesis and the establishment of tumoral microenvironments. The downregulation of specific miRNAs in tumors has been exploited as a mechanism to provide selectivity to oncolytic viruses or gene-based therapies. miRNA response elements recognizing miRNAs expressed in specific tissues, but downregulated in tumors, have been inserted into the 3′UTR of viral genes to promote the degradation of these viral mRNAs in healthy tissue, but not in tumor cells. Consequently, oncolytic virotherapy-associated toxicities were diminished, while therapeutic activity in tumor cells was preserved. However, viral infections themselves can modulate the miRNome of the host cell, and such miRNA changes under infection impact the normal viral lifecycle. Thus, there is a miRNA-mediated interplay between virus and host cell, affecting both viral and cellular activities. Moreover, the outcome of such interactions may be cell type or condition specific, suggesting that the impact on normal and tumoral cells may differ. Here, we provide an insight into the latest developments in miRNA-based viral engineering for cancer therapy, following the most recent discoveries in miRNA biology. Furthermore, we report on the relevance of miRNAs in virus–host cell interaction, and how such knowledge can be exploited to improve the control of viral activity in tumor cells.

Keywords: oncolytic viruses, microRNA, gene regulation, detargeting, host–virus interaction

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INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules (~22 nt) that can negatively regulate the expression of large networks of genes (1). Not surprisingly, miRNA dysregulation impacts virtually all cancer-related processes (proliferation, cell death, migration, and cell cycle, among many others). Such dysregulation provides clear hallmark miRNA signatures that can distinguish between normal cells and the tumor cells of many different types of malignancy (1).

In this regard, therapeutic strategies rely either on the reintroduction of the individual miRNAs involved in tumor suppression functions, such as miR-34 (2), or on reducing oncogenic miRNAs with antisense oligonucleotides—"antagomirs" (3). Interestingly, Brown and coworkers exploited the differences in miRNA expression between tissues and proposed a novel mechanism to control transgene expression, based on the differential expression of miR-142 among lineages of hematopoietic cells (4). Selectivity was achieved by the introduction of engineered target sites, or miRNA response elements (MREs). Later, oncolytic virotherapy also incorporated MREs to control the expression of viral or suicide genes. MREs can attenuate oncolytic viruses in non-tumoral tissue

and therefore avoid the undesired toxicity associated with viral tropism when administered systemically or locoregionally (5–8). Posttranscriptional targeting with MREs could be complemented with transcriptional or transductional targeting to enhance the selectivity of oncolytic viruses.

The study of viral miRNAs and host responses has also shown the relevance of miRNAs in the regulation of viral replication. There are, in fact, several examples of how viral infection modulates cellular miRNome, with consequences on both viral activity and host cell functionality.

This review discusses the latest developments with respect to fine-tuning oncolytic viruses, based on the viral engineering of MREs, and looks at the functional consequences of the interplay between miRNAs and viruses.

THE MECHANISM OF ACTION OF miRNAs

Most miRNAs are transcribed from miRNA genes and follow a canonical miRNA biogenesis pathway. miRNAs are transcribed to primary miRNA transcripts (pri-miRNAs) that are processed by the RNase III enzyme Drosha in the nucleus to generate precursor miRNA that are exported by Exportin-5 to the cytoplasm. There they are recognized and cleaved by another RNase III enzyme, Dicer, to give rise to ~22 nt miRNA duplexes. They are then loaded onto the RISC complex, where the Ago proteins will help with the unwinding of the miRNA duplexes to form a functional miRNA-induced silencing complex that will recognize target mRNAs and interfere with their expression. Target mRNA recognition will be based on partial complementarity of miRNA sequences and the 3′UTR of the mRNAs (9).

miRNAs modulate gene expression through mechanisms of translational inhibition, mostly at the initiation step, and mRNA destabilization as a consequence of mRNA deadenylation and mRNA decay. These mechanisms may occur sequentially, with mRNA destabilization as the dominant effect. In consequence, the repression of miRNAs target genes can be evidenced by depletion of the mRNA content (10, 11).

CONTROLLING VIRAL REPLICATION THROUGH MREs

The selectivity of oncolytic viruses can be determined through the introduction of MREs, preferentially in the 3′UTR of viral genes (12). Noticeably, MREs can be inserted into virtually any viral mRNA. Comparable efficiencies have been observed with MREs targeting early phase transcription factors or late phase capsid structural proteins. Examples of the elements targeted are ICP4, ICP27, and glycoprotein H in herpes simplex virus (13–15), E1A and L5 (fiber) in adenovirus (15, 16), or M and L in vesicular stomatitis virus (17, 18). Interestingly, targeting early phase proteins reduces toxicity derived from its own expression and that of downstream genes, offering a greater safety margin than when targeting late phase proteins (16). However, the use of MREs to target both genomic and messenger RNA in RNA viruses showed efficient repression of mRNAs only. The efficacy of miRNA repression in genomic RNAs is reduced due to secondary structures and scaffold proteins that protect the viral genome (5, 19, 20).

The base pairing of the miRNA and target genes usually displays partial complementarity, restricted to nucleotides 2–7 of the miRNA, and known as the "seed" sequence (21, 22). However, partial complementarity can only mediate translational repression and mRNA decay (10, 23–25). To achieve a fast and robust effect on the control of viral replication, MREs must be designed to trigger direct cleavage in the viral mRNA. Although all human Argonaute proteins (Ago1–4) are capable of promoting a translational repression pathway, only Ago2 has endonucleolytic activity (26, 27). In order to trigger Ago2-mediated direct cleavage, the base-paring miRNA:MRE between nucleotides 10 and 11 must be complete (23). This mechanism of action exploits the same mechanisms as RNAi silencing (shRNA and siRNA) (28). The factors that can influence the efficacy of the MRE include the expression levels and profiles of Ago proteins between tissues (29) and the modulation of their activity by covalent modification (30, 31).

Elements of the design of MRE that contribute to its efficacy include the number of target sites, the distance between target sites, the sequence composition and local RNA structure surrounding its location in the 3′UTR (**Figure 1**). The optimal design for MRE should include a range of target sites, usually from 2 to 8, allowing a dose-dependent response to miRNA concentration (32), and a seed separation of between 13 and 35 nt to avoid steric hindrance (33). Access to target sites can usually be estimated through the secondary structure, with the minimum free energy (mfe) and AU-richness surrounding the site (34–36). Moreover, sites for RNA-binding proteins should be avoided since they may also mask MREs and hamper recognition. Hence MRE engineering might benefit from a computed selection of spacers, to promote optimal separation and secondary structure.

The miRNAs considered as regulators to bind to MREs are selected from those that present abundant expression in normal tissue, but a significantly decreased expression in tumors, such as the ubiquitously expressed miRNA let-7 family (18, 37, 38). Other studies have exploited tissue-specific miRNAs. Some examples of tissue-specific MREs are miR-122 for liver (6, 7), miR-7 for brain (39), miR-148a for pancreas (39, 40), and miR-192 for heart (41). In this regard, miRNA belonging to the same family can be used to increase the efficacy of MRE. The extensive homology presented by miRNA of the same family allows sub-optimal recognition of the MRE (**Figure 1**). An increased level of complexity in the selection of miRNA candidates arises with the design of MREs for multiple organs detargeting, usually non-tumoral cells surrounding the tumor and tissues with native viral tropism (42). This can be achieved by using miRNAs present in both organs, or by combining multiple miRNAs (40). In this context, the extensive miRNome data in The Cancer Genome Atlas (https://cancergenome.nih.gov/) for 33 types of cancer and normal tissue constitutes an invaluable resource for the selection of candidate miRNAs (43). Of special consideration when seeking to fine-tune oncolytic virus activity is the diversity of cell types in tissue, especially in approaches using locoregional administration. Here, MREs could also provide the desired level of selectivity, for example, miR-375 has been described almost exclusively in the beta and alpha cells present in pancreatic islets (44, 45).

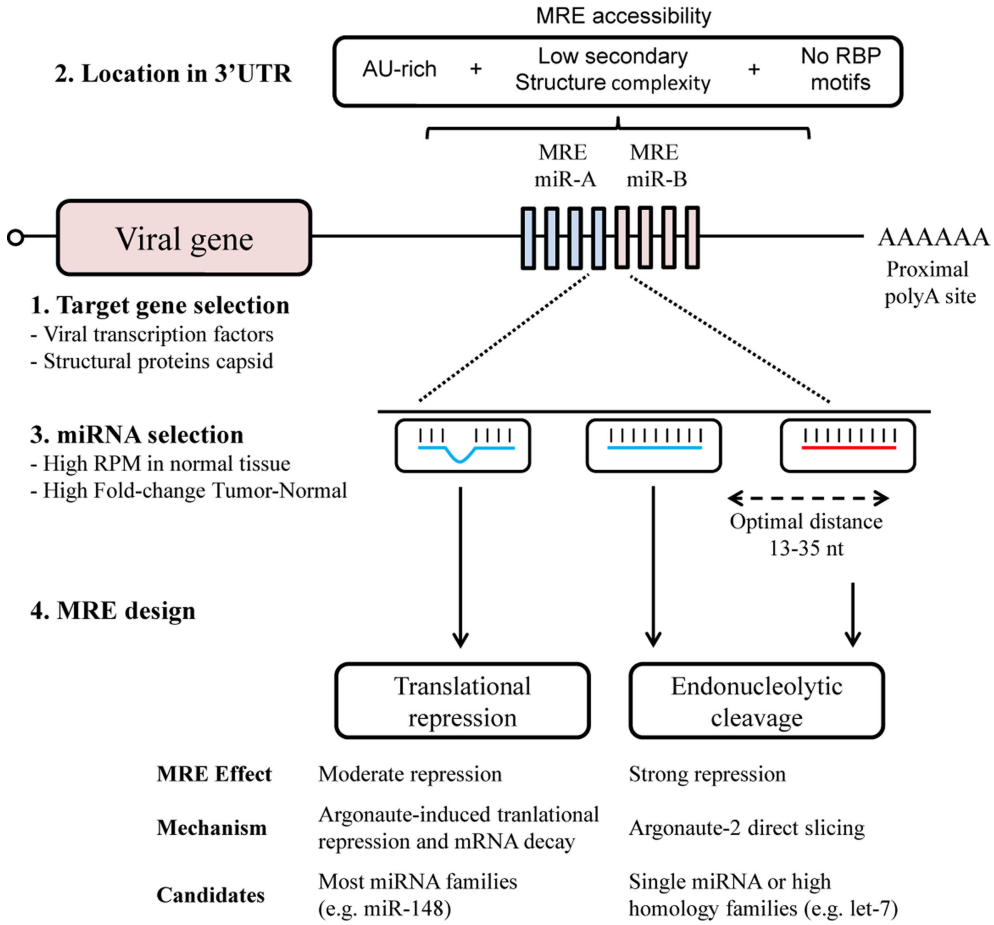

FIGURE 1 | MicroRNA response element (MRE) design for an optimal selectivity of oncolytic viruses. The design of MRE in oncolytic virotherapy has to take into account several elements regarding the biology of the virus and the microRNA (miRNA) regulatory pathways. Although all viral genes with 3′UTR are susceptible to be targeted though MRE (1), it is of notice the toxicity of some viral proteins. An adequate location in the 3′UTR (2) together with a sufficient miRNA expression (3) will contribute to the target recognition. Perfect miRNA–MRE complementarity will trigger Ago2 slicing activity (4); however, imperfect base-paring with other miRNA family members will also contribute to the regulation though translational repression.

An aspect of MRE design, aside from selectivity, that has yet to be tested for oncolytic viruses, is the incorporation of MREs as miRNA sponges or decoys. In other areas of gene therapy, sponge MREs have been used to reduce the effective amount of miRNA content (46–48). These particular MRE designs are characterized by a bulge that impairs the slicing activity of Ago2, while promoting miRNA degradation by way of trimming and tailoring (49). Sponge MREs could be incorporated into oncolytic viruses to downregulate the miRNAs involved in tumor progression (50), increase viral replication, or attenuate host antiviral response (51).

HOST miRNAs RESPONSE TO VIRAL INFECTION

When a virus infects a cell, a host–virus relationship is established, creating an intricate network of interactions characterized by the massive reprogramming of cellular gene expression. The expression profile of cellular mRNAs and miRNAs is affected during viral infection (52, 53). Changes in the host miRNA profile have been reported after infection with adenoviruses (54–56), influenza viruses (57, 58), HIV-1 (59), Epstein–Barr virus (EBV) (60, 61), human cytomegalovirus (HCMV) (62), human herpes virus 1 (HSV-1) (63), and respiratory syncytial virus (64).

By merely observing miRNA profile change, it is difficult to discern whether miRNA deregulation is the consequence of a host-immune response to the infection or if it is triggered by the virus to favor replication. Comparative studies of the expression profiles of different viral infections and the analysis of miRNA targets can help elucidate the significance of deregulation.

The miRNA profile after adenoviral infection has been studied for adenovirus type 3 in human laryngeal epithelial cells (56), adenovirus type 2 (Ad2) in human primary cells (54), and, more recently, for adenovirus type 5 (Ad5) in prostate cancer cells (55). Ad2 infection studies showed that a correlation between the progression of the infectious cycle and the level of miRNA deregulation could be established. Changes in the profile extend

from more upregulated, during the early stages of infection, to more downregulated miRNAs at the later phases (54). Massive miRNA downregulation could be the consequence of the expression of the VA (viral associated) RNAs codified by the virus at the later stages of the infection, competing with endogenous miRNA biogenesis (55) (**Figure 2A**). In fact, the same phenomenon was observed following Ad5 infection (55). Ad2 and Ad5 infection triggered miR-155 upregulation, an effect also observed after VSV (65) and EBV infections (61), suggesting that miR-155 could act as a host antiviral miRNA. It is also known that miR-155 is induced in macrophages, in response to interferon pathway activation (66).

Another miRNA that has been reported to be upregulated in cells infected by several viruses is miR-132. It has been found to overexpress after adenovirus (54, 55), HSV, KSHV, and HCMV infection (67). In contrast to miR-155, miR-132 acts by limiting host antiviral response since it exerts a negative effect on the expression of interferon-stimulated genes. This is a viral strategy which seeks to evade host antiviral response and promote viral replication (67, 68) (**Figure 2B**).

It is probable that viruses have evolved to induce the downregulation of interference miRNAs and favor the upregulation of miRNAs that can facilitate viral replication (54) (**Figure 2B**). Several examples illustrate this view. HSV-1 causes a series of changes in the miRNA profile and antagonizes host defenses by inducing miR-23a and miR-649 expression. These miRNAs, respectively, target *IRF1* and *MALT1* genes, involved in the antiviral signaling pathway (63, 69). In turn, the HSV-1 ICP4 protein induces the expression of miR-101, which limits virus replication to ensure the survival of host cells and therefore support persistent HSV-1 infection (70). In cells infected with Reolysin, a reovirus currently being tested for the treatment of several cancers (71), Nuovo et al. observed a modulation of certain miRNAs, with clear downregulation of let-7d, facilitating

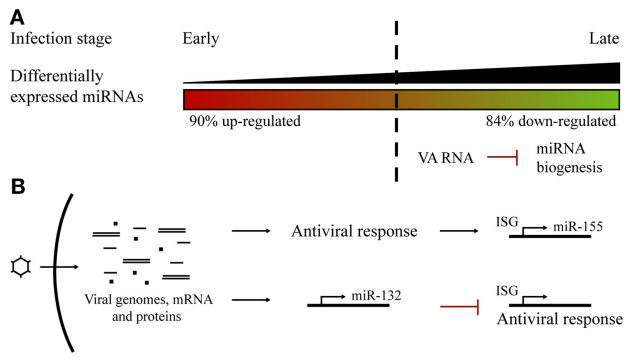

FIGURE 2 | Host microRNA (miRNA) profile alterations in adenoviral infections. **(A)** Changes in the miRNA profile during the course of an adenoviral infection. There was a switch from more upregulated miRNAs at early steps to more downregulated at late phases, probably as a consequence of VA RNAs competition with host miRNA biogenesis. **(B)** Adenoviral infections trigger the overexpression of specific miRNAs. Host miR-155 expression is induced as a consequence of the cell antiviral response. Viral infection promotes the expression of miR-132 to counteract the antiviral interferon response.

the productive viral infection and apoptosis-related death of the cancer cells (72). Hepatitis C virus (HCV) inhibits type I IFN production by upregulating the expression of miR-21 (73), while influenza virus activates the expression of miR-485, which targets the cytosolic sensor of viral RNA RIG-1 (57), and HIV-1 actively suppresses the expression of miR-17 and miR-20a that act against the virus (74).

Regardless of the miRNA changes triggered by viral infection, most cells are already equipped with miRNAs that will interact with viral genes. Antiviral miRNAs, such as miR-24 and miR-93, have been described to inhibit viral replication by directly targeting viral genes. Otsuka and coworkers described miRNA targeting VSV L and P protein genes, therefore inhibiting VSV replication (75). Such is also the case for cellular miR-32, which targets a sequence in the genome of primate foamy virus type 1 (76), or host miR-214, which is capable of inhibiting adenovirus replication by targeting the 3′UTR of E1A mRNA (77). By contrast, there are pro-viral miRNAs, such as miR-122, highly expressed in the liver, which interacts with the HCV genome to positively regulate the accumulation of RNA (78).

Thus, ever more experimental data regarding virus–host interactions are currently being generated. In an attempt to provide some clarity with respect to the complex analysis of the significance of the data, Li and coworkers generated an approach that defines potential regulatory networks of viral proteins, human miRNAs, and putative miRNA transcription factors between host targets (79).

DEREGULATION OF miRNAs IN CANCER WITH IMPLICATIONS FOR VIRAL ACTIVITY

As already mentioned, miRNA signatures can not only distinguish between normal and cancer cells but also between cancer subtypes, and even between the cell types conforming the tumor itself. Studies have shown that lower expression, or even loss of miRNAs, is commonly found in tumor cells (80, 81), where most of them are recognized as tumor suppressors. On the other hand, fewer miRNAs are overexpressed in cancer cells and are considered oncomiRs, since they tend to be involved in tumorigenic processes. Both oncomiRs and tumor suppressor miRNAs contribute to different stages of carcinogenesis (82). On this basis, attempts to modulate miRNA expression are an important area of therapeutic development (83). Since many miRNAs are involved in tumorigenesis, the action of expressing or interfering with a single miRNA may have limited anti-cancer effects. The combination of multiple miRNAs with complementary mechanisms may impact on several signal transduction pathways, leading to an improved outcome. In this respect, multiple long non-coding RNAs have been designed for an adenovirus, aiming to cause it to bind to oncomiRs, instead of otherwise binding to endogenous targets, and thus achieving the interference of multiple miRNAs (84).

Cancer cells are coupled with abnormal signaling pathways and this has consequences for viral replication. For example, adenoviruses use interferon signaling to inhibit lytic virus replication

in normal cells. However, they fail to inhibit it from replicating in cancer cells (85). The loss of interferon defenses in tumor cells is one of the mechanisms involved in the cancer selectivity of reovirus (86). Exploiting interferon deregulation in cancer is also a strategy employed to provide oncoselectivity for complex viruses (87, 88). Alterations to a variety of other pathways in cancer have constituted the principle option when seeking to confer cancer selectivity to viruses, with a view to cancer treatment (89, 90). Thus, although very little is yet known, one could speculate that the dysregulation of miRNAs in cancer may impact viral activity in tumor cells. Although the simplest rationale could claim that the more dysregulated miRNAs would be the first candidates when seeking to influence viral replication, recent observation illustrates that this might not always be the case and, in fact, functional interrogation would always be required. This is a point that was raised by the studies of Hodzic and coworkers, in which they showed that miR-26b, an abundant miRNA in prostate cancer cells, promoted adenovirus propagation and spread, leading to increased cell death (55). Further studies in this direction will provide a clearer view of the relevance that miRNA dysregulation in tumor cells may have with respect to modulating viral activity. Such a body of knowledge could constitute a novel platform in our quest to optimize oncolytic virotherapy.

CONCLUDING REMARKS

Investigation of miRNAs has strongly impacted the field of oncolytic virotherapy. Many studies have shown their potential in precisely detargeting viral protein expression. The expression of viral protein in normal tissue is an undesired effect. They are highly immunogenic proteins that the body tends to eliminate, and can cause inflammation and cell death. Thus, the incorporation of MREs to regulate viral proteins has been key to improving the safety profile and therapeutic index of oncolytic virotherapy. Fine-tuning the design of the MRE has improved the efficacy of both cleavage and detargeting effects.

On the other hand, our understanding of the importance of the role of miRNAs in viral infections is increasing. Virus–host cell interaction impacts cellular miRNAs and alters their miRNome. Viruses take advantage of host cell miRNAs to promote virus replication, but cells react to viral infections by upregulating antiviral miRNAs. Interestingly, biological responses to the viral infection of cancer cells with abnormal signaling pathways are not the same as they would be with normal cells, and miRNAs would also seem to play a role in this differential response.

Up until now, much progress has been made in the engineering of oncolytic viruses with MREs in the attempt to provide improved selectivity and safety for their use. Future research may concentrate on further understanding the relationship between host miRNAs and viral replication, and how this may differentially impact normal and cancer cells. Such knowledge could prove fundamental and serve as the basis for exploiting newly engineered oncolytic viruses with enhanced antitumor potency.

AUTHOR CONTRIBUTIONS

CF coordinated the study and wrote some parts. XB-DR and MR-R wrote part of the manuscript.

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REVIEW
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Oncolytic Viruses—Interaction of Virus and Tumor Cells in the Battle to Eliminate Cancer

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Oncolytic viruses (OVs) are an emerging treatment option for many cancer types and have recently been the focus of extensive research aiming to develop their therapeutic potential. The ultimate aim is to design a virus which can effectively replicate within the host, specifically target and lyse tumor cells and induce robust, long lasting tumor-specific immunity. There are a number of viruses which are either naturally tumor-selective or can be modified to specifically target and eliminate tumor cells. This means they are able to infect only tumor cells and healthy tissue remains unharmed. This specificity is imperative in order to reduce the side effects of oncolytic virotherapy. These viruses can also be modified by various methods including insertion and deletion of specific genes with the aim of improving their efficacy and safety profiles. In this review, we have provided an overview of the various virus species currently being investigated for their oncolytic potential and the positive and negative effects of a multitude of modifications used to increase their infectivity, anti-tumor immunity, and treatment safety, in particular focusing on the interaction of tumor cells and OVs.

Keywords: oncolytic virus, tumor cells, selectivity, cancer treatment, host factors

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INTRODUCTION

One of the most promising developments in cancer therapy to emerge over the past few decades is oncolytic virotherapy (OVT). Many of the more traditional treatment options routinely used to combat cancer in the clinic are not efficacious enough and have considerable side effects for patients. Although these treatments, such as chemotherapy and radiotherapy, are advancing and becoming more tolerable, we are yet to discover an alternative treatment option that has a high level of potency with minimal side effects that will dramatically change the overall survival of cancer patients.

Oncolytic viruses (OVs) have the potential to deliver this goal and much effort has been put into improvement of their efficacy and safety profiles in recent years. There are numerous viruses which either have naturally oncolytic properties or have been engineered to specifically lyse tumor cells. The great advantage of this therapy is that these viruses are able to specifically target tumor cells and therefore healthy tissue is not damaged during the course of the treatment. There are various ways to improve the specificity of OVs, for example, taking advantage of pathways which are upregulated in tumor cells and not healthy cells and engineering a virus which relies on such a pathway for successful infection thereby rendering the virus incapable of infecting healthy tissue (see **Figure 1**).

Another factor of oncolytic viral therapy that makes it a promising candidate is that while viral infection can directly lyse tumor cells, the resultant immune response will be generated not only

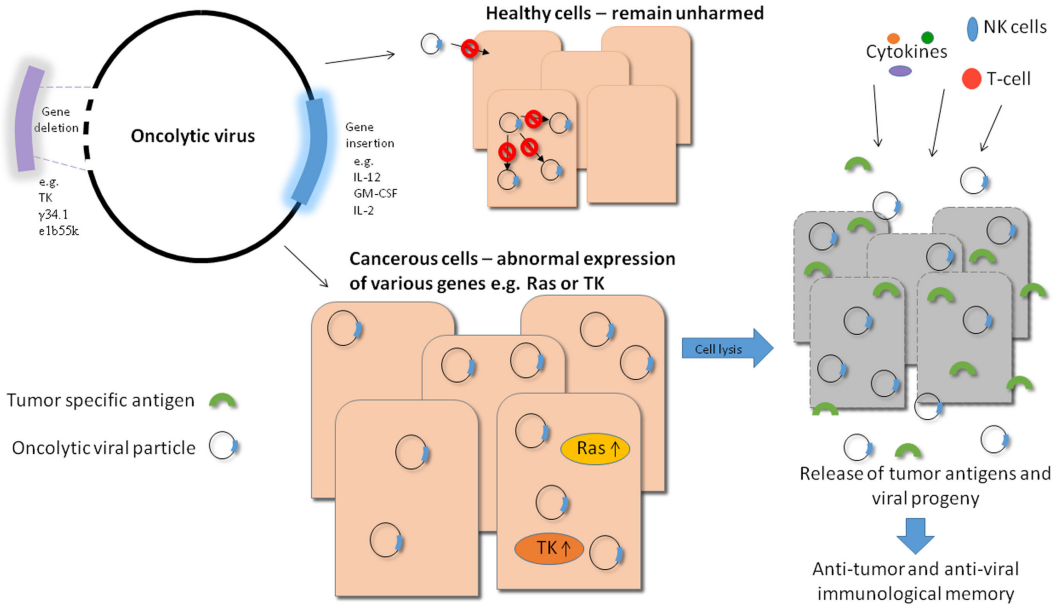

FIGURE 1 | Overview of oncolytic viral therapy. Genetic engineering has made it possible to modify oncolytic viruses (OVs) to make them safer and more effective against tumor cells. Various genes can be deleted to produce a safer virus, e.g., thymidine kinase (TK), while others can be inserted into the viral genome to increase efficacy, e.g., immune stimulators like IL-12. By carrying out these modifications, OVs can be made safer as they require target cells to provide the essential deleted gene, e.g., TK which is upregulated in tumor cells and not in healthy cells. They can also generate long lasting immunity as a result of stimulation of a potent immune response in which tumor cell antigens (along with viral antigens) can be targeted by T-cells. Also, when the virally infected tumor cells die, they release progeny virions into the tumor microenvironment which can in turn infect neighboring cells, improving the efficiency of viral treatment. Systemically delivered OVs also have the potential to eliminate metastatic tumor cells.

to viral antigens but also to tumor cell antigens. This unique feature of oncolytic viral therapy makes it a very exciting avenue of research as not only can the viruses act to eradicate existing tumors but they can also potentially generate lasting immunity in the form of memory T-cells which are primed against tumor cell antigens (see **Figure 1**).

In addition to causing direct lysis of cells, OVs can also be used as delivery vectors for therapeutic genes. In this case, the virus can be genetically modified to include the gene of interest which upon infection and viral replication will be produced at high levels in infected tumor cells where it can exert its function. There are a multitude of anti-cancer genes that can be incorporated into OVs in this way in order to maximize the efficacy of the virus and improve the anti-tumor response generated (see **Figure 1**).

Also, OVs have great potential as combination therapies used together with more traditional approaches. In this way, various treatment options can be used synergistically to combat cancer from more than one angle at a time which will likely give rise to a more positive response to treatment. This combination therapy approach can lead to improved tolerance of treatment in patients as the synergistic effect allows lower doses of each individual therapy to be used to gain similar effects compared with the use of one treatment alone.

As stated, much research effort has been put into improving this area of cancer therapy and the various viruses used and important advances made in their development are discussed here.

HISTORY OF OV THERAPY

The use of OVs was first conceived following the observation of the fact that during or after an infection, tumor regression is occasionally observed (1–3). Based on this observation, patients with Hodgkin's lymphoma were treated with serum containing hepatitis virus (4).

In the following years, a lot of effort was put in to achieving better and safer results. For 30 years, from 1950 to 1980, many studies were performed without reaching good clinical outcomes or providing long-term results (5–7). This was mainly due to the fact that viral treatments were unsafe because there were no methods to control virulence nor to obtain tumor specificity. Finally, in the late 1980s, with the advent of genetic engineering, a renewed interest for OVT rose again and in recent years many advances have been made in this field.

SELECTIVITY OF OVs

There are various ways in which different OVs are able to infect cells. Some viruses, like vaccinia virus (VV) or Newcastle disease virus (NDV) lack specific receptors for attachment so enter cells *via* endocytosis. Other viruses have a specific receptor that they use to enter host cells; for example, adenoviruses (Ads) are able to bind coxsackie and adenovirus receptor (CAR), integrins, or cluster of differentiation 46 (CD46). Measles can also use CD46 for entry, whereas herpes simplex virus (HSV) uses nectin or herpesvirus entry mediator (8, 9). Despite the observed tendency

of tumor cells to upregulate some of these receptors, they are also expressed on many normal cells.

There are a variety of ways in which OVs can be targeted to tumor cells in order to minimize damage to healthy cells. These include exploitation of various pathways which are aberrantly expressed in tumor cells to ensure engineered viruses are only capable of productive infection in cells which have abnormal levels of certain genes. Also, control of viral replication with microRNA differentially expressed in tumor cells compared with healthy cells can restrict viral replication specifically to tumor cells. Viral coat proteins can also be manipulated to ensure viral infection only occurs in cells with certain receptors, e.g., receptors found on tumor cells only. These strategies will be discussed in more detail here.

As it is essential that OVs only successfully infect tumor cells to avoid the spread of virus in healthy tissue, many different approaches have been investigated to increase specificity. One of these is to take advantage of the aberrant expression of various proteins in pathways which can have an effect on viral replication. Of these pathways, OVs commonly exploit aberrant expression of proteins involved in the Ras pathway. This pathway is generally silent in normal cells but activated in tumor cells and the downstream effects of this can be beneficial for OV infection (10, 11). There are a number of ways in which upregulation of the Ras pathway in tumor cells can influence the outcome of oncolytic viral infection. For example, it has been shown that the Ras/MEK pathway can downregulate specific interferon-inducible genes which may have an effect on anti-viral responses and apoptosis control (12). It has also been seen that apoptosis can be involved in the increased efficiency of OVs in tumor cells. In the case of Reovirus, this OV can cause an accumulation of Ras within the Golgi body which leads to triggering of apoptosis signaling pathways and subsequent release and spread of progeny virions (13).

Because of aberrant expression, genes involved in the Ras pathway (among others) can favor replication of viruses in tumor cells and many viruses have been engineered to exploit this to increase their selectivity for transformed cells. For example, engineering viruses which are only able to express certain critical viral proteins upon upregulation of transcription factors downstream of the Ras pathway renders the virus only able to replicate in cells with an upregulated Ras pathway (14).

Other strategies used to produce tumor-targeted replicating OVs include control of certain genes using microRNA. Hikicki et al. have shown that it is possible to place critical viral genes under the control of an miRNA which has low expression levels in tumor cells. This renders the virus unable to successfully infect healthy cells where normal levels of this miRNA are expressed, facilitating interference with production of the critical viral gene (15).

Also, modification of viral coat proteins can be used to specifically direct viral infection to tumor cells. There are various ways to achieve this, for example, covering the viral surface with polymer to "cloak" the existing receptor and addition of epidermal growth factor (EGF) to target the virus to tumor cells which tend to have upregulated EGF receptor (EGFR) expression (16). This strategy not only reduces the broad tropism conferred by the existing viral receptor but also replaces this with tumor specific receptors to direct oncolytic viral infection to target cells, leaving healthy tissue unharmed.

Another approach involves the use of antibodies to target OVs to tumor cells. As an example of this strategy, it was found by Watkins et al. that antibodies can be engineered which contain an Ad fiber protein targeting single-chain variable fragment (scFv), linked to EGF. This facilitated targeting of Ad to EGFR-upregulated tumor cells (17). This antibody focused approach was further developed to allow incorporation of scFv into the viral envelope. For example, HSV type-1 (HSV-1) relies on various glycoproteins for entry into cells and one of these glycoproteins (gD) is responsible for interaction with the viral entry receptors. If an scFv targeting EGFR is fused to this glycoprotein, the virus is then able to use EGFR as an entry receptor which improves tumor targeting (18).

In parallel to the attempt to create safer viruses, a new strategy is developing with the aim of incorporating transgenes within the virus to target the tumor microenvironment or to activate the immune system.

MODIFICATIONS OF OVs

This new strategy is achieved by modification of viral genomes by insertion or deletion of selected genes which aid or hinder oncolytic potential and some of the strategies being explored will be discussed in more detail here.

The ability to modify the genome to our advantage is one of the most promising aspects of OVs. These modifications have a variety of functions including improvement of tumor tropism and increased recruitment of the immune system to aid anti-tumor responses. Also, improved safety and efficacy of OVs are of utmost importance and are another aspect that can be controlled by genetic engineering. Some examples of gene editing include deleting replication-related genes to reduce replication efficiency (as attenuation improves safety) and/or addition of genes that induce pathways to promote tumor cell death, for example, the apoptosis pathway (19).

As previously mentioned, pathways which are alternatively regulated in tumor cells compared with healthy cells can be exploited to produce selective viruses. For example, genes can be deleted resulting in a virus that can only successfully infect certain tumor types which over-express MEK (20). This strategy can also be used with HSV whereby genes can be deleted to produce a virus which preferentially replicates in tumor cells which unlike healthy cells tend to have a constitutively activated Ras pathway (21). As this virus initiates apoptosis in infected and bystander cells and preferentially infects tumor cells, it can be used as oncolytic agent with this deletion (21, 22).

GENE DELETION STRATEGIES

Many OVs have been modified with specific gene deletions to target the virus to tumor cells and inhibit infectivity in healthy cells. An example of this strategy is thymidine kinase (TK) deletion from VV. As the wild-type virus usually encodes this kinase it is able to replicate in healthy cells, however, when the gene is deleted the virus can no longer replicate efficiently in healthy cells. As

tumor cells produce higher levels of TK, even though the gene is deleted the virus is still able to replicate in these cells (23, 24).

Another strategy used to improve tumor specificity is to delete apoptosis-inhibiting genes (usually used with Ad). In wild-type infections, Ad encodes genes which block apoptosis which is an advantage as infected cells then become viral factories given that they will not enter apoptosis in response to infection. As tumor cells often block apoptotic pathways as a survival mechanism, Ads with deleted apoptosis-inhibiting genes can undergo prolonged infection while infection of healthy cells will lead to lysis of the cell and clearance of virus (25).

Gene deletion strategies can also be used to improve the efficiency of virus delivery systems which are designed to deliver OVs to target cells without interference from the host immune system. In these systems, viruses are delivered within host cells (e.g., mesenchymal stem cells) that provide shelter from immune attack and subvert the problem of clearance of virus (by neutralizing antibodies) before they reach their target cells. This method has been improved in Ad by modification of the virus in order to make it more infective in MSC and more efficient at killing tumor cells. Oncolytic Ad can also be engineered through deletion of an anti-apoptotic gene, improving virus release from MSC, and allowing more potent anti-tumoral activity (26).

Although gene deletion often improves efficacy of OVs, the chosen candidates need to be selected very carefully. Various gene deletions have the potential to alter viral infectivity in ways which can either improve or diminish oncolytic potential. For example, deletion of E3-6.7K/gp19K leads to more rapid viral clearance which on one hand improves safety but on the other hand only allows a short time-frame for inserted therapeutic genes to be delivered to target cells and produced in a significant amount. Therefore, this particular deletion can only successfully be used for delivery of genes which can act quickly to have the desired effect (27). Another study has shown that deletion of a combination of viral genes will enhance tumor selectivity but reduce viral potency, highlighting the problems faced in engineering the perfect oncolytic viral therapy (28). There are also genes encoded by OVs that can act to inhibit oncolytic potential, for example, E4orf1 encoded by Ad leads to increased levels of survival in infected cells thereby reducing the ability of the virus to directly lyse infected tumor cells (29). This effect can lead to diminished efficacy and therefore needs to be addressed in order to maximize the potential of this virus to treat cancer.

GENE INSERTION STRATEGIES

Another advantage of gene deletion is the opportunity to insert therapeutic genes in their place without disrupting the reading frame (30). There are a multitude of therapeutic genes and immune stimulators which can be delivered within OVs to combat cancer (for example, the interleukin family of genes used to stimulate the immune system thereby improving anti-tumor immune responses). However, this approach is not perfect and the combination of deleting a gene and inserting a new one can result in problems of its own. For example, deletion of the E1B55K gene leads to improved virus spread (as it facilitates apoptosis), however, this may result in low levels of production of the inserted gene as the cell undergoes apoptosis before high quantities of the gene are expressed. The combinations of deletion and insertion need to be specifically studied in order to ascertain which ones complement each other and which have negative effects on each other (31, 32). Addition of cytokines or other genes may also give rise to toxicity, for example, the IL-12 cytokine has been seen to result in side effects that cause a poor safety profile. This problem has been combated in a range of ways with varying results; these include using a single-chain version of the cytokine and anchoring IL-12 to the membrane of cells through fusion with the CD4 transmembrane region. These methods did not produce the desired reduction in toxicity without reducing anti-tumor efficacy, however, using a helper-dependent Ad vector with an inducible expression system was successful in allowing production of IL-12 without high levels of toxicity (33). Another strategy to reduce potency of the IL-12 cytokine is to deliver it within conditionally replicative Ad rather than replication competent strains. For example, on delivery within an adenoviral vector which can only replicate in hypoxic conditions typical of tumor masses, IL-12 was still effective but resulted in less toxicity and more specific delivery to target cells compared with replication competent viral delivery (34).

OTHER STRATEGIES TO IMPROVE OVs

As well as addition and deletion, control of gene promoters can be used to modify viral behavior. For example, promoters that are activated more highly in tumor cells can be used to control an essential viral gene rendering that virus incapable of replicating in healthy cells (32). They can, however, replicate successfully in tumor cells as these cells have a higher activation of that promoter (e.g., use of Cyclin E promoter to target Ad infection to tumor cells) (35). It is also possible to use promoter control of virus genes in order to attenuate virus and make the therapy safer. If genes essential to virus replication are expressed under the control of a promoter downregulated in tumor cells, then delivery of such a tumor-selective virus into those cells will only allow a low level of infection which results in improved safety with retention of oncolytic activity (although at a lower level than wild-type infection) (15).

A constantly evolving area of research is the combination of OVs with other treatments for synergistic effect. For example, combining oncolytic Ad with a cytotoxic drug currently used in the clinic enhanced the anti-tumor efficacy of this treatment (36). These combinations can improve the activity of OVs in various ways, including promoting better replication or compensating for certain deletions without compromising tumor selectivity. For example, if a deleted gene has more than one function, it can be eliminated to improve selectivity in combination with delivery of a compound that can be administered to improve replication efficiency which may have been lost through deletion; an example is the use of 2-aminopurine to enhance the oncolytic activity of an E1B-deleted Ad (37). Combining treatments can also facilitate administration of each agent at lower and safer doses given their synergistic effect (38). Also, the combination of oncolytic Ad with CAR upregulation can improve the efficacy of this treatment

due to the use of these receptor molecules for Ad infection (39). Various tumor cell types express different levels of CAR and this will have an impact on the ability of the virus to effectively treat different tumors. Therefore, administration of an agent which can upregulate CAR expression before oncolytic Ad therapy could increase the efficacy of adenoviral treatment.

There are numerous viruses which have been found to have natural or engineered oncolytic activity and some of the most promising candidates will be explored in more detail in the following sections.

VACCINIA VIRUS

Vaccinia virus is a naturally oncolytic virus which was found to have a natural tropism for tumor cells due to its sensitivity to type I interferon (40). It is a double-stranded DNA virus of the *Poxviridae* family. There are many different strains of the virus and of these; Lister, Wyeth, and Western Reserve strains are the most used in research. VV is a very promising anti-cancer agent (41) for many reasons including its very short life cycle (around 8 h) and its ability to replicate in hypoxic conditions (42). Moreover, it does not have a specific receptor and viral fusion with the plasma membrane facilitates entry (43) which makes it a potential candidate for treatment of all tumor types. Furthermore, VV does not depend on the host cell for mRNA transcription and its entire life cycle takes place in the cytoplasm, eliminating the risk of genomic integration (44). The virus, which can infect both human and mouse cells, is infectious at four different stages of its life cycle: intracellular mature virion released by cell disruption, intracellular enveloped virion, cell-associated enveloped virion, or extracellular enveloped virion released by endocytosis from the membrane.

To make oncolytic VV safer, two deletions have been made; one in the TK region (45) (as for HSV) and one in the vaccinia growth factor gene region increasing its specificity for tumor cells (23). As well as this, many efforts have been made to generate a strain that results in viral attenuation, rendering the virus harmless in normal tissue. For this purpose, mutations in the F14.5L and A56R genes have been engineered. The F14.5L gene encodes a secretory signal peptide, while the A56R gene generates hemagglutinin.

In addition to these safety measures, many different genes have been integrated into the VV genome in order to increase its anti-tumor efficacy such as cytokines and antibodies, as reviewed by Badrinath et al. (9). As an example, JX-594 is a VV with TK gene deletion and GM-CSF (a cytokine able to stimulate the immune system to kill tumor cells) gene insertion and is currently undergoing phase III trials. Phase I trials have shown promising results and acceptable safety profiles (46) and phase II studies were designed to investigate the optimal dose of intravenously delivered JX-594 (47). The results of a randomized, dose-finding phase II clinical trial reported by Heo et al. showed that high dose JX-594 resulted in higher overall survival duration than low dose administration in patients with advanced hepatocellular carcinoma. This study also demonstrated both anti-viral and anti-tumor immunity generated in clinical patients in response to OV administration (48).

There are many other modifications which can improve the anti-tumor efficacy of VV. For example, the addition of IL-10 was found to improve the oncolytic activity of VV through dampening of anti-viral immunity (prolonging viral infection) without reducing anti-tumor immunity (49).

The major concern in using VV as oncolytic therapy is the fact that it is easily recognized by the immune system. Indeed, the strains of VV exploited in clinical OVT are derived from the vaccine formulations used for smallpox eradication and so there is an activation of the immune system in those patients that were administered with the vaccination. Despite this, Breitbach et al. demonstrate in a clinical study that the intravenous administration of the modified VV JX-594 was safe and that the virus was able to replicate and to express the transgene only in tumor cells (50).

ADENOVIRUS

Adenovirus is one of the most commonly studied viruses in oncolytic therapy and was the first to be given regulatory approval, granted by the State Food and Drug Administration in China in 2005 (51). It is a non-enveloped, double-stranded DNA virus of the *Adenoviridae* family. There are several strains of Ad and of these, Ad5 is the most commonly used in oncolytic therapy. As this virus is widely studied, there are a multitude of various modifications which have been shown to improve its efficacy and safety which will be further discussed below.

It has been seen that addition of various genes to oncolytic Ads can improve their anti-tumor efficacy. For example, the combination of p53 addition to suppress tumor growth with GM-CSF addition to induce the apoptotic pathway elicits a synergistic effect which is effective in combating hepatocellular cancer stem cells (52). This is especially exciting as it potentially provides a mechanism to combat cancer stem cells which are considered to be integral to cancer recurrence after current treatment options. GM-CSF addition has been tested in phase I clinical trials and was shown to be non-toxic and tolerable at the doses used, however, the efficacy showed room for improvement in terms of anti-tumor efficacy and long-term immunity (53).

It is also possible to improve oncolytic Ad by incorporation of a short-hairpin RNA which functions to downregulate Dicer (an endoribonuclease which has a role in processing virus-associated RNA). Downregulation of this protein inhibits the destruction of viral RNA and allows Ad to replicate efficiently and therefore improves the efficacy of this OV (54).

Gene silencing techniques can also be used in order to downregulate certain oncogenes in order to suppress tumor growth. For example, downregulation of EphA3 by insertion of siRNA targeting this gene into the genome of an Ad whose replication is made conditional under the control of TERTp (which increases specificity for tumor cells) results in increased levels of autophagy through inhibition of the AKT/mTOR pathway. This allows the virus to both inhibit tumor cell proliferation and kill infected cells (55).

In terms of important features of OVs on which to focus research efforts, it has been shown that the T-cell immune response to oncolytic adenoviral infection is more efficacious in

combating tumors than direct lysis of tumor cells by viral infection (56).

It has been previously shown that treatment of tumors with immune checkpoint inhibitors (e.g., PD-L1 blockade) allows immune-boosting viral treatments to have a longer lasting effect. It has recently been found that encoding a PD-L1 blocking antibody within Ad vectors leads to production of this antibody in the local vicinity of tumors by host cells and this approach gives rise to better anti-tumor effects than with infusion of the antibody into tumors. When combined with chimeric antigen receptor-modified T-cell treatment targeting HER2 positive tumor cells, this approach to PD-L1 antibody delivery increased treatment efficacy (57).

In addition to investigating methods of improving current Ad strains used in oncolytic therapy, it is also important to find potentially new strains which may prove to be more appropriate for therapy. For example, Ad11 was found to have higher levels of receptor availability and lower levels of neutralizing antibody than Ad5 (which is the most commonly utilized strain in OV engineering). When this strain is modified to replace its E1A enhancer-promoter region with that of Ad5 (leading to higher levels of E1A mRNA) it can become a more potent OV. With additional modifications to increase tumor specificity, the Ad11/Ad5 strain could prove to be a more successful OV than the more commonly studied Ad5 strain (58). Also, another group B oncolytic Ad is enadenotucirev, this virus is able to infect both at apical and basolateral surfaces of polarized cells and results in progeny being released *via* the apical surface which directs them into the tumor mass rather than out into the blood stream (59). This proves to be an improvement on the traditional Ad5 strains used in the design of oncolytic therapy as type 5 Ads seem to infect preferentially *via* the apical surface which could pose a problem when delivered systemically.

Even once a promising candidate has been identified for oncolytic therapy, one of the major obstacles to effective use of OVs is clearance by the host immune response (namely anti-viral T-cells) but it would be extremely useful to use this response to our advantage. In a recent study, it has been found that it is possible to engage and therefore redirect these T-cells to react to specific antigens (in this case EGFR which is often overexpressed on tumor cells) in order to redirect the anti-viral response into an anti-tumor response (60). Also, the generation of T-cell responses both to virus and tumor was found to be more important in viral efficacy than direct oncolysis (56). Therefore, a balance between increasing T-cell production to improve anti-tumor immunity and controlling T-cell response to reduce viral clearance is necessary.

In the constant effort to find new and improved oncolytic therapies, it was found that certain cancer cell types produce a peptide which inhibits the ability of the Ad to escape endosomes and be released (61). This inhibition presents a barrier to effective viral spread and successful oncolytic therapy. However, type 3 Ad has evolved to manufacture a decoy capsid which sequesters HD5 and renders the virus able to escape endosomes (62). This finding suggests that the mechanism used by this strain of Ad can be mimicked in order to improve efficacy of existing or potential oncolytic Ad therapies.

Another obstacle facing oncolytic therapy is the tumor microenvironment and its immuno-suppressive properties. For example, expression of TGF-β in the tumor microenvironment results in diminished ability of virally delivered IL-12 to boost anti-tumor immune responses. One method to overcome this problem is to co-express decorin which leads to attenuated TGF-β expression in tumors (63). This is just one example of therapeutic gene addition which improves the action of oncolytic Ads armed with cytokines aimed at boosting anti-tumor immune responses.

Combination therapy, whereby OVs are used in combination with conventional therapy is a growing area of research with many promising leads. For example, it has been shown recently that Ad encoding pro-inflammatory IL-18 cytokine has a synergistic effect when delivered in combination with dacarbazine, which is conventionally used to treat melanoma by alkylation of DNA strands. When used in combination, it was seen that these treatments together result in inhibition of tumor cell growth and increase in apoptosis (64). A similar strategy was employed in another study whereby IL-12 was used as an immune stimulator and a VEGF-silencing ribonucleic acid was co-expressed in order to overcome the immune suppressive action of VEGF produced by tumor cells (65). Many studies have been undertaken to assess which combinations work best and promising results have been reported, for example, oncolytic Ad combined with Temozolomide treatment (alkylating agent) leads to increased levels of viral replication and tumor cell death *via* various mechanisms thought to include upregulation of autophagy and apoptosis pathways (66). Another combinatorial approach to cancer treatment is to combine OV with anti-tumor antibody treatment. It was found that the gene for an anti-HER2 antibody used in the clinic (Trastuzumab) to combat HER2 positive breast cancers could be inserted into an oncolytic vector and successfully translated into antibody within tumor cells. This was then released upon lysis of infected cells (aiding treatment specificity) and provided an answer to some of the difficulties in delivery of antibodies systemically, specifically the infiltration of tumor masses as virus can infect, and spread throughout the tumor delivering the antibody with it (67).

Another approach to combination therapy is to use two antigenically distinct OVs sequentially (whereby the second to be delivered is not cleared by immunological memory to the first dose). This strategy was reported to be successful when oncolytic VV was used after first administering adenoviral therapy. In this case, it was seen that the increased efficacy was dependent on T-cell activity (68). Subsequent to this finding, various other regimes have also been found to improve the efficacy of OVs. For example, sequential delivery of oncolytic Ad and NDV which are both engineered to express an immuno-stimulatory cytokine leads to significant anti-tumor responses even though when administered alone, each virus showed limited efficacy against tumors (69).

Another exciting avenue being explored to improve the oncolytic potential of Ads is to combine oncolytic therapy with induction of the autophagy pathway. It was found that this pathway is involved in viral antigen presentation and therefore its upregulation could increase presentation of virally delivered tumor-associated antigens (TAAs) at the cell surface in order to

induce a more potent anti-tumor immune response than with antigen delivery alone (70).

Existing neutralizing antibodies pose yet another problem in the design of OVs. Certain viral strains may have been previously encountered/vaccinated against and therefore neutralizing antibodies can be rapidly produced by the host upon treatment (especially when virus is delivered intravenously) and clear the virus before it can have an effect on tumor cells. To combat this problem, it was found that coating virus in albumin provided a protective barrier from neutralizing antibodies in the host and therefore facilitated systemic delivery without the threat of viral clearance due to previously generated immunological memory (71).

One of the main barriers to viral spread in tumor masses is the interstitial matrix (including extracellular DNA). OVs have therefore been modified to encode proteins which will degrade the interstitial matrix along with expression of DNaseI to degrade the extracellular DNA, therefore allowing more efficient spread of OV throughout tumor masses (72).

Oncolytic viruses can also be exploited as carriers for anti-cancer drugs using more than one method. In a recent study, it was found that electrostatic attraction between viral capsid and the drug molecules themselves was an efficient way to deliver anti-cancer drugs which would then act synergistically with the oncolytic adenoviral therapy (73).

HERPES SIMPLEX VIRUS

Herpes simplex virus type-1 is a double-stranded DNA virus belonging to the *Herpesviridae* family. HSV-1 was the first virus in which TK gene mutation was engineered. In 1991, Martuza et al. demonstrated that human glioblastoma cells can be destroyed by HSV-1 carrying a mutation in the TK region and this was observed in cell culture as well as in nude mice (74). A lot of effort has since been put into making HSV more active against tumor cells and safer for normal cells culminating in the approval of Talimogene laherparepvec (T-Vec), an engineered HSV-1 for the treatment of melanoma in 2015 (75). Phase III trials in patients with non-resectable melanoma showed that T-Vec had higher efficacy in patients with stage IIIB–IV melanoma than GM-CSF treatment alone (76). It was also found that T-Vec in combination with a CTLA-4 inhibitor (Ipilimumab) showed encouraging preliminary results in a phase Ib trial (77), though there is much more work to be done to fully evaluate the effects and outcomes of this treatment combination.

Talimogene laherparepvec has two viral gene deletions (one in the γ34.5 gene and one in the α47 gene) and it has the human GM-CSF gene inserted in place of the deleted γ34.5 gene. The function of γ34.5 is to prevent infected cells from switching off protein synthesis upon viral infection. Considering that tumor cells have a defect in this mechanism, a γ34.5-deleted virus is still able to replicate in these cells. This modified virus is therefore safer as it is only able to replicate in tumor cells. The α47 gene is associated with the downregulation of antigen presentation. A deletion in this region has a double function: first, it is able to increase the anti-tumoral immune response, and second, it is associated with the expression of another gene (US11) which results in boosted viral replication in tumor cells. Finally, the insertion of GM-CSF results in increased anti-tumoral immunity as demonstrated in a phase II study. The study reported an increase in tumor specific $CD8^+$ lymphocytes along with a decrease in $CD4^+FoxP3^+$ regulatory T-cells and $CD8^+FoxP3^+$ T-suppressor cells (78, 79).

Another oncolytic HSV is G47Δ, a third generation HSV-1 with three different mutations. It was created by Todo et al. by the deletion of the ICP6 gene from the genome of G207 virus, which already has two mutations (γ34.5 and α47); the *Escherichia coli* LacZ gene was added in place of ICP6 (80). ICP6 encodes the large subunit of ribonucleotide reductase (RR), an enzyme essential for viral DNA synthesis. If this enzyme is missing, the virus fails to replicate. However, tumor cells synthesize a huge amount of RR which can compensate for its deletion from the viral genome. In this way, mutated virus is able to replicate only in tumor cells, becoming safer in normal tissue.

Another example of HSV used as OV is the NV1020 virus which is based on the R7020 construct developed by Meignier et al. (81). NV1020 virus has deletions in the ICP0 and ICP4 gene regions and has only one copy of the γ34.5 gene. Moreover, the α4 promoter which controls TK expression has been inserted, making the virus sensitive to common drugs (such as acyclovir) and improving its safety. This virus has been reported to stabilize metastasis in phase I/II clinical trials involving patients with advanced metastatic colorectal cancer and showed minimal levels of side effects (82).

It has also been found that modification of HSV to include an scFv fragment against HER2 increased viral tropism to HER2 positive tumor cells (83). HSV encodes various glycoproteins to facilitate viral entry and these are: gD, gH/gL, and gB. Usually, receptor recognition leads to modifications in gD and gH/gL which in turn activates gB. Interestingly, this redirected tropism to HER2 positive cells was conferred even when the scFv fragment was engineered into gB (a glycoprotein with a role in virus-cell fusion rather than receptor recognition), thereby bypassing the requirement for receptor mediated activation of gD and gH/gL. This provides a method of improving selectivity and therefore efficacy and safety of oncolytic HSV.

These data indicate a promising route for the clinical use of HSV-1. Many clinical trials have been carried out to treat different types of solid cancer with encouraging results (82, 84, 85). However, some limitations still have to be overcome. A major obstacle for HSV-1 is the way it infects host cells. This virus is able to spread from one cell to another without causing viremia which makes it suitable for intra-tumoral but not intra-venous injection. This could cause some problems in the treatment of tumor lesions which are very difficult to reach directly, like those of pancreatic cancer.

Alongside HSV-1, HSV-2 has also shown promise as an OV. For example, HSV-2 with a deletion of ICP10 to improve selectivity was engineered and found to be even more effective than HSV-1 for treatment of metastatic ovarian cancer in mouse models (86). Building on this initial success, many more studies have been conducted to improve HSV-2 as an OV. These include co-administration of cyclophosphamide, a drug which has chemotherapeutic effects as well as causing a dampening of the innate immune response. This combination works

synergistically as reduced innate immunity can facilitate more potent viral infection. Li et al. showed that this combination of therapies leads to enhanced anti-tumor effects when used to treat lung carcinoma in mice and could prove to be a good combination in clinical trials (87). It has also more recently been found that HSV-2 can act in synergy with adoptive T-cell treatment. Administration of oncolytic HSV-2 directly into tumor sites was found to improve the homing of adoptively transferred T-cells (engineered to target tumor cells) to the tumor mass. This was achieved, at least in part, by elevated levels of various chemokines such as CXCL9 and CXCL10 (88, 89). It was also found that in addition to increasing attraction of these T-cells to the tumor, the various chemokines were also able to maintain persistence of these T-cells at the tumor site.

NEWCASTLE DISEASE VIRUS

A more recently developed, naturally oncolytic virus is NDV. It has been found that NDV can effectively kill a variety of tumor cell types and that this activity occurs by induction of immunogenic cell death which in turn leads to adaptive anti-tumor immunity (90).

The initial suggestion of a mechanism for NDV tumor selectivity was that there is a lack of robust/normal anti-viral response in tumor cells. However, it seems that some tumor cells with intact anti-viral pathways are still killed by NDV. It was then found that the lack of apoptosis in tumor cells is what makes NDV tumor-selective (91). Interestingly, it was found that certain strains of NDV can induce apoptosis in tumor cells (92) and that the apoptosis pathway stimulated in infected tumor cells is p53-independent and perhaps triggered by endoplasmic stress (93). It was also found that apoptosis of infected cells occurs predominantly *via* the intrinsic mitochondrial pathway and is caspase dependent (94). This induction of apoptosis results in tumor cell death.

Although NDV is naturally tumor-selective, there are inherent problems with the virus which need to be overcome in order to improve its ability to infect and spread within solid tumors as viral spread is limited by factors such as the extra-cellular matrix (95). Again, there are many studies looking at ways to modify this virus to make it safer and more efficacious; for example, insertion of the IL-2 gene into the NP/P site has proved effective (96). Inclusion of IL-2 and/or TRAIL has been shown to increase apoptosis levels in infected cells resulting in the tumor-selective parental virus becoming an even more potent anti-cancer agent (97–101). Further improvement of oncolytic NDV is found with IL-2 addition in combination with expression of TAA. This combination, delivered by oncolytic NDV, improves tumor-specific T-cell responses leading to higher efficacy than delivery of TAA alone (102). Another strategy to boost the efficacy of oncolytic NDV is to insert the ICOS gene (usually upregulated by viral infection) in order to induce higher levels of T-cell infiltration into the local tumor and distant tumor sites (103).

As well as arming viruses with immune stimulators, other therapeutic genes are also able to increase anti-tumor effects of viral therapies. For example, NDV engineered to encode TNF receptor Fas shows greater oncolytic effect as Fas is responsible for increased apoptosis of infected cells *via* both the intrinsic and extrinsic apoptosis pathways, thereby increasing cell death and in turn anti-tumor efficacy (104). NDV's naturally oncolytic properties could also be augmented by arming the virus with GM-CSF (105).

In addition to arming the OV with various therapeutic genes and immune stimulators, it is also beneficial to work toward increased virulence (within safety limits). Using a more infectious strain of NDV can produce better cytotoxic effects in tumor cells than arming less virulent strains with immune-modulating genes (106). However, a potential problem encountered with NDV is its ability to infect avian species. This introduces an added difficulty in that any oncolytic therapy involving NDV needs to ensure attenuation of the virus in avian hosts without reduced potency in mammalian cells (107).

It is also important to consider that modification of OVs does not always produce the desired result, for example, viral attenuation to improve safety needs to be carefully tested as production of an attenuated virus which may establish persistent infection could lead to tumors which are not effectively killed by OV and thereby become resistant to treatment. It has previously been reported that NDV has the potential to cause persistent infection in certain cell types (108). This highlights the fact that it is important to carefully engineer OVs in order to maximize efficiency and broaden tropism for a range of cancer types.

Frequently, viral therapies are able to target tumor cells but are not efficacious enough when administered alone or they are potent but toxic at high doses. These problems could potentially be overcome by combining various viral therapies which act synergistically to combat tumor growth. For example, two antigenically distinct viruses which both encode immune stimulators can be sequentially administered and allow two cycles of transgene expression without interference from neutralizing antibodies. This approach allows lower doses to be used (making the treatments safer) and allows a multi-faceted approach which is likely to be more effective than single treatments (69). Oncolytic NDV can also be used in combination with traditional therapies to gain synergistic effects which enhance its action and the overall effect of treatment (109). Also, localized NDV therapy was shown to sensitize distant tumors to treatment with immune checkpoint inhibitors through induction of inflammatory immune infiltrates in these distant sites (110). This study provides a strong basis for developing this combination treatment with potential for entry into clinical trials.

RETROVIRUS

In order to overcome the problem of viral infection causing lysis of infected cells and potentially harmful inflammatory responses, a method has been developed whereby retroviral particles which retain their replicative ability can be delivered and will selectively replicate only in cells which are undergoing proliferation (tumor cells) and are compromised in their ability to trigger innate immune responses (again, tumor cells are often unable to trigger innate immunity due to disruption in the signaling pathway). The ability of these particles to integrate into the host genome and replicate without causing lysis of the cell makes them efficient and

long lasting producers of the therapeutic gene they are delivering without the consequences of productive viral infection (111).

As is the case for traditional oncolytic viral therapy, this method can be designed using a variety of retroviruses and with the addition of various therapeutic genes. For example, suicide genes which trigger cell death can be delivered to tumor cells *via* particles from various leukemia viruses with varying levels of success (112).

As well as delivering therapeutic genes, replicating retroviral vectors can also be used in order to enhance the response to anti-cancer drug therapy. For example, delivery of an activator of a therapeutic drug by replication competent retroviral vector resulted in significant anti-tumor effect and prolonged survival time in a murine model of malignant mesothelioma (113).

These vectors can also be delivered within a "gutted" Ad genome and the outcome of this combination is improved transfer efficiency of the retroviral genome into the tumor tissue and therefore increased production of the therapeutic gene and better treatment efficacy (114).

MEASLES VIRUS (MV)

Measles virus is a single-stranded, negative sense enveloped RNA virus of the *Paramyxoviridae* family. There are a number of receptors that can be utilized by MV to successfully infect cells including CD150, CD46, and nectin-4. Of these, CD46 has been attributed to increased specificity of MV to tumor cells that express increased levels of this receptor compared with healthy cells. This increased expression leads to increased levels of cell lysis upon infection of tumors compared with healthy tissue (115). Selectivity can also be increased by engineering a MV which is blinded to its usual receptors and redirected to recognize specific tumor cell markers as target antigens (116). Also, as tumor cells often have a defective interferon system they tend to be more susceptible to viral infection leading to increased lysis of these cells by MV in comparison with healthy cells (117). Another method of increasing oncolytic MV selectivity is to engineer miRNA sensitive viruses which can only successfully infect cells in which certain miRNAs are downregulated (i.e., cancer cells). For example, a virus has been developed which shows sensitivity to three host miRNAs through insertion of specific miRNA target sites into the viral genome, rendering the virus incapable of infecting healthy cells which express one or more of these miRNAs but still able to infect specific cancer cells which have downregulated levels of these miRNAs (118).

As well as showing selectivity to tumor cells, oncolytic MV therapy has been shown to recruit certain aspects of the host immune response including neutrophils (119) and dendritic cells (120) to augment tumor cell lysis by also stimulating anti-tumor immune responses to clear the tumor mass. This discovery has prompted development of oncolytic MVs which are engineered to stimulate the immune system at the tumor site in order to exploit the role it plays in anti-tumor immunity. For example, molecules known to stimulate potent immune responses, such as neutrophil-activating protein (NAP) derived from *Helicobacter pylori*, can be engineered into the MV genome to enhance anti-tumor effects generated by oncolytic MV (121). Immune responses can also be manipulated by encoding immune checkpoint blocking antibodies in oncolytic MV genomes. These viruses will result in soluble antibodies against inhibitory immune checkpoints being produced in infected cells which will act to dampen the ability of the immune response to limit itself. Blocking of inhibitory immune checkpoints allows OVs to exploit the immune system to a greater extent than would occur naturally as the immune cells are deprived of negative signals which usually regulate the immune response. This approach has been explored by Engeland et al. with promising results warranting further investigation (122).

One potential problem encountered with oncolytic MV is the widespread immunity in the population, gained from measles vaccinations. This immunity could dampen the effect of oncolytic MV therapy by rapidly clearing the virus before it can take effect on tumor cells. There are a number of potential ways to overcome this, for example, administering immunosuppressants along with oncolytic MV, however, this approach has drawbacks as immuno-suppression must be carefully managed to ensure patients do not become susceptible to infection by otherwise harmless agents. The most promising solution so far is to "hide" the virus within mesenchymal stem cells, allowing delivery of the virus to the target site without recognition by the immune system and thereby bypassing the effect of neutralizing antibodies (123). It is also possible to exchange measles coat proteins (which are recognized by existing neutralizing antibodies) for those of a virus to which patients are not immune. For example, Miest et al. successfully replaced measles envelope glycoproteins with those from a related virus (canine distemper virus) which allowed the MV genome to be transported without detection by existing neutralizing antibodies against MV envelope proteins (124).

Much progress has been made with MV over the years and this has resulted in many clinical trials being started to ascertain the suitability of oncolytic MV as a clinical treatment. Various cancer types have been targeted for clinical trials of oncolytic MV including myeloma (125) and ovarian cancer (126). Encouraging results have been obtained, especially with regard to safety profiles, and this warrants further studies into optimal oncolytic MV treatments (127).

OTHER OVs

Reovirus is a double-stranded, non-enveloped RNA virus of the *Reoviridae* family and is considered a naturally occurring OV. Reoviruses are thought to selectively infect tumor cells because their oncolytic functions depend on the activation of the Ras pathway (128) which tends to be upregulated in transformed cells. Reolysin is a type 3 Reovirus and is so far the only wild-type Reovirus undergoing studies for use as a therapeutic agent. Many clinical trials have been performed or are ongoing and are being conducted on various tumor types as discussed by Gong et al. (129). In 2015, the FDA and EMA granted Reolysin an orphan drug designation for various cancers including gastric, pancreatic, and ovarian cancer (130).

Other viruses are now being tested as therapeutic agents for cancer treatment. As melanoma is easily targeted, many current

studies are focused on this neoplasm (131). In recent years, two promising viruses have come out as possible OVs: coxsackievirus and echovirus. The former is responsible for the common cold and enters target cells *via* ICAM-1, while the latter is a positive sense single-stranded RNA virus from the *Picornaviridae* family responsible for many human disorders. As these two strains are the cause of very common diseases in humans, it is very important to ensure their safety.

TUMOR CELL BIOLOGY AND VIRAL THERAPY

In the search for the most effective viral therapy to treat cancer, alongside the focus on how to improve the virus we can also attempt to influence tumor cell biology to our advantage. This strategy has been adopted by various groups and research has so far shown that there are many tumor cell genes which can be manipulated to increase the efficacy of OVs.

For example, tumor cell genes can play a role in the targeting of OVs to tumor cells. It was found by Cuddington and Mossman that a certain OV (Bovine herpesvirus-1) is better able to infect cells which have increased levels of KRAS expression (e.g., tumor cells) (132). This represents a method of tumor targeting which relies on tumor cell factors to ensure oncolytic therapy is delivered to tumor cells, leaving healthy cells unharmed. This knowledge could potentially be applied to other oncolytic viral therapies by engineering entry mechanisms specific to tumor cells.

The aberrant expression of components of the Raf/MEK/ERK pathway in tumor cells can also have an effect on the regulation of Ad receptor and therefore levels of viral infectivity. As this pathway tends to be upregulated in tumor cells compared with healthy cells, it can have a significant effect on oncolytic viral therapy. It was found that this pathway plays a role in downregulation of CAR (Ad receptor) and therefore oncolytic Ad will be less able to infect target tumor cells. To overcome this, it is possible to inhibit MEK either directly or indirectly in order to inhibit the Raf/MEK/ERK pathway and re-establish expression of CAR on the cell surface (133). However, it has subsequently been shown that phosphorylation of ERK during the later stage of adenoviral infection can actually play a role in facilitating the sustained levels of viral protein within the cell required to produce enhanced levels of progeny virions (134). Taken together, this evidence highlights the need to balance initial inhibition of this pathway to increase CAR expression with later enhancement of the same pathway to facilitate sustained progeny production.

As well as mutations, regulation of certain genes using microRNA can also be used to enhance viral specificity for tumor cells. For example, using an miRNA which is downregulated in tumor cells (such as let-7a) to control expression of an essential viral gene in VV (such as B5R which increases both pathogenicity and oncolytic activity) results in a virus which can only express sufficient amounts of B5R in cells which have low levels of let-7a expression, i.e., tumor cells (15).

Another gene found in tumor cells that can influence OV therapy is VEGF. Our group has demonstrated that VEGF-A increases VV internalization and in turn replication levels (135). Therefore, oncolytic VV can take advantage of the increased expression of VEGF by tumor cells to increase delivery of therapeutic genes which in turn increases the efficacy and potency of the treatment. In addition to this, Arulanandam et al. found that the increase in VEGF expression upon infection with VV leads to upregulation of PRD1-BF1 (a transcription repressor) which increases sensitivity of tumor vascular endothelial cells to infection with vaccinia *via* repression of type-1 interferon anti-viral signaling. This increase in viral tropism in turn allows the OV to spread through the tumor more efficiently and therefore increases the efficacy of this oncolytic therapy (136). This natural repression of interferon signaling highlights the potential of using interferon inhibitors to increase the efficacy of oncolytic viral therapy, as seen by Stewart et al. (137).

It has also been found that a properly functioning host interferon response pathway is a critical factor in measles infection of malignant pleural mesothelioma. It was seen that in cell lines, there is a correlation between sensitivity of cells to measles infection and an inability of the cell to elicit a full interferon response in the presence of MV (138). This warrants further investigation as it suggests that inhibition of the interferon pathway could prove to be critical in ensuring the efficiency of oncolytic therapy. Previous to this study, it was also found that VV infection is greatly increased through downregulation of c-Jun NH2-terminal kinase (JNK). Inhibition of the JNK signaling cascade leads to lower levels of double-stranded RNA dependent protein kinase which in turn allows increased replication of VV genomes (139). This knowledge provides an avenue of exploration for improvement of OV efficacy.

Also, it has been reported that another gene found in tumor cells (CEACAM6) has an effect on oncolytic viral therapy. This tumor-associated gene has various functions including a role in promotion of tumor adhesion and invasion among other factors (140). It was shown by Wang et al. that over-expression of CEACAM6 did not have an effect on Ad receptor expression or at attachment and internalization steps of infection but did interfere with cytoplasmic virus trafficking to the nucleus *via* reduced expression of cytoskeletal proteins (141). As this cell adhesion molecule is able to inhibit adenoviral infection of tumor cells, systemic pre-treatment with siRNA targeting this protein could significantly enhance the anti-tumor response generated by adenoviral vectors.

Another host system which can have a significant effect on oncolytic viral efficacy is the stress response pathway. In the case of oncolytic rhabdovirus, inhibition of certain ER stress response factors can significantly increase efficacy of subsequently delivered oncolytic rhabdovirus (142). This method of pre-conditioning tumor cells to improve subsequent viral infection warrants further study as a potential method of increasing efficacy of oncolytic therapy in patients.

In addition to this, induction of the unfolded protein response in tumor cells has been found to increase the efficacy of oncolytic Ad by improving viral spread and tumor cell killing (143).

Recently, it has been reported that host microRNAs are able to regulate infection of cells with various viruses and in various ways. One of these is the control of Ad replication by miR-27a/b which downregulates SNAP25 and TXN2. This leads to a reduction in

Ad entry into cells and cell cycle arrest, respectively (which in turn reduce replication of Ad within the cell) (144). Controlling expression of host miRNA by inhibition of miRNA processing factors could therefore be a promising way to ensure maximal efficacy of oncolytic Ad therapies.

These examples open new insight in the field of oncolytic viral therapy revealing the possibility to manipulate virus–host interaction. We are still far from the optimum and new studies should be focused on the long-term effects of this interaction and the possible side effects.

CONCLUSION AND THE FUTURE OF THE FIELD

Oncolytic viral therapy is a promising treatment for cancer. New knowledge regarding both viral biology and tumor cell biology has made it possible to improve several aspects of OV therapy including safety, potency, and delivery methods. Also, the possibility of relying not only on direct lysis of tumor cells by viral infection but also mounting a multi-faceted approach involving viral lysis, immune stimulation, and gene therapy has become an important aspect of research in this field.

As the current treatment options for cancer tend to rely on only one method of attack, formulating a therapy which has the ability to act in multiple ways against cancer is an extremely exciting prospect. In order to effectively kill tumor cells and also reduce the chance of recurrence, it is necessary to utilize both the ability of virus infection to lyse the cells which are infected along with the ability of virus infection to stimulate a potent immune response, generating long lasting immunity against the antigens present on tumor cells.

If we can also bring into play gene therapy which can act in multiple ways, ultimately leading to reduction in tumor volume, we can make oncolytic viral therapy an even more formidable weapon in the fight against cancer.

The next step in this area should be to improve efficacy through arming with immuno-modulatory genes. These genes can influence the host response to viral infection, stimulating long-term immunity in the form of memory T-cells and attracting immune cells to the tumor mass through stimulation of cytokine production. In addition, combined therapy with conventional agents should be another relevant field of research, in order to increase viral action. This area of research involves a multitude of possibilities including ways to increase the host response to virus (for example, blocking immune checkpoints) and also the use of oncolytic therapy to augment the action of conventional therapy like chemotherapy and radiotherapy.

We also need to focus on exploiting the range of genes found to be differentially expressed in tumor cells which have recently been seen to play a role in potency of OVs. If we are able to gain a better understanding of the interaction between virus and tumor cells, we can overcome some of the obstacles in OVT by modulating the expression of tumor cell genes to enhance viral infectivity and efficacy.

Ultimately, the long-term goal is to formulate a treatment which can target solid tumors and circulating tumor cells in a number of ways simultaneously (145), with the aim of mounting a response that is effective even if the tumor cells become resistant to one approach. In order to do this, we need to take into consideration the interactions between tumor cells, virus, and the host. The most effective treatments will be designed to improve viral infection of tumor cells, boost immune responses to tumor antigens and manipulate tumor cell gene expression and pathways to favor successful viral infection. This can improve on current therapies which are ineffective once the tumor cells gain resistance, an issue which occurs often given the heterogeneous and rapidly mutating nature of many cancers.

AUTHOR CONTRIBUTIONS

YW conceived the study, interpreted the data, and finalized the manuscript. AH and GM designed the study, searched references, collected data, and wrote the manuscript. NL participated in interpretation of some data and critically reviewed the manuscript.

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REVIEW
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Targeting Nucleotide Biosynthesis: A Strategy for Improving the Oncolytic Potential of DNA Viruses

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The rapid growth of tumors depends upon elevated levels of dNTPs, and while dNTP concentrations are tightly regulated in normal cells, this control is often lost in transformed cells. This feature of cancer cells has been used to advantage to develop oncolytic DNA viruses. DNA viruses employ many different mechanisms to increase dNTP levels in infected cells, because the low concentration of dNTPs found in non-cycling cells can inhibit virus replication. By disrupting the virus-encoded gene(s) that normally promote dNTP biosynthesis, one can assemble oncolytic versions of these agents that replicate selectively in cancer cells. This review covers the pathways involved in dNTP production, how they are dysregulated in cancer cells, and the various approaches that have been used to exploit this biology to improve the tumor specificity of oncolytic viruses. In particular, we compare and contrast the ways that the different types of oncolytic virus candidates can directly modulate these processes. We limit our review to the large DNA viruses that naturally encode homologs of the cellular enzymes that catalyze dNTP biogenesis. Lastly, we consider how this knowledge might guide future development of oncolytic viruses.

Keywords: adenovirus, herpes simplex virus-1, nucleotide metabolism, oncolytic virus, cancer, ribonucleotide reductase, thymidine kinase, vaccinia virus

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INTRODUCTION

Oncolytic viruses are those that preferentially replicate in, and kill, cancer cells. Most wild-type viruses are naturally oncolytic in that they generally grow best in transformed and dividing cells, but this level of specificity is rarely stringent enough for therapeutic use in cancer patients. To improve tumor specificity, researchers have modified a wide range of viruses to take advantage of dysregulated control pathways and altered signaling cascades characteristic of cancer cells. One pathway exploited in the development of oncolytic DNA viruses is that controlling the level of nucleotides available for DNA replication.

It is important that normal cells maintain the proper ratios of all four dNTPs as well as control the concentration of the dNTPs throughout the cell cycle. Otherwise they face a risk of increased mutations and genomic instability [reviewed in Ref. (1–3)]. Although the reported intracellular dNTP concentrations vary greatly depending upon the assay, cell line, and/or tissue examined, one can observe some common themes (2). Most importantly, dNTP concentrations change throughout the cell cycle in non-transformed cells, with the lowest concentration seen in resting (G0) and

early G1 cells. These concentrations rise toward S-phase and then decrease as cells enter late G2 and undergo mitosis (2).

In a natural infection, viruses are most likely to encounter host cells in G0 or G1, since that is most common state of cells *in vivo*. The low level of dNTPs present at that time is a significant barrier to virus replication. Consequently viruses have evolved a number of strategies to increase the availability of dNTPs. For example, a classic strategy is one employed by small DNA viruses such as SV40, which promotes the degradation of the tumor suppressor proteins p53 and pRb, and thus drives entry into S phase. Other small viruses, including human parvovirus (4, 5) and human papilloma viruses (6), encode proteins that can cause already dividing cells to arrest at stages of the cell cycle more favorable for virus replication (i.e., S or G2). Some large DNA viruses do this as well. For example, Orf and HCMV encode proteins which disrupt the activity of the anaphase-promoting complex (APC), also leading to alterations in cell cycle progression [reviewed in Ref. (7)].

Many herpes viruses and poxviruses encode enzymes that can directly catalyze dNTP biogenesis. This is probably the most effective strategy for obtaining these critically important metabolites, as it avoids the necessity of perturbing the cell cycle, which can often trigger antiviral defenses. Numerous research groups including our own have engineered viral genomes to alter expression of viral proteins involved in dNTP synthesis in order to target virus replication specifically to tumors. Disabling the capacity of the virus to stimulate nucleotide biosynthesis in infected cells forces the virus to rely on pre-existing levels of dNTPs, which are very low in non-dividing cells but elevated in cancer cells poised for proliferation (1). A thorough understanding of nucleotide biosynthesis in normal cells and dysregulation of this pathway in infected cells and cancer should aid in the rational design of novel oncolytic viruses.

dNTP BIOGENESIS AND REGULATION IN NORMAL CELLS

Mammalian cells employ several mechanisms that must work in concert to provide the extra dNTPs needed for S-phase genome replication, while also maintaining the dNTP pools needed throughout the cell cycle for mitochondrial replication and for DNA repair (**Table 1**). At the same time, an intricate system of feedback regulation ensures that dNTP pools remain balanced. This section highlights key aspects of these pathways. **Figure 1** outlines the reactions catalyzed by these cell enzymes.

Three of the four cellular dNTPs (dATP, dGTP, and dCTP) are products of pathways largely dependent upon the activity of ribonucleotide reductase (RNR). Several types of RNRs exist. Mammalian RNRs are class I enzymes, comprising a heterotetramer consisting of two large subunits (R1 or RRM1) and two small subunits (R2 or RRM2) [reviewed in Ref. (8)]. RNR reduces NDPs (ADP, GDP, CDP, and UDP) to their respective dNDP forms, which are subsequently converted to dNTPs by cellular NDP kinases. RNR regulation is complex and involves an intricate system of feedback inhibition which allows RNRs to sense specific dNTP levels and alter the rate of reduction of other NDPs so as to maintain balanced dNTP pools [interested readers are invited to read (8)].

Cellular RNR protein levels are regulated by a combination of transcription and posttranslational processes. Few R1 or R2 mRNA transcripts are found in G0 cells, but these then rise as cells enter G1 and S-phase. The R1 protein exhibits a long

TABLE 1 | Key proteins, discussed in this review, catalyzing dNTP biogenesis.

Gene or protein	Natural substrate(s)	Product(s)	Comments
Ribonucleotide reductase (RNR)	ADP, CDP, GDP, UDP	dADP, dCDP, dGDP, dUDP	
R1			Protein levels remain relatively constant throughout cell cycle
R2			Cell cycle regulated, rate limiting for *de novo* nucleotide metabolism
P53-R2			Low levels throughout cell cycle, induced in response to DNA damage
NMP kinases			
Thymidylate kinase (TMPK)	dTMP, dUMP	dTDP, dUDP	Mitochondrial and cytoplasmic isoforms exist
Cytidine/uridine monophosphate kinase (CMPK)	CMP, dCMP, UMP, dUMP	CDP, dCDP, UDP, dUDP	Mitochondrial and cytoplasmic isoforms exist
Guanylate kinase (GMPK)	GMP, dGMP	GDP, dGDP	Mitochondrial and cytoplasmic isoforms exist
Adenylate kinase (AK)	AMP, dAMP, CMP, dCMP	ADP, dADP, CDP, dCDP	Multiple isoforms and tissue-specific species exist
NDP kinases	NDPs, dNDPs	NTPs, dNTPs	
Thymidine kinase (TK)			
TK1	dT, dU	dTMP, dUMP	Cytoplasmic, cell cycle regulated
TK2	dT, dU, dC	dTMP, dUMP, dCMP	Mitochondrial localization, expressed at low levels throughout cell cycle
dCMP deaminase (DCTD)	dCMP	dUMP	
dUTPase	dUTP	dUMP	Mitochondrial and cytoplasmic isoforms exist
Thymidylate synthetase (TYMS)	dUMP	dTMP	Cytoplasmic. Unclear if mitochondrial isoform exists
Deoxycytidine kinase (DCK)	dA, dG, dC	dAMP, dGMP, dCMP	Cytoplasmic only
Deoxyguanosine kinase (DGUOK)	dA, dG	dAMP, dGMP	Mitochondrial only

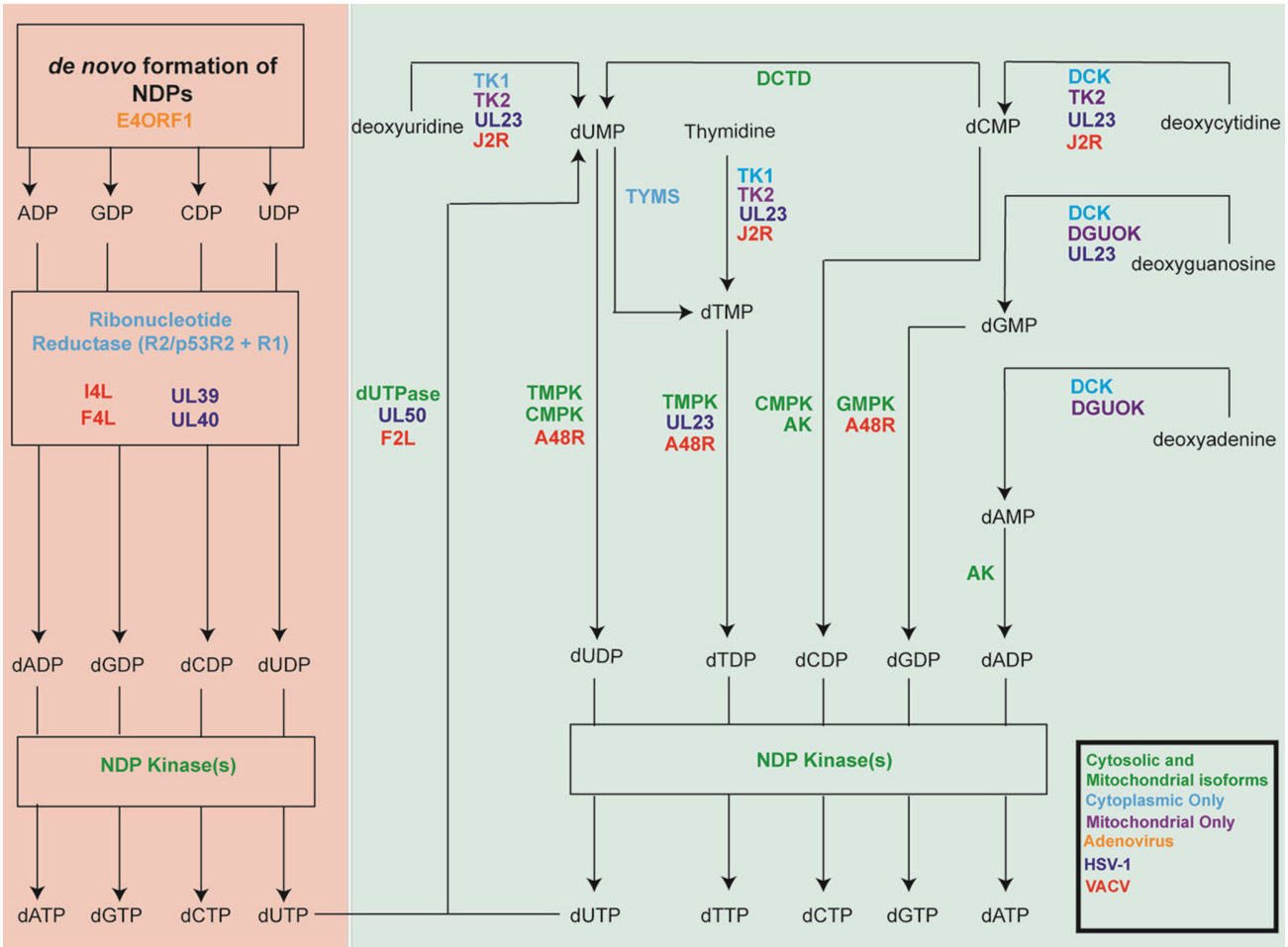

FIGURE 1 | Key cellular nucleotide metabolism enzymes and their herpes simplex virus-1 (HSV-1) and vaccinia virus (VACV) homologs. Shown are the cellular enzymes that catalyze key steps in nucleotide metabolism. Green lettering indicates proteins that are expressed as both cytoplasmic and mitochondrial isoforms, while proteins found only in the cytoplasm are shown in light blue and those found only in the mitochondria are shown in purple. The figure also shows the viral genes encoding homologous proteins. HSV-1 genes are shown in dark blue, VACV in red and adenoviruse (Ad) in orange. Note that the figure over simplifies the biology because it is not possible to display all of the many different isoforms and enzymes, which sometimes also exhibit overlapping catalytic specificities.

half-life (~20 h), thus R1 protein levels remain relatively constant throughout the cell cycle. In contrast, the R2 protein is much less stable with a half-life of only ~3 hr. As the demand for dNTPs declines at the end of S-phase, the R2 protein is targeted for degradation by the Skp1/Cullin/F-box complex during G2, and by Cdh1-APC during mitosis. These reactions are promoted by interactions with the "KEN box" domain found near the R2 N-terminus (9). Because of these processes, the levels of the R2 subunits are thought to rate-limit the formation and stability, and therefore activity, of the RNR complex.

Mammalian cells also encode a second R2 species called p53R2 (or RRM2b). R1 can complex with p53R2, and although active, this RNR isoform exhibits lower catalytic activity than RNRs composed of R1 and R2 (10, 11). The p53R2 protein is expressed throughout the cell cycle and its presence is thought to ensure that sufficient dNTPs are available to support mitochondrial genome replication outside of S-phase (11, 12). As the name implies, p53R2 expression can also be upregulated in a p53-dependent manner in response to DNA damage (13). This is thought to increase the availability of the dNTPs needed for DNA repair.

Although *de novo* synthesis from NDPs is thought to be the major pathway responsible for dCTP, dGTP, and dATP production for nuclear DNA synthesis, salvage enzymes exist that can also contribute to their biosynthesis. This includes deoxycytidine kinase (DCK), which is not cell cycle regulated and can phosphorylate dC, dG, and dA (14). The resulting dCMP, dGMP, and dAMP can be further phosphorylated to dNDPs by a variety of nucleoside monophosphate kinases including the many adenylate kinases, uridylate-cytidylate kinases (UMP-CMPK), and guanylate kinases (GMPK) reviewed in Ref. (15). The resulting dNDPs are converted to triphosphates by the same NDP kinases used for *de novo* synthesis of dNTPs.

Cells do not have a source of rTDP that could serve as an RNR substrate for dTDP production and so other enzymatic routes are used to manufacture dTTP (**Figure 1**). The production

of dTTP requires thymidylate kinase (TMPK), which converts dTMP to dTDP. NDP kinases then convert dTDP into dTTP. There are two sources of dTMP in mammalian cells. One route employs thymidylate synthetase (TYMS) to convert dUMP into dTMP. Most of this dUMP is thought to come from deamination of dCMP by dCMP deaminase (DCTD) (16) although dUMP can also be produced from the pyrophosphorolysis of dUTP by dUTPase. The latter route has the dual function of reducing the incorporation of uracil into DNA, by DNA polymerases, which if not repaired by uracil N-glycosylases (UNG, also designated UDG) can be mutagenic. The second source of dTMP is through thymidine kinase (TK) catalyzed phosphorylation of thymidine. Cells express both cytoplasmic (TK1) and mitochondrial (TK2) forms of the enzyme (17). Both forms can convert thymidine to dTMP and deoxyuridine to dUMP, while TK2 can also convert deoxycytidine to dCMP (17). The availability of thymidine is influenced by the activity of thymidine phosphorylase (TYMP). Although the enzyme can interconvert thymine and thymidine, it enzymatically favors the catabolism of thymidine (18).

Enzymes catalyzing dTTP formation can be cell cycle regulated in a variety of ways. The E2F-pRb pathway that drives entry into S phase has been shown to regulate the transcription of TYMS, dUTPase, TK1, and TMPK (19–22). TK1 activity is also subject to additional levels of regulation, with its transcription and translation efficiency also promoted by p107 and cyclinA/Cdk-2 (22). After DNA replication, TK1 activity is reduced following phosphorylation by Chk1 kinase (23, 24). Furthermore, TK1, TMPK, and TYMS are degraded at the end of mitosis, in a process that is mediated by APC (25, 26).

Balancing these biosynthetic pathways, are enzymes that catabolize unwanted dNTPs, including pyrophosphatases like the dUTPases. These may help maintain balanced dNTP pools and reduce the availability to virus replication machinery. Recently, a dCTPase, DCTPP1, was identified in human cells, which is thought to regulate and sanitize dNTP pools by degrading dCTP or dCTP analogs such as 5-methyl-dCTP (27). DCTPP1 levels vary with the cell cycle, but in a manner opposite to the behavior of anabolic enzymes (27). Another enzyme that catabolizes dNTPs is SAMHD1 (28, 29). In addition to being a ribonuclease, SAMHD1 has a dNTP triphosphorylase activity. In the presence of dGTP, SAMHD1 hydrolyzes dNTPs releasing a deoxyribonucleoside and triphosphate. The enzyme is subject to complex regulatory schemes that differ in different cell types.

Mitochondrial DNA replication is not subject to cell cycle control, so there must be mechanisms to maintain mitochondrial dNTP pools that are separate from those used for nuclear DNA synthesis [reviewed in Ref. (2, 30)]. This is done through two mechanisms. First, transport proteins can move dNMPs, dNDPs, or dNTPs from the cytoplasm of cycling cells into the mitochondria, where mitochondrial NMPK/NDMKs can generate dNTPs. Cytoplasmic and mitochondrial dNTP pools appear to mix in dividing cells (31), and while dNDPs and dNTPs cannot passively diffuse across mitochondrial membranes, some nucleotide transport proteins are capable of exchanging nucleotides across the membrane (32). Alternatively, in non-dividing cells, mitochondria are thought to mostly generate dNTPs using mitochondrial enzymes and salvage pathways, while in the cytoplasm catabolic reactions degrade dTMP to thymine. Besides the aforementioned TK2 which is encoded by a separate gene from TK1, mitochondrial isoforms of TMPK (33), dUTPase (34), and UNG (35) have been identified. Unique to the mitochondria is deoxyguanosine kinase (DGUOK), which converts dG and dA to dGMP and dAMP, respectively (36). While no evidence of mitochondrial TYMS activity has been reported, some groups have detected mitochondrial RNR activity suggesting that *de novo* dNTP synthesis may also be possible (30).

The differences between the mechanisms of cytoplasmic and mitochondrial dNTP biogenesis may contribute to the differences in the relative abundance of each nucleotide. For example, while dGTP is the least abundant nucleotide in the cytoplasm, it is the most abundant in mitochondria (37). Mutations in genes required for nucleotide biosynthesis (e.g., p53R2, TK2, and DGUOK) produce defects in mitochondrial DNA replication and have been linked to several inherited human diseases (38–40).

ALTERED dNTP BIOGENESIS IN CANCER

A number of excellent reviews have summarized what is known about the links between altered dNTP metabolism and its relationship to cellular transformation and cancer (2, 29, 41). Here, we summarize some of the key points relating to how the genes and proteins outlined above influence proliferation of cancer cells, how these systems can be targeted by anticancer therapies, and how alterations in nucleotide metabolism can affect the outcome of cancer treatments. Lastly, we provide a comprehensive analysis of the changes in relevant gene expression patterns that are seen in many different cancer types, using the Oncomine database (**Figure 2**). These changes provide insights into how one might align the properties of an oncolytic virus with the cancer it targets.

The best-known and most exhaustively studied links between nucleotide metabolism and cancer concern RNR, in part because the enzyme is a target of many widely used chemotherapeutic agents. Mutations in both R2 and p53R2 have also been linked to tumorigenesis, while R1's role is less clear and R1 has been reported to function as a tumor suppressor (41). These seemingly contradictory roles reflect the different ways that RNR activity can affect normal and transformed cell processes, and the differing perspectives clinicians and researchers apply to studying this enzyme in health and disease. As a general rule, any increase in dNTP biosynthesis is expected to favor tumor growth and also decrease the therapeutic effectiveness of a nucleotide analog that competes with these dNTPs. As noted above, the fact that the R1 and R2 subunits are differentially regulated through the cell cycle adds additional complexity to interpreting the links between RNR and cancer.

Although the mechanism is not clear, R1 overexpression has been shown to induce PTEN expression and to reduce transformation and tumorigenesis in animal models (42–44). Furthermore, R1 overexpression correlated with better survival in patients with non-small cell lung carcinoma following surgery alone (45). However, R1's role in supporting DNA repair has also been suggested to explain why elevated R1 expression is linked to increased resistance to platinum drugs and gemcitabine (whose target includes RNR), and has been suggested to be used as a

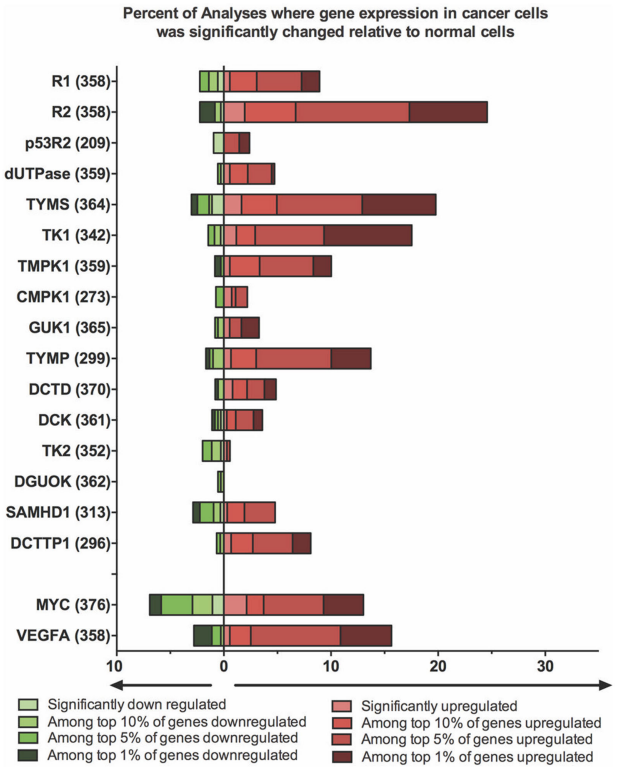

FIGURE 2 | Expression of deoxynucleotide biosynthesis genes is altered in a significant portion of cancer cells. A differential analysis of mRNA expression between cancer and normal tissue was performed for each gene using Oncomine Research Edition (Version 4.5) software. A change in mRNA expression greater than twofold, with a *P*-value $<10^{-4}$, was considered significant. The percent of analyses where gene expression was significantly changed in cancer cells was then calculated and plotted. The fraction of samples where gene expression was among the top 1, 5 or 10% of genes whose expression was altered was also calculated. The total number of analyses surveyed in the data, are denoted in brackets beside each gene. Data were retrieved and analyzed on April 6, 2017. Two genes (Myc and VEGFA) whose expression is frequently altered in cancer cells are included for comparison.

biomarker for tailoring an individual's cancer treatment plan to these drugs [reviewed in Ref. (41)].

In contrast to R1, R2 dysregulation is clearly associated with oncogenesis. Increased R2 expression is linked to increased DNA replication, and this is associated with greater uracil incorporation leading to more DNA breakage and mutation (46, 47). Elevated R2 levels have also been associated with increased cell proliferation, invasiveness and tumor vascularization (41, 48–50). The role of p53R2 is less clear. It is induced in response to signaling through p53 and increased p53R2 levels correlated with better prognosis in some cancer patients (41). However, like R2, p53R2 overexpression also increased the rate of spontaneous tumor formation in transgenic mice (47). Using the Oncomine database, Aye et al. found that a significant fraction of cancer specimens exhibited increased R1 and R2 levels, but alterations in expression of p53R2 were less common (41). Our own analysis supports these earlier findings (**Figure 2**).

The enzymes involved in dTTP biosynthesis (TYMP, TYMS, TK1, and TMPK) are also upregulated in transformed cells facilitating increased rates of DNA synthesis, and several chemotherapeutic strategies target these pathways (19, 51, 52). Fluoropyrimidines like 5′-fluorouracil (5-FU) are activated by TYMP and TK1 and then bind to and inhibit TYMS. The active metabolites can also serve as DNA polymerase α substrates and are then incorporated into DNA (53). Antifolates, like methotrexate, also reduce dTTP production by blocking production of the co-factors used by TYMS. Upregulation of TYMP, that interconverts thymine and thymidine, promotes tumorigenesis, angiogenesis, and metastasis and has been implicated in suppressing innate immune response in tumors and perturbing energy metabolism [reviewed in Ref. (18)]. At the same time, increased TYMP levels can enhance sensitivity to some fluoropyrimidines (18). Although chemotherapeutic agents targeting these pathways may initially be effective, the high mutation rate in cancer cells coupled with strong selective pressure can lead to the development of resistance. For example, 5-FU treatment can drive selection for increased TYMS levels, leading to 5-FU resistance and poorer prognosis (19, 51, 54). A combination of metformin and 5-FU treatment can also induce 5-FU resistance, although this creates gemcitabine sensitivity probably due to a concurrent increase in DCK activity (55).

TK1 levels are elevated in cells dysregulated in several pathways commonly associated with cancer, including the E2F-pRb pathway. A classic example of dysregulation of this pathway is that induced by the SV40 large T antigen that binds to and inactivates pRb (56). SV-40-transformed TK-deficient cells were less able to form tumors in hamsters, compared to those with a functional TK, suggesting that TK also promotes tumorigenesis (57). Furthermore, it has recently been shown that upon re-entering the cell cycle, terminally differentiated muscle cells did not express sufficient TK1 activity to support efficient proliferation, suggesting that low levels of TK1 may play a role in preventing aberrant replication (58). At the same time, TK1-dependent nucleotide biosynthesis is required for DNA repair. Increased TK1 activity can enhance the excision of genotoxic damage and thus promote resistance to many cancer therapies (59). TK levels are elevated in many cancer cells, regardless of the cell's proliferation state (60), and since this can sometimes be detected in serum it has been suggested that TK might be used as a prognostic marker (61, 62). TK activity also increases the efficacy of fluoropyrimidines (54).

Thymidylate kinase activity can reduce the amount of dUTP incorporated into DNA during repair by providing sufficiently high levels of dTTP at the damaged site (61). It has been suggested that this may reduce the risk of double-stranded breaks produced by UNG-mediated DNA repair. In agreement with this, inhibiting TMPK sensitizes cells to the DNA damaging agent doxorubicin, and treatment with this combination produced superior tumor control (63). Cancer cells have also been shown to exhibit altered levels of other monophosphate kinases (CMPK1, GMPK) and this sometimes correlates with differences in cancer prognosis and the response to some chemotherapeutics (64–66).

Deoxycytidine kinase phosphorylation of the chemotherapeutic agents AraC and gemcitabine is required for their inhibition of

DNA polymerase, and the loss of DCK activity has been linked to AraC and gemcitabine resistance (67–69). An analysis of pancreatic cancer patients treated with gemcitabine found that DCK levels correlated with prolonged survival (70, 71), but little else has been reported linking DCK to cancer.

Altered expression or mutation of dNTPase enzymes have been seen in several cancers. SAMHD1 has been suggested to have tumor suppressor activities, as its overexpression can decrease cellular proliferation, and mutation, and loss of SAMHD1 has been reported for several cancers including leukemias, lymphomas, breast, and colorectal cancer (29, 72). SAMHD1 levels also differentially affect common chemotherapeutics. SAMHD1 can contribute to AraC resistance, with lower levels of SAMHD1 correlating with increased AraC sensitivity (73). Furthermore, a retrospective analysis of AraC-treated AML patients found that individuals with lower SAMHD1 levels experienced better outcomes (73). While decreased SAMHD1 levels increase AraC sensitivity, SAMHD1 overexpression increases sensitivity to other DNA-damaging agents such as etoposide, mitomycin C, or camptothecin (72). These observations can be explained by the effects SAMHD1 has on dNTP pools. High levels of enzyme activity would decrease dNTP concentrations, thus slowing cell proliferation, decreasing rates of error-prone DNA replication, and inhibiting repair of lesions caused by direct acting DNA damaging agents. Conversely loss of the enzyme would promote dNTP-dependent replication and cell proliferation. The way SAMHD1 activity modulates AraC sensitivity is explained by the recent observation that it catabolizes a variety of nucleoside analogs (74).

Both DCTPP1 and dUTPase have been linked to tumor invasiveness. DCTPP1 expression has been reported to be elevated in breast and gastric cancers, and these expression levels correlated with poorer prognosis (75, 76). The expression of dUTPase is regulated by p53, and elevated in p53-mutated cells (21). In Huh7 cells, siRNA-mediated silencing of dUTPase decreased both invasiveness and cell growth, suggesting that dUTPase levels may affect tumor establishment or progression (77). However, dUTPase activity also seems to be important in the context of R2 levels. Decreased dUTPase levels increase the rates R2-induced genomic instability. A study examining the survival of colorectal cancer patients revealed that patients with high R2 and low dUTPase levels faced a poorer prognosis than patients with elevated levels of both R2 and dUTPase (46), perhaps due to the enhanced flux of dUTP that would be produced relative to dTTP formed by TMPK (63). Both DCTPP1 and dUTPase contribute to cellular resistance to the chemotherapeutics 5-FU and decitabine (76–78). 5-FdUTP is a known substrate for cellular dUTPases, suggesting an obvious mechanism of action (79).

Using publicly available data sets in the Oncomine database, we have examined changes in expression of genes involved in nucleotide metabolism in different tumor types compared to normal tissues (**Figure 2**). The microarray data searched on April 6, 2017, comprised 19 major cancer types (plus an "other category") and surveyed ~350 unique tests per gene. It is clear from these analyses that it is very common for genes involved in dNTP synthesis to be highly upregulated in cancer. In particular, TK1, R2, and TYMS mRNAs were found more frequently than *MYC* among the top 1% of overexpressed genes. The same conclusions are supported by histochemical protein analyses and tissue microarray data posted by the Human Protein Atlas (http://www.proteinatlas.org). A search of these data (v.17) showed that the TK1, R2, and TYMS proteins are expressed at high or medium levels in 25, 79, and 83% of the 20 cancer types surveyed, respectively. The high frequency with which a protein like R2 is overexpressed in so many cancer types highlights the tumor specificity that could be afforded by exploiting this pathway when designing oncolytic viruses.

ADENOVIRUS (Ads)

Adenoviruses are non-enveloped double-stranded DNA viruses that replicate in the nucleus of infected cells. They have been widely tested as gene therapy vectors and oncolytic agents and an early region 1b (E1b)-deleted Ad (H101/Oncocrine) was licensed in China in 2006 for use in treating head and neck cancer. While numerous strategies for engineering Ad-tumor specificity have been described [reviewed in Ref. (80, 81)], this section will focus on how Ads alter dNTP levels, and how this might be used as way of generating oncolytic variants.

Wild-type Ad infection has been reported to increase dNTP levels and its growth is influenced by dNTP availability (82, 83). However, Ads do not encode homologs of the cellular enzymes that catalyze nucleotide biosynthesis; therefore, they likely increase dNTP levels by indirect methods. For example, Ads encoding *E4-ORF1* mutations grow more poorly than wild-type virus, and cells infected with such mutants exhibit reduced dNTP levels compared to wild-type virus infected cells (83). Although E4-ORF1 shows some resemblance to the dUTPase genes encoded by humans, herpes simplex virus-1 (HSV-1) and VACV, the purified Ad protein lacks dUTPase activity (84). Instead, E4-ORF1 seems to indirectly increase dNTP levels by activating the MYC and AKT signaling pathways that upregulate enzymes involved in the formation of ribose (83). When combined with mutations in *E1b*, a virus bearing an *E4-ORF1* deletion showed an increased ability to kill cancer cells in culture (85). Whether *E4-ORF1* mutant viruses might have independent oncolytic properties, or could improve the efficacy of other existing oncolytic Ad strains *in vivo,* is unknown.

A ΔE1ΔE3 Ad expressing human R1 exhibited a superior ability to control the growth of human colon adenocarcinoma tumors in CD-1 nude mice following intratumoral virus administration of Ref. (86). Since this is a non-replicating virus the effect would have to be attributed to R1's other tumor-suppressor activities.

VACCINIA VIRUS

Poxviruses are large double-stranded DNA viruses which replicate in the cytoplasm of infected cells. While capable of attaching to and entering a wide variety of cells, postentry factors are thought to define cellular and host tropism (87). A number of poxviruses have been tested for their potential as oncolytic agents (**Table 2**), but the two in the furthest state of development are the *Leporipoxvirus* myxoma virus (MYXV) and the *Orthopoxvirus*

TABLE 2 | Oncolytic vaccinia viruses with mutations in nucleotide metabolism genes.

Virus	Strain	Mutation(s)	Transgene(s)	Status	Reference
JX-594[a]	Wyeth (NYCBOH)	TK$^-$	GM-CSF *lacZ*	Currently being evaluated in a phase III trial for HCC	(103, 107, 115, 118–120, 123, 206, 262–267)
vvDD-CDSR (JX-929)[b]	Western reserve	TK$^-$, VGF$^-$	Yeast cytosine deaminase, *lacZ*, human somatotropin receptor 2	Completed phase I trial	(105, 109, 127–129, 133, 268)
GL-ONC1 (GLV-1h68)[c]	Lister	TK$^-$, *A56R*$^-$, *F14.5L*$^-$	*Renilla* luciferase-GFP, *lacZ*, *gusA*	Currently in phase I/II trial	(134–136, 138, 269–271)
VV-FCU1	Copenhagen	TK$^-$	FCU1	Preclinical	(114)
VV-FCU1	Western reserve	TK$^-$, R1$^-$	FCU1	Preclinical	(157)
ΔF4L	Western reserve	R2$^-$	Neomycin, *gusA*, and mCherry	Preclinical	(104, 150)
ΔF4LΔJ2R	Western reserve	TK$^-$, R2$^-$	*lacZ*, neomycin, *gusA*, and mCherry	Preclinical	(104, 150)
TK$^-$/ΔJ2R[d]	Various	TK$^-$	Various	Preclinical	

[a]*The JX-594 backbone of contains a natural truncation in the B18R gene not found in other recombinant VACVs (116).*

[b]*While VVDD-CDSR is the lead candidate and only version of a TK$^-$/VGF$^-$ VACV in clinical trial, other viruses that contain the same mutations, but have additional modifications exist. Some examples include a version that expresses GFP or luciferase, a version (JX-963) that expresses GM-CSF in lieu of CDSR, and a version (VVDD-A34R$_{K151E}$) which encodes a mutation that enhances viral spread throughout tumors (91, 129, 130, 268, 272).*

[c]*While GL-ONC1 is the lead candidate, and only version of a TK$^-$/A56R$^-$/F14.5L$^-$ VACV in clinical trials, other modifications to this backbone have been made and are being tested in preclinical settings. Some examples include versions which express MMP-9, Cdc6, hEPO, or hNIS [reviewed in Ref. (139)].*

[d]*The TK (J2R) loci is disrupted in a number of recombinant VACVs and has been used as a comparator for evaluating many other recombinant VACVs.*

vaccinia virus (VACV). MYXV infects rabbits and hares, but naturally has oncolytic properties in some cancers. It is currently being developed for use in the treatment of hematological cancers (88–90).

Vaccinia virus has a well-established safety record based on its extensive use as a smallpox vaccine. While VACV naturally displays preferential growth in cancer cells (91), it can productively infect a wide variety of animals and cell types, including non-dividing cells. This is facilitated by a combination of specific host range factors as well as genes that support general replication in a variety of cell types. Such proteins are attractive targets for improving the oncolytic selectivity of the virus. Some examples include immunoregulatory proteins, viral growth factors (VGFs), and the subject of this review—enzymes involved in nucleotide metabolism.

The repertoire of nucleotide metabolism genes encoded by poxviruses varies by genus. VACV and other orthopoxviruses encode the largest array of these genes including catalytically active homologs of R1, R2, TK, TMPK, dUTPase, and UNG. Some VACV strains also encode a GMPK, although whether this is functional, and contributes to GDP production, is unknown (92).

VACV TK and TMPK Mutants

Vaccinia virus encodes two separate enzymes that catalyze biosynthesis of dTTP—a TK homolog encoded by the *J2R* gene, and a TMPK homolog encoded by the *A48R* gene. Like mammalian TK1, VACV TK is a class II enzyme, forms a homotetramer, and phosphorylates thymidine (93–95). It is also inhibited by dTTP and can phosphorylate thymidine analogs such as AZT, AraT, and BrdU (95, 96). Unlike mammalian TK1, VACV TK has a limited capacity to phosphorylate deoxycytidine (95). Mammalian cells can become BrdU-resistant through the loss of TK activity (97), and the availability of TK$^-$ cells led to the discovery that VACV must encode a TK activity (96) and also permitted the isolation of BrdU-resistant/TK-deficient viruses (98). This method facilitated the identification of the gene responsible for TK activity in the early 1980s (99–101) and led to the widespread use of the TK locus as a site for incorporating transgenes. Compared to cells infected with wild-type VACV, those infected with TK mutants show decreased levels of dTTP and dCTP (102).

Cellular TK activity can complement mutations in viral TK and provides a basis for producing tumor-specificity. This is best highlighted by studies using shRNA- or siRNA-mediated silencing of cellular TK in HeLa cervical carcinoma cells. Although wild-type and TK$^-$ viruses grow equally well in HeLa cells under ordinary conditions, when cellular TK is silenced the growth of a TK mutant (JX-594) is reduced about fivefold, whereas the wild-type virus is unaffected (103). Serum-starvation has also been reported to decrease the levels of cellular TK in normal cells, but to a lesser extent in cancer cells (91). While cancer cells cultured under normal or serum-starved conditions support equal growth of both TK-mutant and wild-type virus, one sees decreased transcription and yields of TK mutants relative to wild type in many untransformed cells cultured under serum-starved conditions (91, 104). It should be noted that this differential virus growth is not seen with all serum-starved normal cell lines (104), which could be due to the fact that dTTP can also be generated in a TK-independent manner, through TYMS. The selective replication of TK mutants in cancerous tissue has also been shown in animal models (105, 106), in explanted patient tumors, and in biopsies taken from patients receiving virus as part of a clinical trial (107).

TK mutants are considerably less virulent than wild-type VACV in animal models; however, the degree of attenuation depends upon the administration route and genetics of both virus and host. In a mouse BALB/c intracranial infection model, TK mutations increased the LD_{50}'s ~25-fold using a VACV Wyeth strain (3.2×10^6 versus 9.1×10^7 PFU) and ~4,000-fold higher using a Western Reserve (WR) strain (10 versus 4×10^4 PFU) (108). TK mutants also showed decreased pathogenesis in rhesus macaques, as evident by decreases in the size of necrotic lesions after intradermal administration (109). However, in immune compromised animal models, TK mutants can still be virulent. McCart et al. compared the pathogenesis of wild type and TK

mutants in a WR strain background following intraperitoneal administration to nude mice. They found that while wild-type virus killed the mice by day 5, mice injected with TK mutants survived to day 17 (105). The TK mutants showed a ~100-fold reduction in titer relative to wild-type virus in the brain, but differences were insignificant in other tissues including spleen, ovaries, testes, and bone marrow (105). Our laboratory has also examined the pathogenicity of a TK mutant in tumor-bearing immune compromised animals at doses sufficient for oncolytic activity and found significant virus titers in normal tissues, particularly ovaries, as well (104).

In general, while TK mutations promote increased safety and selectivity and are the basis of all the VACV oncolytics that have entered the clinic, TK deletions have always been combined with mutations in other viral genes and/or transgenes. TK mutants exhibit increased safety and/or efficacy when combined with mutations in the VGF (105), R1 or R2 (104, 110), or the type I interferon binding protein (*B18R*). TK has also served as a site for integration of transgenes encoding granulocyte-macrophage colony-stimulating factor (GM-CSF, described below), hydroxyprostaglandin dehydrogenase (111), the pattern recognition receptor DNA-dependent activator of IFN-regulatory factors (DAI) (112, 113), TIR domain-containing adaptor inducing IFN-β (TRIF) (112), recombinant antibodies such as those targeting PD-1 (110), a fusion of yeast cytosine deaminase and uracil phosphoribosyltransferase (FCU1) (114), and many antigens used in recombinant vaccines.

JX-594 (Pexastimogene devacirepvec or Pexa-Vec) is the oncolytic VACV in the furthest state of clinical development. This Wyeth strain of TK$^-$ virus also encodes human granulocyte-macrophage colony-stimulating factor (GM-CSF) and beta-galactosidase (115). The *B18R* gene in this recombinant also naturally encodes a truncated protein (116), which is also known to reduce virulence (117). GM-CSF has many functions including the ability to stimulate the development of hematopoietic cells and has been shown to improve the efficacy of a TK$^-$ virus in rabbits bearing VX-2 liver tumors (91).

JX-594 is being developed by Silagen and has been tested in at least 11 clinical trials. Over 300 patients with a wide variety of cancers including melanomas, and hepatocellular, colon, pancreatic, lung, and ovarian carcinomas have been enrolled, and the virus has been administered by both intratumoral and intravenous routes (103, 107, 115, 118–124). JX-594 is generally well tolerated, with most patients experiencing transient flu-like symptoms. Maximum tolerable doses of 10^9 PFU have been reported when administered intratumorally (124), while a maximum feasible dose of 10^9 PFU has been reported for intravenous administration (107, 119). A phase I trial in seven children with stage III or IV cancers showed similar safety profiles to that seen with adults (121). Evidence of tumor-specific replication, transgene expression, development of virus neutralizing antibodies, and efficacy have been observed in subsets of patients in these studies.

In a phase II dose-finding study of JX-594 in 30 patients with advanced hepatocellular carcinoma (HCC), patients receiving the highest dose of virus (10^9 PFU) exhibited longer survival than those receiving 10-fold less virus (14 versus 6 months) (122). However, in the 120-patient phase IIb part of the study, JX-594, administered to HCC patients who had failed sorafenib therapy, did not significantly increase overall survival compared to individuals receiving standard of care therapy (125). A phase III trial in treatment-naive HCC patients, in combination with sorafenib, is currently recruiting.

Vaccinia virus "double-deleted" virus (VV-DD) combines a TK mutation with a mutation in the VACV growth factor (VGF) gene in a WR strain background (126). VGF$^-$ VACV showed pathogenicity in nude mice similar to that of TK$^-$ VACV, but when these deletions were combined, the resulting virus showed little ability to replicate outside of tumors (105). VV-DD exhibited a better safety profile compared to wild-type WR in rhesus macaques (109) and increased tumor selectivity compared to TK$^-$ mutants *in vitro* (91). It also showed antitumor effects in several tumor-bearing animal models (91, 105, 127, 128). A version of this virus called JX-963, which also encodes GM-CSF, showed improved efficacy over VV-DD in rabbits bearing VX2 liver tumors (91). VV-DD's oncolytic efficacy might also have been improved by the introduction of a point mutation in A34R, which promotes increased viral spread (129, 130).

Another version of this virus (also called VV-DD (131), VVDD-CDSR, or JX-929), encodes TK and VGF mutations as well as yeast cytosine deaminase and human somatostatin receptor type 2. It has been tested for safety in two phase I clinical trials. Sixteen patients with solid tumors (breast, colon, pancreatic carcinomas, and melanoma) were enrolled in the first trial after having received multiple prior alternative treatments. These patients received intratumoral doses of virus up to 3×10^9 PFU (132). In a second trial, 11 patients with treatment-refractory solid tumors received up to 3×10^9 PFU of virus as a single intravenous dose (133). In both instances the virus was well tolerated, and no maximum tolerable dose was seen. Evidence of viral replication was observed, along with the appearance of virus-specific antibodies, and some very preliminary evidence of efficacy (132, 133). While not used in either study, the virus-encoded somatostatin receptor would permit tracking virus distribution using ^{111}In-pentetreotide (128). The encoded yeast cytosine deaminase catalyzes conversion of 5-fluorocytosine to the cytotoxic 5′-FU, enabling combination suicide gene/prodrug/oncolytic virus therapy (127). In an immunocompetent animal model of ovarian carcinomatosis, this combination approach prolonged survival (127).

A Lister-based strain of recombinant VACV called GLV-1h68 or GL-ONC1, combines a TK mutation with mutations in the viral hemagglutinin (*A56R*) and *F14.5L* genes (131, 134). This virus demonstrated reduced virulence in nude mice (134), and oncolytic efficacy in several immune compromised animal models (135–138). Phase I/II trials in patients with head and neck cancer, lung cancer, peritoneal carcinomatosis, and additional solid tumors are currently underway [reviewed in Ref. (139)]. GLV-1h68 has been further modified to express transgenes which permit monitoring virus distribution, or aim to improve oncolytic efficacy (139).

Vaccinia virus TMPK is a functional but non-essential early gene, with viral homologs only found in other orthopoxviruses

(140, 141). The catalytically active dimer is structurally related to human TMPK (141, 142). Like human TMPK, the VACV enzyme phosphorylates dTMP, dUMP and a number of dUMP analogs (143). However, unlike human TMPK, VACV TMPK can also phosphorylate dGMP and several different dGMP analogs. This includes O^6-Me-GMP, which is not a substrate for human TMPK or GMPK (143, 144). This additional activity suggests that VACV TMPK might be a potential target for antiviral drugs. Moreover, given that TMPK functions downstream of TK, it is possible that TMPK mutants would display similar oncolytic properties to TK mutants. Further studies should investigate this possibility.

VACV RNR Mutants

Vaccinia virus encodes homologs of both the small and large subunits of RNR, products of the *F4L* (145, 146) and *I4L* genes (147), respectively. These virus and human genes are sufficiently similar that some antibodies cross-react with the encoded proteins. VACV R1 and R2 can form functional complexes with each other, and also with cellular R1 or R2. Interestingly, complexes composed of viral R2 and mouse R1 show greater specific activity than cellular complexes, while viral R1 and cellular R2 complexes show reduced activity (148, 149). Like cellular R2, VACV R2 interacts with R1 subunits through its C-terminus, and the residues required for RNR activity are highly conserved (150). Like its cellular counterpart, VACV RNR is also subject to allosteric regulation and is inhibited by hydroxyurea (HU) (145, 146, 148, 151, 152). However, unlike cellular RNR, VACV RNR exhibits little ability to reduce UDP (148). In addition, the virus R2 also bears an N-terminal truncation (150) that deletes sequences corresponding to a region spanning the KEN box motif in the cellular enzyme. The KEN box is recognized by Cdh1-APC, and this leads to its degradation during mitosis (9). The absence of this element in viral R2 would explain the relative stability of this protein during infection (150).

Both the VACV *F4L* and *I4L* genes are non-essential. However, R1 and R2 mutants show different phenotypes, which suggest that the R2 subunit is a more important determinant of viral fitness. There is little difference in growth properties between wild-type VACV and R1 mutants, even in serum-starved (i.e., non-proliferating) cells. In contrast, R2 mutants replicate poorly in serum-starved cells. Although mutations in either gene will enhance virus sensitivity to HU or cidofovir (a nucleoside analog that competes with dCTP) (150), the effect is magnified in R2 mutants. These viruses also greatly differ in virulence. R2 mutants are highly attenuated following intranasal or intravenous administration, while R1 mutants show only mild attenuation (150, 153, 154). However, R1 mutants are attenuated when administered intracranially (153, 155) suggesting that VACV R1 may play some role in neurovirulence (R2 mutants were not tested in these studies). The relative difference in the importance of viral RNR subunits is further illustrated by bioinformatics analysis. R2 homologs are encoded by most poxviruses, while only a subset of genera (*Orthopoxviruses* and *Suipoxviruses*) encode the R1 subunit (150, 156). This suggests that natural selection pressures favor R2 over R1.

Transgene has generated a recombinant WR-based VACV, VV-FCU1, with R1 deleted and the TK locus replaced with a gene encoding yeast cytosine deaminase fused to uracil phosphoribosyl transferase (FCU1) (157). This enzyme fusion converts the prodrug 5-flurocytidine to 5-flurouracil (158), delivering the chemotherapeutic to virus-infected cancer cells. While combining 5-flurocytidine treatment with VV-FCU1 resulted in more sustained control of Renca tumors in immune competent mice, it did not significantly prolong survival over virus alone (157). These studies did not evaluate whether an R1 mutation alone, or combined with other mutations (e.g., TK), affected the oncolytic properties of the virus. Note that VV-FCU1 has unfortunately also been used to designate another FCU1-expressing TK mutant (114). However, the latter is $R1^+$ and was generated from the Copenhagen strain of VACV.

R2 deletion reduces the yield of VACV ~1,000-fold compared to wild-type WR, when grown in serum-starved non-transformed cells (e.g., N60 fibroblasts or normal kidney cells) (104). This difference is less apparent in cycling cells (~10-fold), and in some cancer cell lines VACV R2 mutants grow almost as well as the wild-type strain (104, 150). This effect is linked to levels of cellular R2. HeLa cells exhibit elevated amounts of dNTPs (159) and support growth of R2-deficient VACV at levels much like that of wild-type VACV. However, when cellular R2 levels were reduced using siRNA silencing, the yields of R2-deleted VACV mutants were reduced ~10-fold while growth of wild-type VACV was not significantly affected. Furthermore, in BSC-40 or CAPAN-2 cell lines, where VACV R2 mutants grow relatively poorly, virus expression of cellular R2 can compensate for the deficiency (150). The p53-R2 cellular enzyme does not complement viral R2 mutants, perhaps because of the reduced activity relative to R2 (10, 12, 150).

Our laboratories have been investigating whether R2-deficient VACVs (strain WR) can be used as oncolytic agents for treating bladder cancer. These R2-deficient VACVs promote oncolysis in immune compromised mice bearing either subcutaneous or orthotopic human bladder cancer xenografts. Oncolytic activity is also seen in immune competent rats bearing orthotopic bladder tumors (104). In the rat model, long-term complete responses were achieved, and these animals showed evidence of induced antitumor immunity. These R2-deficient VACV strains also appear to be safer (i.e., less virulent) than the TK-deficient strain. Although neither virus induced overt toxicity following administration to the bladder of tumor-bearing immune competent rats, *ex vivo* analysis demonstrated that TK-deficient virus was recoverable from the ovaries, kidneys, and lungs, while the spread of R2-deficient strains was limited to the tumor site. An even greater safety advantage has been seen in immune compromised mice. When TK-deficient virus was administered by either intravenous or intratumoral routes, weight loss and pox lesions were observed. In contrast, R2-deficient viruses did not cause these classic signs of poxvirus virulence.

One can also combine R2 and TK mutations without impairing the antitumor activity of VACV. In fact this combination of mutations (ΔF4LΔJ2R) produces a virus that lacks the virulence of ΔJ2R strains in immune compromised animals while still being able to cure xenografted tumors. Interestingly, fewer virus-specific

antibodies were detected in rats infected with R2-deficient VACV relative to TK-deficient virus. This most likely reflects the poorer growth of R2 mutant strains in rat tissues.

VACV Uracil-N-glycosylase and dUTPase Mutants

Vaccinia virus encodes two proteins that minimize uracil incorporation into virus genomes: a dUTPase (encoded by *F2L*) and a uracil-N-glycosylase (UNG, also designated UDG, encoded by *D4R*). It has been hypothesized that these viral proteins benefit VACV when it infects quiescent cells, which express low levels of these enzymes and have relatively high ratios of dUTP-to-dTTP (160). RNRs can also reduce UDP to dUDP, creating a flux of undesirable dUTP (**Figure 1**).

The VACV dUTPase is functional (161) and structurally related to mammalian dUTPase (162), but has little influence on viral fitness. Mutants grow to similar levels as wild-type VACV in both dividing and non-dividing cells and exhibit little alteration in virulence (160, 163).

Vaccinia virus UNG has two known functions. It is a functional UNG and catalyzes uracil excision from DNA. However, VACV UNG also has an essential role in DNA replication, distinct from its glycosylase activity. Together with the viral DNA polymerase (E9L) and processivity factor (A20R), VACV UNG forms a virus holoenzyme [reviewed in Ref. (164)]. This role is important because although catalytically inactive UNG mutants are viable, knockouts are not unless cultured in a complementing cell line (165, 166). These catalytically dead mutants show modest reductions in DNA synthesis and viral yields when grown in both dividing and non-dividing cells (160, 165). UNG activity is clearly important *in vivo*, as catalytically inactive UNG VACVs are attenuated (160, 165).

While neither UNG nor dUTPase mutants alone have properties that would be desirable in oncolytic viruses, a virus bearing mutations in both genes might be more useful. A catalytically inactive UNG/dUTPase double mutant was shown to grow selectively in dividing cells, where it grew to similar levels as wild-type VACV. In quiescent cells, this virus grew to 2–3 logs lower levels than wild-type or single mutant viruses (160). This virus might also be more attenuated in normal mouse tissues than a virus bearing mutations in either gene alone (160).

While these observations would suggest that a UNG/dUTPase double mutant may be an oncolytic candidate, two characteristics would argue against this scheme. First, to preserve the essential structural role of UNG in VACV DNA replication, the catalytic domain must be inactivated by point mutation rather than by clean deletion. This creates the possibility of reversion to wild type. Second, given that uracil incorporation into DNA can be mutagenic, a virus that has a reduced ability to repair uracil could be prone to higher mutation rates, resulting in a greater number of defective viral genomes.

HERPES SIMPLEX VIRUSES

Members of the *Herpesviridae* infect a wide range of animals. They are large double-stranded DNA viruses that replicate in the nucleus of infected cells, and are characterized by abilities to undergo lytic replication, as well as to establish latency. While most herpes viruses are species-specific, the tissue-tropism of different viruses ranges widely from relatively broad to very narrow—in some cases infecting only a single cell type (167). To date, only human-specific members of the *Alphaherpesvirinae* subfamily have been investigated for their oncolytic properties (**Table 3**). This includes HSV-1, herpes simplex virus-2 (HSV-2) (168–170), and varicella zoster virus (VZV) (171). Several recombinant HSV-1's have entered clinical trials, and to date the only FDA-approved oncolytic virus is a modified HSV-1 (Talimogene laherparepvec or T-Vec), which was licensed based on the results of a phase III clinical trial in patients with advanced melanoma (172, 173). T-Vec is deleted in the genes encoding ICP34.5 and ICP47, is engineered to have early expression of *US11*, and encodes the human GM-CSF transgene (174). These alterations in the viral genome do not directly affect nucleotide metabolism but are discussed in more detail below in the context of other oncolytic HSVs.

Herpes simplex virus-1 and HSV-2 can infect both resting and dividing cells and encode R1, R2, TK, dUTPase, and UNG homologs (167). While these proteins can catalyze nucleotide metabolism, some promote reactions not catalyzed by their cellular counterpart, or have additional immune regulatory functions. Because of this, development of viruses bearing mutations in these genes for oncolytic virotherapy requires additional considerations.

Herpes Virus RNR Mutants

An increase in RNR activity is seen early in HSV-1 infection (175, 176) and HSV-1 encodes homologs of both the R1 and R2 subunits (genes *UL39* and *UL40*, respectively). These viral subunits interact with each other (177, 178) and catalyze the same reactions as cellular RNR. However, HSV-1 RNR activity is regulated differently. Unlike cellular RNR, HSV-1 RNR is subject to ATP inhibition, but is not inhibited by dATP or dTTP (179, 180).

The R1 protein encoded by HSV-1, and other alpha- and beta-herpes viruses, bears an additional ~400 amino acid N-terminal domain incorporating a receptor-interacting protein (RIP) homotypic interaction motif. This domain, while dispensable for R1-R2 binding and RNR activity (177, 181), promotes an interaction between viral R1 and the cellular kinase RIP3 (182). In human cells the interaction between viral R1 and RIP3 disrupts formation of the RIP1/RIP3 necrosome complex, thus preventing activation of antiviral programmed necrosis. In contrast, in mouse cells the viral R1-RIP3 interaction leads to necrosome activation and subsequent cell death (182, 183). This domain also promotes an R1 interaction with eIF4G, which is thought to serve as a scaffold for further interactions with eIF4E, and aid viral mRNA translation (184, 185). This importance of this domain for pathogenesis is confirmed by the observation that murine cytomegalovirus R1 mutants are severely attenuated, despite the fact that MCMV R1 is non-functional as an RNR (186). The fact that R1 serves an additional role in immune regulation and is perhaps needed earlier in the infection cycle than are dNTPs, may rationalize the otherwise puzzling fact that R1 is expressed earlier than R2 (187).

TABLE 3 | Oncolytic herpes viruses with mutations in nucleotide metabolism genes.

Virus	Strain	Viral mutation(s)	Transgene(s)	Status	Reference
Herpes simplex virus-1					
G47Δ[a]	F	R1$^-$, ICP34.5$^-$, ICP47$^-$, *US11* under control of ICP47 promotor	*lacZ*	Completed phase II trial	(204, 205)
NV1020 (R7020)	F	TK$^-$ (also disrupts *UL24* expression), 15 kb deletion of joint region (deletes one copy of ICP0, ICP4, LATs, and ICP34.5 genes)	Extra copy of TK placed under ICP4 promoter, HSV-2 glycoproteins inserted in the joint region	Currently in phase II trial	(238–241, 273–275)
rRP450	KOS	R1$^-$	CYP2B1	Currently in phase I trial	(189, 191, 195)
hR3	KOS	R1$^-$	*lacZ*	Preclinical	(192)
GL207	F	R1$^-$, deletion in both copies of ICP34.5 gene	*lacZ*	Preclinical	(197–203, 276–278)
Δ68H-6	17syn+	R1$^-$, ICP34.5 beclin1 binding domain deletion	*lacZ*	Preclinical	(209)
dlsptk[b]	KOS	TK$^-$ (deletion maintains *UL24* expression)		Preclinical	(193, 229, 230)
KOS-SB	KOS	TK$^-$ (deletion maintains *UL24* expression)		Preclinical	(279)
NV1066	F	TK$^-$, deletion of internal repeat region (deleted in one copy of ICP0, ICP4, ICP34.5 genes)	eGFP	Preclinical	(233–237)
Herpes simplex virus-2					
FusOn-H2	wt186	Deletion of PK domain in R1, RR domain fused to eGFP and under control of CMV promoter	eGFP	Preclinical	(213, 215)

[a]*G47Δ has been further modified to express transgenes such as IL-18, IL-12, platelet factor 4, or angiotensin (207, 280–282).*
[b]*The HSV-1 TK gene overlaps with UL24. Depending on the strategy taken to disrupt TK, some HSV-1 TK mutants also have mutations in UL24.*

Herpes simplex virus-1 R1 mutants have properties that make them promising oncolytic agents. The R1 mutant hR3 is attenuated in animal models (188, 189) and has also been reported to exhibit defects in reactivation from latency (189). While R1 mutants show reduced replication compared to wild-type HSV-1, they also grow more selectivity in dividing cells. In serum-starved cells, R1 mutants produce 10- to 1,000-fold lower yields of virus and only a third of the DNA synthesis of wild-type virus (153, 190, 191). Cancer cells often bear p16 mutations, which result in increased RR activity (detectable as R2 mRNA), and an R1 HSV-1 mutant was shown to replicate to higher levels in these cells, regardless of whether these cells were dividing or not (192). R1 mutants also show increased sensitivity to the TK substrate prodrugs acyclovir (193) and ganciclovir (194), which may provide additional safety benefits.

An oncolytic HSV-1 (KOS strain) virus called rRP450 was engineered to incorporate a rat *CYP2B1* gene replacing the virus R1 sequence. *CYP2B1* encodes a cytochrome P450 that converts cyclophosphamide into its cytotoxic product. While rRP450 controlled the growth of tumors in a number of model systems, including rat 9L gliosarcomas, human U87 gliomas, murine MC26 liver metastases, and human Rh30 rhabdomyosarcomas, oncolytic efficacy was significantly enhanced when the animals were also given cyclophosphamide (189, 191, 195). This suggests that R1 may serve as an appropriate site for the expression of transgenes in HSV-1. A phase I clinical trial (NCT01071941) in patients with primary liver tumors and metastases is currently underway.

Herpes simplex virus-1 R1 mutations have also been combined with other viral mutations. The HSV ICP34.5 protein serves many functions including preventing host protein shutoff, disrupting the type I interferon response, and inhibiting autophagy [reviewed in Ref. (196)]. It is also a key neurovirulence factor and many oncolytic HSV-1 strains bear ICP34.5 mutations including T-Vec. HSV-1 clone G207 (an F strain) was assembled bearing mutations in R1 and in both copies of the gene encoding ICP34.5. It is attenuated in mice and non-human primates (197, 198) and showed efficacy in many animal tumor models including xenografts of U87 gliomas (199) and F5 meningiomas (200). G207 was well tolerated in a clinical dose-escalation study, where 21 patients with recurrent glioblastomas, gliosarcomas, or anaplastic astrocytomas were enrolled and given a single intratumoral dose of virus (up to 3×10^9 PFU), at 1–5 sites (201). In a subsequent phase Ib trial, patients with recurrent glioblastomas were given two doses of virus totaling 1.15×10^9 PFU, one prior to and one after surgical resection (202). Collectively both trials showed that G207 was well tolerated, replicated *in vivo*, generated neutralizing antibodies, and showed some evidence of antitumor effects in some patients (201, 202). A phase I clinical trial (NCT02457845) in children with progressive or recurrent malignant brain tumors is currently underway (203).

G207 (like T-Vec) has been further modified by deleting the open reading frame encoding ICP47, generating a virus called G47Δ. This deletion has two consequences. The first is to increase immune recognition of infected cells, since the ICP47 protein inhibits MHC-I antigen presentation. Second, this deletion places the nearby *US11* gene under the control of the ICP47 promoter, thus increasing virus yield by preventing premature termination of protein synthesis resulting from loss of ICP34.5 (204, 205). This virus has shown efficacy in a number of tumor models [reviewed

in Ref. (206)] including tumor models established using brain or breast cancer stem cells (207, 208). G47Δ has been evaluated in phase I and II clinical trials. The Japanese Ministry of Health, Labor and Welfare gave G47Δ "Sakigake" or breakthrough status in February 2016, based on the positive outcomes of a phase II trial in individuals with recurrent or residual glioblastoma (206).

It has been suggested that deleting genes encoding R1 (*UL39*) and ICP34.5 (γ_1*34.5*) would compromise the efficacy of these mutant HSV-1 viruses. Kanai et al. studied a combination of a complete *UL39* deletion with partial mutations in γ_1*34.5* in the 17syn$^+$ strain (209). They found that the virus still exhibited the reduced neurovirulence characteristic of strains completely deleted of γ_1*34.5* while this virus (called Δ68H-6) also exhibited increased efficacy in experimental glioma models (209).

Herpes simplex virus-2 R1 mutants are also being investigated for their oncolytic properties, although from a perspective different from the role the enzyme plays in dNTP biosynthesis. A HSV-2 mutant lacking part of the R1 N-terminal domain (ICP10ΔPK) replicated less efficiently in serum-starved cells and exhibited reduced virulence in animals, despite still being able to complex with R2 and catalyze dNTP synthesis (210, 211). This virus is being tested as a candidate vaccine against genital herpes (212) but can also control the growth of human melanoma xenografts in nude mice (213, 214). A similar version of this virus, called FusOn-H2, also exhibited growth restriction in serum-starved cells and controlled growth of xenografted human MDA-MB-435 melanomas (168) and EC9706 esophageal tumors (215) in nude mice.

Few studies have explored whether HSV-1 R2 mutants might also exhibit oncolytic properties. Unlike the wild-type virus, an HSV-1 R2 mutant (*ts1222*) was unable to replicate in serum-starved BHK21 cells (216). This mutant is also dramatically attenuated in mice (188).

Herpes Virus TK Mutants

An HSV-1-encoded TK was discovered when it was shown that the virus could productively infect a cell line lacking TK activity (217, 218). HSV-1 TK is encoded by the *UL23* gene and is expressed early in infection (219). Besides phosphorylating pyrimidine nucleosides (dT, dC, dU), HSV-1 TK also exhibits TMPK activity, converting dTMP to dTDP (220, 221). Importantly, HSV-1 TK can also phosphorylate deoxyguanosine and its analogs (222) much more efficiently than cellular TK, and it is this property that has made it a target of many antiviral drugs. The deoxyguanosine analogs acyclovir and ganciclovir are phosphorylated by HSV-1 and other herpes virus TKs. Cellular dGMP kinase and NDP kinases then catalyze further phosphorylation steps, creating the triphosphorylated products. These are then incorporated into DNA and cause chain termination (222–224).

Herpes simplex virus-1 and HSV-2 TK mutants grow to near wild-type levels in cells under normal serum conditions, but growth is reduced 10- to 100-fold in serum-starved cells (225–227). When administered intracranially in BALB/c mice, a TK-deficient HSV-1 strain showed a 100-fold reduction in LD_{50}, while a five-log reduction was observed with HSV-2 TK mutants (225). Defective reactivation in infected ganglia was also observed, and these TK defects could be complemented with either human TK1 or TYMS, but not DCK (228, 229). Collectively these data suggest that TK-deficient herpes viruses would selectivity replicate in rapidly dividing cancer cells.

An HSV-1 TK mutant (*dlsptk*) exhibited oncolytic properties in a number of immune compromised animal models. These include human U87 gliomas (230), medullablastomas derived from human Daoy cells, and malignant meningiomas from human M3 cells (231). While showing oncolytic efficacy, HSV-1 TK mutants still exhibit some degree of neurovirulence, with some mice succumbing to virus-related events, presumably encephalitis (230, 232).

NV1066 is a recombinant HSV-1 (F-strain) that combines a *UL23* (TK) deletion (which also disrupts *UL24* expression), with a deletion spanning the internal repeat region. This deletes one copy of each gene encoding ICP0, ICP4, and ICP34.5 (233). NV1066 selectively kills stem-like tumor initiating cells (234), and replicates in animal models of cancers including lymphatic metastases (233), peritoneal carcinomas (235), esophageal adenocarcinomas (236), and a metastatic pleural cancer model derived from A549 cells (237).

NV1020, was originally designed as an HSV-1/-2 vaccine (238). It is similar to NV1066, but it also has an insertion of genes encoding a set of HSV-2 glycoproteins as well as HSV-1 TK (the latter under control of the ICP4 promoter). A number of animal models have been used to show it can control tumor growth including an orthotopic model of bladder cancer (239) and A549-derived tumors established in the pleural cavity of athymic rats (240). NV1020 was studied in a phase I/II trial in patients with advanced metastatic colorectal cancer (241). The virus was well tolerated, with fever and chills being commonly reported. The phase I component of the study did not identify a maximum tolerable dose, and a dose of 10^8 PFU was used in the phase II arm. Median survival for patients following treatment was 11.8 months.

Despite the promising safety profile and tumor-selective features of HSV TK mutants, TK mutations have not been widely incorporated into oncolytic herpes viruses. The fact that HSV TK is required to bioactivate acyclovir and ganciclovir, providing an important safety net should an adverse response to the virus be observed, may explain why the development of oncolytic herpes viruses has favored retention of a functional TK. On the other hand, while HSV-1 TK mutants may not have gained wide spread use as oncolytic agents, various forms of the HSV-1 TK gene have been used to track virus distribution or used as a suicide gene, in both HSV-1 and other viruses such as Ad (242–244).

Herpes Virus UNG and dUTPase Mutants

Herpes simplex virus-1 encodes both UNG (*UL2*) (245) and dUTPase (*UL50*) (246) enzymes. Herpes UNG can be coprecipitated with the viral DNA polymerase and the presence of UNG causes the complex to pause upstream of uracil residues in the template strand (247). This could permit DNA repair in a manner linked with DNA synthesis. Unlike VACV, herpes virus UNG-polymerase interactions are not essential for viral viability, as mutant viruses grow nearly normally in dividing NIH 3T3 or BHK C13 cells (245, 248). However, UNG mutants are attenuated in neural tissue, with LD_{50} about 10-fold higher than wild-type

virus when administered by intracranial injection and more than 10,000-fold higher when administered peripherally (248).

While no research concerning the oncolytic properties of a virus bearing only an UNG mutation has been reported, a virus bearing mutations in the UNG gene and $\gamma_1 34.5$ has been studied. This doubly mutated herpes virus, called 3616UB, showed efficacy comparable to a virus bearing $\gamma_1 34.5$ mutations in Daoy or SK-M tumor cells xenografts in SCID mice (249). However, 3616UB was more attenuated than either wild type or $\gamma_1 34.5$ mutant viruses after intracranial administration and was also more sensitive to ganciclovir (249).

Herpes simplex virus-1 dUTPase mutants also show decreased neurovirulence and exhibit defects in exit from latency (250, 251). The need for a functional dUTPase appears to be cell type or tissue dependent and is likely affected by the availability of cellular dUTPase. In cycling cells, dUTPase is dispensable for viral replication, with mutant viruses growing to titers as high as wild-type viruses (227, 250). However, in cells with either naturally low dUTPase levels (e.g., SK-N-SH), or where dUTPase levels have been reduced by shRNA-mediated silencing, the yields of dUTPase-deficient HSV-1 are reduced approximately 10-fold. These defects can be complemented by human dUTPase, either overexpressed by the host cell or encoded by the virus (252). The dUTPase also helps maintain the integrity of the virus genome, as dUTPase mutants isolated from brains of infected mice exhibited greater numbers of mutations per genome than wild-type viruses (251).

The effect of the viral dUTPase on neurovirulence is partly regulated by the virus-encoded *US3* kinase, which activates dUTPase by phosphorylating the enzyme at Ser187 (252, 253). Mutating Ser187 attenuates the virus upon intracranial administration but does not affect virulence when administered to the periphery (254). In contrast, a mutant lacking the gene entirely is attenuated via either route of administration. The way virulence is affected by the route of administration could be partially explained by differences in dUTPase activity in different tissues. The fact that HSV-1 dUTPase mutants grow more poorly in cells with low dUTPase levels suggests that these viruses might have superior oncolytic potential. However, the increased mutation rates caused by dUTPase mutations may argue against using these viruses as therapeutics.

SUMMARY AND CONCLUSION

Large DNA viruses such as VACV and HSV-1 encode a remarkably similar repertoire of nucleotide metabolism genes. Both viruses encode RNR R1 and R2 subunits, a UNG, and dUTPase. Although VACV encodes distinct TK and TMPK enzymes, these functions are consolidated in the TK enzyme of HSV-1. Over the course of virus evolution, the collective activities and substrate specificities of these enzymes may even have played a role in driving drift in the base composition of these viruses. Relative to the human genome, VACV genomes are A + T rich (66%), while HSV-1 genomes are G + C rich (68%). However, not all herpes viruses are G + C rich and not all poxviruses are A + T rich. The more balanced G + C content in VZV (46%) and HHV-8 (53%) can perhaps be explained by the fact that these herpes viruses encode a TYMS in addition to the biosynthetic genes encoded by HSV-1 and HSV-2 (255). Conversely, poxviruses like Orf and molluscum contagiosum lack R2 and TK homologs and in this case oddly exhibit a strikingly *higher* G + C content (~64%) (256, 257).

Vaccinia virus and HSV-1 illustrate an interesting situation that can be productively exploited to assemble cancer-selective viruses. These viruses have followed an evolutionary path that permits the very successful exploitation of a widely encountered biological niche in the form of non-replicating cells. However, in order to efficiently infect non-replicating cells, this infection strategy renders large DNA viruses dependent upon a complement of virus-encoded nucleotide metabolism genes. By mutating these genes, one creates a requirement for the cell to provide the complementing activities and this environment is typically one found in cancer cells. On the other hand, Ads have evolved mechanisms to induce the expression of these dNTP biosynthetic pathways by manipulating systems like those regulated in the cell cycle. Producing cancer specific oncolytic Ads requires an entirely different mutational strategy. However, it should be noted that there is no one particular approach for acquiring dNTPs that has been adopted specifically by any given virus family. For example, as noted above Orf virus does not encode TK or R2 proteins like VACV. Instead it encodes a protein called PACR that stabilizes cellular R2 by preventing its degradation by the APC at the end of G1/S phase (258). All DNA viruses require some source of dNTPs for replication, but evolution has produced different ways of accessing these metabolites and that strategy determines how one goes about manufacturing an oncolytic virus.

Although these observations suggest the possibility that deleting genes encoding TK and RNR might alter the genetic stability of oncolytic viruses, there is little evidence to support this hypothesis. There is some evidence that HSV-1 dUTPase mutations create a mutator phenotype (259), but the situation is less clear with TK mutants. These may exhibit an antimutator phenotype but that phenotype depends upon the host cell type (248, 260). We have passaged these viruses extensively and seen no significant differences in the rate of accumulation of mutations, compared to wild-type virus, as judged by whole genome sequencing.

Further studies are required to shed more light on these questions, especially relating to how virus infection (whether mutant or wild type) perturbs the dNTP pools in infected cells. It is becoming increasingly apparent that nucleotide pools not only directly affect cell and virus replication but also play a key role in modulating immune responses to infection. Space precludes an extensive review of these still emerging investigations, but we would note how SAMHD1 negatively affects the replication of both HSV-1 and VACV in human primary monocyte-derived macrophage (102). This can be attributed to the destruction of dNTPs by the SAMHD1-encoded phosphohydrolase, and the effect of this on VACV replication is exacerbated by virus-encoded TK mutations (102). SAMHD1 is expressed in a variety of cell types (28), including most hematopoietic cells, and this constitutive activity is generally resistant to signaling by a diversity of proinflammatory cytokines (261). Given this situation, one is left to speculate how newly replicated viruses might affect the lymphocytes recruited to sites of infection, and whether deleting the virus genes that are needed to support nucleotide metabolism

would protect these newly recruited immune cells from secondary infections. This could perhaps enhance antiviral responses in normal infections and antitumor responses where oncolytic viruses are employed.

AUTHOR CONTRIBUTIONS

CI researched and wrote the manuscript. MH and DE provided guidance in manuscript preparation and subsequent editorial input. All authors agreed to be collectively responsible for the work.

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REVIEW
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Monitoring the Efficacy of Oncolytic Viruses *via* Gene Expression

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With the recent success of oncolytic viruses in clinical trials, efforts toward improved monitoring of the viruses and their mechanism have intensified. Four main gene expression strategies have been employed to date including: analyzing overall gene expression in tumor cells, looking at gene expression of a few specific genes in the tumor cells, focusing on gene expression of specific transgenes introduced into the virus, and following gene expression of certain viral genes. Each strategy presents certain advantages and disadvantages over the others. Various methods to organize the dysregulated genes into clusters have provided a window into the mechanism of action for these viruses. Methodologically, the combined approach of looking at both overall gene expression, the tumor cells and gene expression of viral genes, enables researchers to assess correlation between the introduction of the virus and the changes in the tumor. This would seem to be the most productive approach for future studies, providing much information on mechanism and timing.

Keywords: oncolytic viruses, gene expression, oncolytic virotherapy, microarray analysis, transgenes

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INTRODUCTION

With the development of recombinant deoxyribonucleic acid (DNA) technology in the early 1990s, the possibility of genetically engineering oncolytic viruses to improve virotherapy became a reality (1). Capitalizing on what was known about the mechanisms for oncolytic viruses, research focused on improving the safety profile, attenuating direct tumor lysis, and modulating the immune response. Beyond its potential therapeutic benefits, genetic engineering has generated new ways of monitoring and predicting sensitivity or resistance to oncolytic virotherapy (2). Various strategies, including animal models, cell lines, and even human *in vivo* gene expression studies, have been implemented to assess the effects of the oncolytic virus in a personalized way.

In 2015, the Food and Drug Administration approved the first oncolytic virus based on the results of a phase 3 trial for melanoma (3–5). Many other viruses are now being tested in clinical trials for various indications. This necessitates careful consideration of endpoints and creative new ways of monitoring therapeutic success.

Abbreviations: ACC, adenoid cystic carcinoma; βhCG, beta-human chorionic gonadotropin; CEA, carcinoembryonic antigen; CDV, canine distemper virus; DAI, DNA-dependent activator of IFN– regulatory factors; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; GBM, glioblastoma multiforme; GM-CSF, granulocyte-macrophage colony-stimulating factor; GnRH, gonadotropin releasing hormone; HSV, herpes simplex virus; IL, interleukin; IFN, interferon; IPA, Ingenuity Pathways Analysis; ISVP, infectious subviral particle; mRNA, messenger ribonucleic acid; MOI, multiplicity of infection; MV, measles virus; NDV, Newcastle disease virus; NOS/SCID, non-obese diabetic/severe combined immunodeficient; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; ruc-GFP, Renilla luciferase-Aequorea-green fluorescent protein; SEA, superantigen *Staphylococcus* enterotoxin A; TNBC, triple negative breast cancer; TNF, tumor necrosis factor; VACV, vaccinia virus; VSV, vesicular stomatitis virus.

Four main strategies for monitoring oncolytic viruses were surveyed: overall gene expression in tumor cells, gene expression of a few specific genes in the tumor cells, gene expression of specific transgenes introduced into the virus, and gene expression of certain viral genes.

Monitoring Overall Gene Expression of Tumor Cells (See Tables 1–3)

The first method of assessing the impact of oncolytic virotherapy involves monitoring overall gene expression of tumor cells before and after virotherapy. To identify genes whose expression was altered in tumor cells during infection with an oncolytic virus, genome-wide expression profiling needs to be performed. The studies surveyed included both animal studies and studies performed on human cell lines. Kurozumi et al. used a rat intracranial glioma model in immune competent rats (6). They introduced a type one herpes simplex virus (HSV-1) and used quantitative real-time polymerase chain reaction (PCR)-based microarrays to monitor tumor gene expression. They found that oncolytic virus treatment induced at least a twofold increase or decrease in the expression of 50 genes when compared with the control. More specifically, they found numerous genes were upregulated from the chemokine family (see **Table 1**). Unfortunately, Kurozumi et al. did not analyze gene clusters for these genes. Similarly, Zhang et al. also looked at tumor gene expression using an animal model. In this case, they used nude mice injected with metastatic human breast adenocarcinoma GI-101A cells (7). They monitored tumor gene expression after treatment with a vaccinia virus (VACV) using a mouse genome array and a human genome array. They found 681 genes differentially expressed when compared with controls. As opposed to the Kurozumi group, Zhang et al. used gene ontology to evaluate clustering and found upregulated genes related to the immune system, and downregulated genes related to enzymatic function (see **Table 1**).

However, some reservations must be expressed regarding the use of Affymetrix mouse arrays. Since they are relatively species specific, genes that are identified as differentially expressed may primarily represent host cells infiltrating the tumor. When assessing the changes of potential cross hybridization of human genes to the mouse chip, less than 50% of the genes identified with the mouse arrays were differentially expressed according to the human chip. Furthermore, the immune-related genes that were differentially expressed displayed very low fluorescence intensities in human as compared with very high intensities displayed by the same genes in the mouse array. This would suggest that perhaps comparisons of gene expression between species should be evaluated with caution.

Balogh et al. used a rat adrenal tumor cell line, namely, pheochromocytoma cells, as the model, and looked at gene expression after infection with a recombinant oncolytic viral strain of Newcastle disease virus called MTH-68/H (8). They used a rat specific microarray chip to monitor gene expression and confirmed changes *via* quantitative reverse transcriptase PCR. They found that 729 genes were upregulated and 612 genes were downregulated with oncolytic viral treatment compared with controls. Balogh et al. relied on DAVID functional annotation-clustering tool to group the genes according to function. This clustering tool was far more elaborate than the method used by Zhang. They found pathways including receptor signaling, apoptosis, and cellular stress to be involved (see **Table 1**). The detailed report they provided was extremely helpful in beginning to understand the mechanisms involved in oncolytic viral therapy. In addition, Pfankuche et al. used a canine sarcoma cell line as the tumor model and injected a canine distemper virus (CDV) called DH82-Ond-pi (9). They visualized the cells using immunofluorescence and used a canine specific microarray chip to evaluate tumor gene expression. They identified 892 upregulated genes and 869 downregulated genes when compared with controls. They analyzed these results using WebGestalt and DAVID and found that upregulated genes were primarily related to immune processes, cell migration, apoptosis, and blood coagulation (see **Table 1**). Certainly, the role of oncolytic viruses in impairing angiogenesis demands further attention based on the mechanism related clues provided by this gene expression study.

Beyond the studies looking at animal cell lines, there are a few studies that looked at tumor gene expression in human cell lines. Saito et al. used two human oral squamous cell carcinoma cell lines called tHSC-4 and HSC-3 and injected the Sindbis SIN AR399 oncolytic virus (10). Using real-time quantitative reverse transcription (RT)-PCR to monitor the tumor gene expression after treatment with the virus, they found that Caspases 7, 8, and 10 were upregulated in both HSC-3 and HSC-4 cells, but that Caspases 3 and 9, cytochrome *c*, NF-κB, and IKK were only upregulated in HSC-3 cells. The likely interpretation of these data is that SIN induced oncolysis in HASC-3 cells by activating a few apoptotic pathways. Similarly, Lacroix et al. looked at six human medulloblastoma cell lines (MB) and how they are affected by treatment with the oncolytic parvovirus H-1 (H-1PV) (11). They used microarray and quantitative real-time PCR (QRT-PCR) to evaluate gene expression after oncolytic virus treatment. They focused on the 25 most significantly upregulated and the 25 most significantly downregulated genes. They used KEGG pathway analysis to identify clusters of genes and found that five pathways were particularly impacted by H-1PV infection. These included the pathways for steroid biosynthesis, ether lipid metabolism, TGF-beta signaling, Wnt signaling, gonadotropin releasing hormone signaling, and Jak-STAT signaling. One advantage in the methodology employed by the Lacroix group was focusing on the top 25. As opposed to the other groups that tried to find patterns from all the up and downregulated genes, Lacroix et al. potentially eliminated noise and were able to better identify the most important pathways. Finally, Haddad et al. looked at the PANC-1 human pancreatic cancer cell line infected with GLV-1h153, an oncolytic VACV (12). They used cDNA microarray chips to monitor gene expression after infection with the virus and used a cutoff of a twofold change to identify the most relevant genes. At 6 h postinfection, they found that 139 genes were up- or downregulated, but by 24 h after infection 5,698 genes were dysregulated. They analyzed the pathways using the Ingenuity Pathways Analysis (IPA) software and found that downregulated genes clustered around pathways associated with cell death, cell cycle, and DNA repair. Upregulated genes were associated with mechanisms related to

TABLE 1 | Tumor Genes.

Reference	Harvest time point: postinfection/other notes	Top upregulated genes	Top downregulated genes	Pathways/functional groups most affected
Haddad et al. (12)	6 h	SLC5A5, HIST2H4A, AK026847, HIST1H4E, HIST1H4B	BHLHB2, CX3CL1, G0S2, SOCS1	HMGB-1, interleukin (IL)-2, IL-6, IL8, Janus kinase/signal transducer and activator of transcription (JAK/STAT), interferon (IFN), and ERK 5 signaling
	24 h	SLC5A5, AK026847, HSPA6, HIST2H4A	IL8, ICAM1, SFRP1, CCL20, RSU1	P53- and Myc-induced apoptotic processes, pancreatic adenocarcinoma signaling, and phosphoinositide 3-kinase/v-ask murine thymoma vial oncogene homolog 1 (PI3/AKT) pathways
Balogh et al. (8)	12 h	Rsad2, Cxcl11, 10869879, Ddx60, Ifnb, Ifih1, Ifnb2, 10720237, Isg15, Herc6, Usp18, Oasl2, Oasl, Oas1b, Gbp5, Gbp1, Mx1, Irgm, Ifit1, Ifit2, Ifit3, Ifi47, Cxcl9, TRAIL, Tnf, Atf3	Tradd, Fadd, etnk2, trpc3, p2ry12, galr2, rpa3	Toll-like receptor signaling, RIG-I-like receptor signaling, IFN signaling, IFN effector pathways, apoptosis pathways, endoplasmic reticulum stress pathways, and cell cycle regulation
Lee et al. (15)	0, 24, 48, and 72 h	LEF1, PVRIG, SLFN11, LPP, CECR1, ARHGEF6, IRX3, STAMBPL1, IGFBP2, CD1D	CD151, AHNAK, TRIP6, LGALS1, MGST1, SRGN, CCND2, CCDC50, ITGB7, PDLIM1	Phosphoprotein, mutagenesis site, regulation of programmed cell death, lysosome, regulation of apoptosis, and surface antigen
Lacroix et al. (11)	72 h	EFTUD1, MMP1, PPM1F, LAMB3, TMEM200C, SIRPA, THEG, VPS18, RBM22, FOLR2, COX17, TFPI2, ACTL8	ZIC1, FLRT3, MYC, FOXG1, MAPT, NFIA, PHLPP1, ZNF671, FZD3	Steroid biosynthesis, ether lipid metabolism, TGF-beta signaling pathway, Wnt signaling pathway, gonadotropin releasing hormone signaling pathway, and the Jak-STAT signaling pathway
Reinboth et al. (29)	Early (2 h)	114 human genes strongly correlating with viral genes	Networks: posttranslational modification, free radical scavenging, gene expression, cell death, and cellular growth and proliferation. Molecular functions: cell cycle, cellular movement, development, growth and proliferation, and cell-to-cell signaling	
	Intermediate/late (10 h)	84 human (early) genes strongly correlating with viral genes (intermediate/late)	Cell death, cell cycle, lipid metabolism, small molecule biochemistry, and cellular development	
	48 h			Cell death, cellular growth and proliferation, protein synthesis and folding, infectious disease, genetic disorder, cell cycle, and deoxyribonucleic acid replication, recombination, and repair
Kurozumi et al. (6)	3 days	Cxcl11, Ifnγ, Cxcl9, Ccl12_ predicted, Cxcl10, Ccl4, Il1b, Ccl5, Ccr6, Cxcr3	Spp1, Il6st	
Pfankuche et al. (9)	1 day	DDX60, DLA-79, CXCR7, F13A1, LOC100685890, CCR5, TRIM22, LOC100686473, GPR34, ENPEP	SERPINB2, TPM2, SCIN, VEGFB, THBS2, COL4A1, DMD, S100P, LOC608476, GSTA3	WebGestalt (UP): immune response-activating signal transduction activation of immune response; immune response-regulating signaling pathway; positive regulation of immune response; response to other organism; regulation of immune response; positive regulation of immune system process; regulation of immune system process; immune response; immune system process DAVID (UP): activation of innate immune response; cell migration; leukocyte proliferation; positive regulation of programmed cell death; positive regulation of leukocyte activation; regulation of leukocyte proliferation; blood coagulation

(*Continued*)

TABLE 1 | Continued

Reference	Harvest time point: postinfection/other notes	Top upregulated genes	Top downregulated genes	Pathways/functional groups most affected
				WebGestalt (DOWN): blood vessel morphogenesis; positive regulation of cell migration; positive regulation of cell motility; cardiovascular system development; positive regulation of cellular component movement; circulatory system development; regulation of cell adhesion; positive regulation of locomotion; localization of cell; biological adhesion DAVID (DOWN): blood vessel development; protein amino acid glycosylation; organic acid metabolic process; regulation of neurological system process; regulation of transferase activity; blood coagulation; nucleobase, nucleoside and nucleotide metabolic process; antigen receptor-mediated signaling pathway; leukocyte proliferation
Josupeit et al. (14)	Most significantly expressed in susceptible cells	FAM49B, B4GALNT1, COL4A5, SLITRK4, SLC26A10, IFITM3, ASAP1, LAYN, NTRK2, ARHGEF25, CTGF, NXPH1, UGT8, NCAN, NAP1L3		
	Most significantly expressed in resistant cells	CTHRC1, RPS4Y1, EIF1AY, DDX3Y, DPYD, PNMAL1, S100A10, TXLNG2P, TRIM38, SPP1, KDELR3, SPARCL1, MPPED2, FABP6, CCDC71L, EDNRB, TSPAN31, FAM213A		
Garcia et al. (30)	0, 2, 6, 12, 18, 24, 30, 36, and 48 h postinfection	TNFAIP8		
Tanaka et al. (16)	0, 6, 24, and 48 h postinfection	SAMD9		
Kurozumi et al. (17)	12 h postinfection	CYR61, Ang-2	TSP-1	
Zhang et al. (7)	3 and 6 weeks (from the mouse chip microarray)	Ly6a, Plac8, Ly6c, Ccl8, Ifitm3, Ms4a4c, Clec4e, Ly6e, Tgtp, Ifit1, Rsad2, Ccl2, Ifi27, Ifi47, Ccl7, Dck, Ifit3, Irf7, Gas1, Gbp2, Cd69, Il18	Elavl2, Lmcd1, Arr3, Trip4, Crmp1, Hpd, Ewsr1, Ociad2, Cox15, Hmgn3, Nfia, Cables1, Rfxank, Tusc4, Cnot3, Magi1, Mrg2, Stag1, Sca2, Pdcd2, Tub, Ndrg1, Pigl	UP: major histocompatibility class I, chemokine receptor binding, chemokine activity, and cytokine activity; down: peptidases, proteases
Jiang et al. (22)	24 h	Tumor necrosis factor		
Li et al. (20)	48 h		MYCN	
Ma et al. (19)	3 days	Dm-dNK		
Saito et al. (10)	14, 18, and 22 h	CASP3, CASP7, CASP8, CASP9, CASP10, CYCS, IKK, NF-κB		
Han et al. (18)	12, 24, and 48 h	*Staphylococcus* enterotoxin A		

infection. In contrast to the groups surveyed so far, Haddad et al. also labeled the virus with green fluorescent protein (GFP) and used this to determine if GFP-marker gene expression can be correlated with viral copy number. Analyzing GFP expression levels in the cells infected by the virus was shown to be both time and multiplicity of infection dependent. Considering that by 24 h postinfection almost 70% of live cells expressed GFP, and that the amount of dysregulated genes was significantly higher by 24 h, it seems reasonable to assume that the virus significantly affected the tumor. The pathway analysis enabled Haddad et al. to go a step further and begin to understand the method by which the viruses affect the tumor cells. More robust examples of combined testing of both tumor gene expression and viral levels will be reviewed in a later section.

The human studies reviewed until this point compared between tumor cells and normal cells. A slightly different methodology was employed in a few studies in which gene expression was compared between susceptible and resistant cancer cells. For example, Carey et al. capitalized on the fact that vesicular stomatitis virus (VSV) replicates selectively in cancer cells that

TABLE 2 | Methods.

Reference	Virus type	Virus name	Type of samples	Gene expression analysis	Viral analysis	Pathway analysis
Haddad et al. (12)	Vaccinia	GLV-1h153	Human pancreatic cancer cells	HG-U133A cDNA microarray chips	Green fluorescent protein (GFP) expression	Ingenuity Pathways Analysis (IPA)
Balogh et al. (8)	Newcastle disease virus	MTH-68/H	Rat adrenal tumor cells	Affymetrix exon chip/ microarray, quantitative reverse transcriptase PCR		DAVID functional annotation-clustering tool
Lee et al. (15)	Vaccinia	Pexa-Vec	Human hematologic malignant cells	Microarray	qPCR	DAVID functional annotation-clustering tool
Lacroix et al. (11)	Oncolytic parvovirus	H-1PV	Human medulloblastoma cells	Microarrays, quantitative real-time PCR (QRT-PCR)	QPCR-assay, dot blot assay	KEGG pathway analysis
Reinboth et al. (29)	Vaccinia virus (VACV)	GLV-1h68	Human melanoma cell lines	Microarray	Customized Affymetrix platform, GFP expression	IPA software
Kurozumi et al. (6)	HSV-1	hrR3	Implanted Rat glioma cells intracranially into immune competent rats	Quantitative real-time polymerase chain reaction-based microarrays, enzyme-linked immunosorbent assay (ELISA) for interferon-gamma expression by ELISA		
Alain et al. (25)	Reovirus	Dearing strain of reovirus serotype 3	Human glioma cells and Ras mouse embryo NIH3T3 cells	Northern blotting	Immunofluorescence	
Carey et al. (13)	Vesicular stomatitis virus (VSV)		Human LNCaP and PC3 cells	Real-time reverse transcription (RT)-PCR, microarray analysis		IPA software
Gholami et al. (27)	Vaccinia	GLV1h-153	Human triple negative breast cancer cell lines		GFP expression	
Pfankuche et al. (9)	Canine distemper virus (CDV)	DH82-Ond-pi	Canine histiocytic sarcoma cell line and *in vivo* SCID mice model	Microarrays	Immunofluorescence	WebGestalt and DAVID
Josupeit et al. (14)	Oncolytic parvovirus	H-1PV	Human NCH421k cells and the NCH421R and NCH421I subclones	Affymetrix human genome-U133 plus 2.0 microarray	Dot blot assay, immunofluorescence	
Garcia et al. (30)	CDV		Human mammary tumor and canine-derived adenofibrosarcoma cell lines	Quantitative polymerase chain reaction (qPCR)	qPCR	
Tanaka et al. (16)	Inactivated Sendai virus particle	HVJ-E	Human glioblastoma cell line U251MG	Real-time quantitative PCR, microarrays		
Hirvinen et al. (21)	VACV	vvdd-tdTomato-hDAI	Human melanoma HS294T and human monocyte THP-1 cells	Whole Genome sequencing	Fluorescence	BACA, David, and IPA analysis
Kurozumi et al. (17)	HSV-1	hrR3	Human U343, U87, U87ΔEGFR, and LN229 glioma cell lines, rat glioma D74/HveC cells, Fischer rats 8–10 weeks of age, and athymic nude mice 6–8 weeks of age	QRT-PCR		
Zhang et al. (7)	VACV	GLV-1h68	Human ductal adenocarcinoma GI-101A cells were injected into 6- to 8-week-old female, nude mice	GeneChip mouse genome array and human genome U133 plus 2.0 array	GFP and fluorescence microscopy	Gene ontology (GO)
Jiang et al. (22)	Adenovirus	SG502-TNF	Human A549 lung cancer cell line and human TE-1 esophageal cancer cell line	SYBR green I PCR	GFP and fluorescence	
Li et al. (20)	Adenovirus	ZD55-shMYCN	LA1-55N human neuroblastoma cell line	QRT-PCR		

(*Continued*)

TABLE 2 | Continued

Reference	Virus type	Virus name	Type of samples	Gene expression analysis	Viral analysis	Pathway analysis
Ma et al. (19)	Adenovirus	ZD55-Dm-dNK	HCT-116 and SW620 Human colorectal cancer cell lines	RT-PCR and enzyme assay	Western blot analysis	
Saito et al. (10)	Sindbis virus	SIN AR399	HSC-3 and HSC-4 human oral squamous cell carcinoma cell lines	Real-time quantitative RT-PCR	Viral titers, immunoblot analysis	
Han et al. (18)	Adenovirus	PPE3-SEA	MB49 mouse bladder cancer cells	RT-PCR	Western blot analysis	
Sato et al. (26)	Adenovirus	OBP-301 and OBP-401	Acc2 and AccM human salivary gland adenoid cystic carcinoma cell lines	Quantitative real-time RT-PCR analysis (viral gene)	GFP and fluorescence	
Guse et al. (24)	Adenovirus	Ad5/3-Δ24, Ad5-Δ24pK7, Ad5-Δ24RGD, Ad5-Δ24E3, Ad300wt, Ad5LacZ	HEY human ovarian cancer cells, 786-O human renal cancer cells, and 4- to 5-week-old female nude mice		Real-time quantitative PCR was done with a SYBR green assay using a RotorGene system and fluorescence	
Shin et al. (23)	VSV	rVSV-IL12, rVSV-F	SCC 09 and FaDu human squamous cell carcinoma cell lines, and SCC VII murine squamous cell carcinoma cell line, and 6-week-old female C3H/HeJ mice	Real-time reverse transcriptase-polymerase chain reaction assays		

TABLE 3 | Gene Overlap.

Upregulated genes	Reference	Gene overlap by papers				
		Downregulated genes	Reference	Mixed genes	Upregulated	Downregulated
Rsad2	Balogh et al. (8), Zhang et al. (7)	MYC/MYCN	Lacroix et al. (11)/Li et al. (20)	SPP1	Josupeit et al. (14)—resistant	Kurozumi et al. (6)
Cxcl11	Balogh et al. (8), Kurozumi et al. (6)	NFIA	Lacroix et al. (11), Zhang et al. (7)			
Ddx60	Balogh et al. (8), Pfankuche et al. (9)					
Ifit1	Balogh et al. (8), Zhang et al. (7)					
Ifit3	Balogh et al. (8), Zhang et al. (7)					
Ifi47	Balogh et al. (8), Zhang et al. (7)					
Cxcl9	Balogh et al. (8), Kurozumi et al. (6)					
TNF	Balogh et al. (8), Jiang et al. (22)					
IFITM3	Josupeit et al. (14)—susceptible, Zhang et al. (7)					

have defects in the interferon (IFN)-I pathway (13). They looked at two different lines of prostate cancer cells. The first, human LNCaP cells, possess a defective IFN-I response, making them sensitive to VSV infection. The second, human PC3 prostate cancer cells, on the other hand, have functional IFN-I signaling, making them resistant to VSV infection. They employed real-time RT-PCR analysis and found that primary transcription, secondary transcription, and viral protein synthesis were delayed in PC3 cells compared with LNCaP cells. To look at gene expression, they used microarray and found that PC3 cells expressed many antiviral gene products compared with LNCaP cells. Furthermore, they looked at 80 different signaling pathways using IPA software and found specific pathways to be associated with a difference in gene expression between the two cell lines. Predictably, the IFN pathway had the highest percentage of differentially expressed genes. This research suggests the possibility of sensitivity markers for VSV treatment and hints at the mechanism of action of the virus. Similarly, Josupeit et al. looked at human NCH421k glioblastoma multiforme cells, which are susceptible to infection by parvovirus H-1 (H-1PV) and compared its response to H-1PV with NCH421R cells, which are a subclone resistant to H-1PV (14). They used "stem like" cell lines in NOD/SCID mice. They found a decrease in metabolic activity in the sensitive cell line compared with the resistant cell line when treated with H-1PV. When they analyzed gene expression using the Affymetrix Human Genome-U133 plus 2.0 microarray, they found 201 genes that were differentially expressed by at least threefold. They used unsupervised clustering to group the differentially expressed genes into three different categories. Some of these gene products are involved in regulating the antiviral immune response. A

further example of comparing the response to oncolytic viruses in susceptible and non-susceptible cell lines is the study done by Lee et al. They compared the response of sensitive human myeloid leukemia lines and resistant human lymphoid leukemia cell lines to Pexa-Vec, a VACV engineered to express human granulocyte-macrophage colony-stimulating factor and β-galactosidase (15). Using quantitative PCR (qPCR) they found that 660 genes were upregulated at least twofold and 776 genes were downregulated at least twofold in the lymphoid cancer cell lines. In the case of the upregulated genes, changes were particularly remarkable, with more than 50 genes induced fivefold or higher, and 150 genes that were expressed three or fourfold higher, than the control. They used the DAVID functional annotation-clustering tool to classify the genes into 319 functional gene clusters. Some of the clusters included genes related to: viral replication and regulation of apoptosis.

Gene Expression of Specific Genes in the Tumor Cells

A second strategy currently employed to monitor the efficacy of oncolytic virotherapy is to monitor the expression of specific genes in the tumor cells. Interestingly, to the best of our knowledge, no such human studies have been published. However, a number of human cell-line studies have been done. For example, Tanaka et al. focused on the sterile alpha motif containing domain (SAMD9) gene in the human glioblastoma cell line called U251MG (16). They treated this line with Sendai virus particle (HVJ-E). Using real-time quantitative PCR and microarray analysis they found that SAMD9 gene was upregulated in tumor cells treated with the virus and the SAMD9 messenger ribonucleic acid (mRNA) was upregulated in a time and dose-dependent manner. In a study by Kurozumi et al., they also looked at a limited number of genes in the tumor cells, but they looked at both human and animal cell lines (17). They looked at 10 genes in glioma cell lines following treatment with HSV-1 virus hrR3 and compared the response to controls. When they used QRT-PCR, they found that three genes in particular were dysregulated. The first, the antiangiogenic factor *TSP-1* was downregulated, and the other two, angiogenic factors *CYR61* and *Ang-2* were significantly upregulated when compared with controls. The advantage of this study over the previous one is that they were able to correlate the upregulation of CYR61 gene expression with the presence of the virus in the tumor tissue *in vivo*. CYR61 is a known protein that is involved in apoptosis, angiogenesis, the cell cycle, and extracellular matrix formation. By focusing on known genes but looking at the tumor cell expression in response to viral therapy and looking at the presence of the virus, researchers are able to better pinpoint the mechanisms at work when a virus infects a tumor cell.

Monitoring Transgene Expression

A third and particularly innovative strategy currently employed to monitor the efficacy of oncolytic virotherapy is the introduction of transgenes into the oncolytic virus and then analyzing gene expression of the transgene specifically. As we saw with the specific gene studies, there were no known published human studies. However, we will analyze one animal line study and a number of studies looking at human cell lines. Han et al. developed an oncolytic adenovirus PPE3-SEA that expressed the superantigen *Staphylococcus* enterotoxin A (SEA) and that has improved tumor specificity due to regulating the expression of E1A and E1B genes (18). They tested the PPE3-SEA virus against MB49 mouse bladder cancer cells *in vitro* and *in vivo*. They found that the mice treated with the virus had a significantly lower mean tumor volume than the control group. This seemed to correlate well with the increased expression of the virus mRNA *in vitro*.

In the human cell line studies, for example, Ma et al. constructed an adenoviral vector ZD55-Dm-dNK, containing the *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase—an important suicide gene (19). They looked at the expression of Dm-dNK human in colorectal cancer cells (HCT-116 and SW620) using RT-PCR. They found higher expression of the virus in the colorectal cancer cell lines, and lower levels of expression in the normal cell controls.

Similarly, Li et al. constructed ZD55-shMYCN, an oncolytic adenovirus ZD55 targeting the MYCN gene (20). They treated a p53-null and MYCN amplified human neuroblastoma cell line LA1-55N with the new virus. Using a two-step real-time reverse transcription (RT)-PCR procedure, they found that the virus selectively replicated and significantly downregulated the MYCN expression and that it was capable of effectively silencing the MYCN gene and inducing apoptosis in the tumor cells. Furthermore, they were able to demonstrate that the virus inhibited the growth of xenograft tumor *in vivo*.

In addition, Hirvinen et al. developed an oncolytic VACV that expressed intracellular pattern recognition receptor DNA-dependent activator of IFN− regulatory factors (DAI) to stimulate the innate immune system and to activate adaptive immune cells in the tumor (21). They tested this virus on two different human cell lines: human melanoma cells (HS294T) and human monocyte cells (THP-1). They used the BACA representing tools *via* the DAVID database to analyze the genes. They found that in the THP-1 cell line, there were a lot more upregulated genes than in the melanoma cell line. They used IPA and found that the most upregulated networks involved pathways connected to the activation of the immune system. More specifically, pathways related to dendritic cell growth, communication between innate and adaptive immune systems, and recognition of viruses were dysregulated. Significantly, they also found a sevenfold upregulation of *DAI* in both cell lines.

Finally, Jiang et al. developed a recombinant adenovirus using SG502 and tumor necrosis factor (TNF), yielding SG502-TNF (22). They looked at the effect of the virus on two different human cell lines: human A549 lung cancer cell line and human TE-1 esophageal cancer cell line. With the help of SYBR green I PCR, they found that the expression of TNF protein increased in both cell lines after infection with the virus. Furthermore, they found that the virus attacked the tumor cells specifically, and that they regulated the apoptotic-signaling pathway.

Shin et al. investigated human squamous cell carcinoma cell lines, murine squamous cell carcinoma cell lines, as well as a murine model (23). They used a VSV that was engineered to

express the murine interleukin (IL) 12 gene called rVSV-IL12 and compared it to a non-cytokine carrying VSV virus called rVSV-F. They found that both viruses demonstrated similar infection efficiency. Real-time RT-PCR and enzyme-linked immunosorbent assay were used to look at viral replication and IL12 expression. They found that human squamous cell carcinoma cell lines infected with rVSV-IL12 had a high level of IL12 expression at 48 h postinfection. In the murine model, the animals treated with virus had a smaller tumor area than the control group. The mice treated with rVSV-IL12 had a much greater reduction of the tumor compared with the mice treated with rVSV-F. *In vivo* they showed that by day 30, none of the control mice survived, yet 3 animals injected with rVSV-F and 10 animals injected with rVSV-IL12, survived beyond day 30.

Monitoring Viral Gene Expression (See Table 4)

In assessing the impact of oncolytic virotherapy, a fourth method is to look at the gene expression of the virus itself inside of the tumor cells over time. By marking the virus with GFP and measuring the viral gene expression over time within the tumor cells, there is a clear indicator of viral growth, followed by a decrease in viral presence in the tumor cell as the tumor cell is destroyed. This method has been demonstrated in a number of studies including: animal studies (*in vivo*), and *in vitro* studies with cell lines, but no human studies have been published to date to the best of our knowledge.

Studies Where Human Cell Lines Implanted into Animals (*In Vivo*)

In conducting animal studies, human cell lines are implanted into animals (*in vivo*). For example, Guse et al. used two different murine xenograft models, one for renal cancer and a second for ovarian adenocarcinoma (24). They co-injected a luciferase-encoding virus with eight different adenoviruses. In the ovarian cancer cell model, they found using PCR that the mice infected with some of the adenoviruses had an over 3 log increase in luciferase gene expression, a luminescence gene, compared with mice infected with other adenoviruses. They also found that gene copies of luciferase genes were increased in some of the models and decreased in others. In the renal cancer model, they used qPCR to monitor gene copies and found that they increased by three orders of magnitude in some of the lines but not at all in others. Bioluminescence demonstrated photoemission for all of the tumors that were treated which implies that the virus entered the tumors.

TABLE 4 | Viral Proteins Monitored.

Viral proteins monitored	
Reference	**Protein**
Alain et al. (25)	S1
Garcia et al. (30)	CDVM
Li et al. (20)	E1A
Sato et al. (26)	E1A
Guse et al. (24)	E1A

Human and Mice Cell Line Studies (*In Vitro*)

Shifting to *in vitro* studies, Alain et al. looked at human glioma cells, Ras-transformed mouse embryo NIH3T3 cells, reovirus resistant human glioma cells, and untransformed NIH3t3 cells (25). They infected these four cell lines with mammalian orthoreoviruses. Using Northern blotting, they found that outer capsid protein sigma-1 reovirus transcripts were found only in the Ras-transformed cell line and the susceptible cell line. However, they found that when the cells were treated with an E64 protease inhibitor it successfully blocked the virus. Treating with an infectious subviral particle enabled the virus to be detected even in the resistant cell line and even in the presence of E64. Furthermore they found that the level of active cathepsin B and L was increased in tumors.

Sato et al. looked at human salivary gland adenoid cystic carcinoma cell lines (26). They infected the cells with a telomerase-specific replication-selective adenovirus (OBP-301) and OBP-401, a genetically engineered adenovirus with the GFP gene. Using quantitative real-time RT-PCR, they found that E1A expression increased in infected cells. When using the virus with GFP, the intensity of the fluorescence increased in a dose-dependent manner. Another *in vitro* study was conducted by Gholami et al. They used the human triple negative breast cancer cell lines HCC38 and MDA-MB-468 (27). They infected the cells with a VACV GLV-1h153 that was engineered to express the human sodium iodine symporter gene. Using GFP, they found that the virus infected the cell lines in a time-dependent way that was proportional to the concentration of the virus.

Using Marker Peptides to Monitor Viruses Instead of PCR

Peng et al. developed oncolytic viruses that could be tracked *via* marker peptides (28). They used the Edmonston vaccine strain of measles virus (MV-Edm) to express either human carcinoembryonic antigen (CEA) or beta-human chorionic gonadotropin (βhCG). They injected MV-shCEA or MV-βhCG into two groups of transgenic mice. They compared detection of CEA in serum to RT-PCR for nucleocapsid RNA and found that serum CEA was a more sensitive method than PCR.

Combination Studies (Viral and Host Gene Expression)

Until now we have reviewed studies that primarily looked either at tumor gene expression or viral gene expression. Perhaps the most interesting studies are the studies that combine both. Combination studies can potentially assess the correlation between the viral replication and the host response. Reinboth et al. looked at two different human melanoma cell lines (888-MEL and 1936-MEL) (29). They infected the cell lines with an attenuated VACV GLV-1h68. They used a platform called 36K to monitor human gene expression and they used a customized platform to monitor viral expression. To monitor viral expression, various markers were used including RUC-GFP, gusA, and the viral IFN-α/β-receptor-like secreted glycoprotein. The levels of the first two markers increased after infection, and the glycoprotein was expressed exclusively by GLV-1h68. To

analyze the relationship between host cell transcription and viral replication, they assessed the correlation between viral and human gene expression at 2 h postinfection (early) and at 10 h postinfection (intermediate/late). At 2 h postinfection, they found 7 VACV genes and which were correlated to 114 human genes. Analysis with IPA demonstrated that these genes were related to the following pathways: apoptosis and the cell cycle, posttranslational modification, cellular growth and signaling, and other networks (**Table 1**). They then assessed whether human early gene transcription was predictive of VACV intermediate/late transcription and found 84 human early genes that correlated. These genes were important in processes such as cellular development and death, and lipid metabolism amongst others. Upon looking at expression 48 h after infection (late), there was a significant change in the expression of genes related to cellular growth, cell death, protein synthesis and folding, DNA replication, and DNA repair.

Similarly, Garcia et al. used three human mammary tumor cell lines along with a cell line for adenofibrosarcoma of canine origin, and infected them with CDV (30). They were testing the sensitivity to CDV infection, cell proliferation, apoptosis, mitochondrial membrane potential and expression of tumor necrosis factor-alpha-induced protein 8 (TNFAIP8). Using qPCR they were able to quantify both TNFAIP8 gene, and the virus CDVM gene expression; they found that both TNFAIP8 and CDVM gene expression were positively correlated in all cell lines.

CRITICAL ANALYSIS: ADVANTAGES/ DISADVANTAGES OF FOUR STRATEGIES

In critically assessing the advantages and limitations of each of the four strategies described earlier, various conclusions became clear. For example, when looking at overall gene expression changes of tumor cells, the advantages include that it generates the broadest view of what is happening inside the tumor cells after infection with the virus without the initial bias of inserting a gene and looking specifically at that gene. Of course, it is also provides a much wider scope which is more advantageous than looking at only one or two genes. The disadvantages of this method, on the other hand, is that they provide less specific information about specific pathways or mechanisms through which the tumor increases or decreases due to the wider lens used. In addition, this method provides no information on the viral gene expression or the correlation between the virus and the decrease in tumor size.

Upon analyzing the second method, namely, gene expression of specific genes in the tumor cells, we saw various strengths and weaknesses as well. For example, this method could potentially provide more information on the mechanism for tumor shrinkage or growth. In addition, since the method is more specific it makes it slightly easier to execute. Furthermore, once the specific genes are chosen, it enables a more in depth analysis of how these genes are important and how gene expression changes in the tumor cells after viral infection can have an impact on the cells.

On the other hand, looking at specific genes in the tumor cells might not reflect the overall tumor status since the broad picture of what is going on in the tumor cells is missing. Similar to the limitation of the first method, this method also does not provide any information on the viral gene expression or the correlation between the virus and the decrease in the tumor.

The third method of looking at transgenes is advantageous because it is a relatively simple study to do, allowing for effective monitoring of a specific transgene, and it is not "shooting in the dark" and looking for a wide variety of genes. In this way, researchers can test how effective a specific transgene performs. However, disadvantages include that it lacks the broader perspective of a study looking at overall gene expression and does not provide any indication of how the expression of genes in the tumor cells change after viral infection.

Monitoring viral gene expression is helpful in terms of providing information about how effectively the virus is infecting and replicating in the host cell, but provides no information about gene expression changes in the tumor cell or the mechanisms for tumor growth or shrinkage. Compared with analyzing marker peptides to monitor viral growth and replication, gene expression might not be as sensitive, but this requires further studies to confirm this finding.

Overall, in terms of future directions, it would seem that combination studies are the optimal method for studying gene expression changes. They potentially allow both a broad picture of gene expression changes in tumor cells, and the ability to correlate the tumor shrinkage or growth with viral replication. Furthermore, much information about the precise mechanisms for how the virus attacks the tumor cells can be culled from this type of study. As more combination studies are performed, patterns in the clusters of genes involved will become apparent enabling researchers to pinpoint exactly how various viruses attack tumors and providing fruitful ideas for developing a new generation of recombinant viruses that are more effective.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. AA drafted the article, reviewed the relevant literature, made substantial contributions to conception and design, interpreted the data and approved final version. JR drafted the article, reviewed the relevant literature, made substantial contributions to conception and design, interpreted the data, revised the article critically and approved final version. PZ made substantial contributions to conception and design, participated in revising it critically, interpreted the data, and approved final version. BG participated in revising it critically, interpreted the data, and approved final version.

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