Please stand by: how oncolytic viruses impact bystander cells

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Oncolytic viruses (OVs) do more than simply infect and kill host cells. The accepted mechanism of action for OVs consists of a primary lytic phase and a subsequent antitumor and antiviral immune response. However, not all cells are subject to the direct effects of OV therapy, and it is becoming clear that OVs can also impact uninfected cells in the periphery. This review discusses the effects of OVs on uninfected neighboring cells, so-called bystander effects, and implications for OV therapies alone or in combination with other standard of care chemotherapy.

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By utilizing viruses that are clinically nonpathogenic for humans (including animal viruses) or by strategically attenuating pathogenic viruses, oncolytic viruses (OVs) are ideally innocuous to healthy tissues yet deleterious to malignant tissues (Figure 1A). Normal cells detect virus infection via various innate mechanisms (toll-like receptors, RNA-dependent protein kinase, RIG-I, cGAS) and activate an interferon response, which is often defective in cancer cells. The differential sensitivity between cancer cell and normal cells has even been used in the preclinical setting to selectively purge leukemia cells from bone marrow [1]. However, first-generation OVs often fail to produce clinically relevant tumor regression as a standalone therapy. Second-generation viruses, first-generation viruses armed with antitumor genes [2], have become the norm in efforts to maximize the efficacy of OV therapy. Armed viruses produce 'bystander effects', since they create a scenario where a virus-infected cell can affect the fate and behavior of uninfected neighboring cells. Genetic transfer of the HSV thymidine kinase (HSV-tk) gene and subsequent administration of acyclovir was the first description of bystander effects caused by viruses, where HSV-tk in transduced cells phosphorylate acyclovir or ganciclovir, enabling it to be transferred to adjacent cells via gap junctions, causing cell death in surrounding cells devoid of HSV-tk [3–6]. Intentionally designed bystander effects in second-generation OVs are achieved through direct cytokine secretion, antibody release, prodrug enzymes and other active molecules, and is the subject of multiple literature reviews [7–10]. However, not all bystander effects are intentional. Rather, some are a serendipitous consequence of virus infection. This review highlights some of the occurrences of bystander effects observed in both first- and second-generation OVs, and some of the molecular underpinnings of this bystander activity. This review will focus predominantly on HSV-based OV platforms.

Uninfected cell death

Bystander cell death

The ability of OVs to induce cell death in infected cells is well known, and bolstering the lytic phase of OV therapy is a strategy employed to improve therapeutic efficacy [11,12]. However, less is understood about the paracrine mechanisms through which OV infection causes cell death. Stanziale *et al.* reported human gastric cancer cells

Figure 1. Oncolytic viruses have three ways of mediating antitumor effects. (A) OVs enter tumor cells where they replicate, ultimately leading to virus-mediated cell death or lysis. Lytic infection leads to the release of antigen from both tumor and virus. Healthy cells are able to restrict virus infection. **(B)** Adaptive T-cell immunity is generated against the virus and tumor antigen released by lytic viral infection. **(C)** The process of cell lysis results in the release of cytokines and other factors that are able to mediate cell cytotoxicity on their own and have been reported to augment chemotherapy drugs.

APC: Antigen-presenting cell; MHC: Major histocompatibility complex; OV: Oncolytic virus.

Table 1. Current combination chemotherapy: oncolytic virus clinical trials.

Indication	Oncolytic virus	Virus type	Chemotherapy drug(s)	Clinical trial numbers	Recruitment status
Colorectal cancer	Pexa-Vec	Vaccinia virus	Tremelimumab	NCT03206073	Recruiting
Colorectal cancer	Reolysin®	Reovirus	Irinotecan, leucovorin, 5-FU, bevacizumab	NCT01274624	Active, not recruiting
Glioblastoma	TG6002	Vaccinia virus	5-flucytosine	NCT03294486	Recruiting
Glioblastoma	DNX2401	Adenovirus	Temozolomide	NCT01956734	Completed
Head and neck	Reolysin®	Reovirus	Paclitaxel	NCT01166542	Completed
Malignant glioma	PVSRPO	Poliovirus	Lomustine	NCT02986178	Recruiting
Mesothelioma	ONCOS-102	Adenovirus	Cisplatin, cyclophosphamide	NCT02879669	Recruiting
Metastatic melanoma	Reolysin®	Reovirus	Paclitaxel, carboplatin	NCT00984464	Completed
Multiple	MV-NIS	Measles virus	Doxorubicin	NCT02364713	Recruiting
Multiple myeloma	MV-NIS	Measles virus	Cyclophosphamide	NCT00450814	Recruiting
Non-small-cell lung cancer	Ad-MAGEA3	Adenovirus	Pembrolizumab	NCT02879760	Recruiting
Non-small-cell lung cancer	Reolysin®	Reovirus	Paclitaxel, carboplatin	NCT00861627	Completed
Ovarian	Reovirus	Reovirus	Paclitaxel	NCT01199263	Active, not recruiting
Pancreatic cancer	TBI-1401(HF10)	HSV-1	Gemcitabine, paclitaxel	NCT03252808	Recruiting
Pancreatic cancer	LOAd703	Adenovirus	Gemcitabine, paclitaxel	NCT02705196	Recruiting
Pancreatic cancer	Reolysin®	Reovirus	Gemcitabine, irinotecan, leucovorin, 5-FU, pembrolizumab	NCT02620423	Active, not recruiting
Triple negative breast cancer	Imlygic (T-Vec)	HSV-1	Paclitaxel	NCT02779855	Recruiting
Solid tumors	VCN-01	Adenovirus	Gemcitabine, paclitaxel	NCT02045602	Recruiting

infected with a first-generation HSV-1-based OV (NV1066) die via apoptosis [11]. Interestingly, when examining a mixture of infected and uninfected cells, they reported approximately 10% of the uninfected cells also undergo apoptosis [11]. Hypothesizing that apoptosis in the uninfected cell population would limit viral replication and thus subsequent antitumor efficacy, they showed that inhibiting apoptosis resulted in a significant increase in viral recovery from infected cells *in vitro*, resulting in reduced cancer cell proliferation [11]. Subsequent studies have confirmed NV1066 induces cell death in uninfected bystander cells [13], and suggest tunneling nanotubes that directly connect infected with uninfected cells could be a driving mechanism for bystander toxicity [14].

Bolstering combination therapies

The factors underlying bystander cell death during OV infection may be a detriment to the initial lytic phase and viral spread of OV therapy, but may be therapeutically advantageous by enhancing concomitant therapies. Huang *et al.* showed synergistic antitumor efficacy in mice bearing human HCT-116 tumors treated with oncolytic vaccinia virus strain vvDD in combination with paclitaxel [15]. Virus-free conditioned media from tumor cells infected with vaccinia virus (vvDD) sensitized tumor cells to paclitaxel cytotoxicity *in vitro*. The enhanced paclitaxel sensitivity was mediated by type I interferon released after initial virus infection in addition to high-mobility group box 1 (HMGB1) release following cell death [15]. A recent report by Currier *et al.* highlighted another example of two-drug-synergy with HSV1716 and the small molecule aurora A kinase inhibitor, alisertib, *in vitro* and *in vivo* [16]. Cells infected with HSV1716 secreted unknown factors into the supernatants which increased alisertib cytotoxicity in uninfected neuroblastoma and malignant peripheral nerve sheath tumor cell lines [16]. PhD dissertation work by Lynne Braidwood at the University of Glasgow and Virttu Biologics reported synergy between a variety of targeted inhibitors and chemotherapy drugs in combination with HSV-free conditioned medium [17]. Many publications report OV synergy with chemotherapy drugs, as previously reviewed extensively by others [18–20]. However, it is becoming more evident that OV infection can bolster the efficacy of conventional chemotherapy drugs. A list of ongoing clinical trials combining chemotherapy with OVs is presented in Table 1.

Paracrine mediators of cytotoxicity

Cytokines, T-cell engagers & secreted therapeutics

Cytokines are some of the most well-documented antiviral factors released following virus infection; the interferon response being at the vanguard of the struggle between a virus's goal of persistent infection and the host's intrinsic reflex to eliminate the pathogen [21]. Antiviral cytokine activity is controlled by a complex and interconnected web of pattern recognition receptors and intrinsic viral restriction factors [22]. Cytokine secretion is a primary paracrine mediator of inflammation and sets the stage for an active, or inactive, immune response [23]. Lending credence to the importance of cytokines and their ability to shape the tumor microenvironment and immunity, many second-generation OV's are armed with inflammatory cytokines [7]. For example, the US FDA approved the OV Imlygic (Talimogene laherparepvec, Amgen, CA, USA), which expresses GM-CSF to potentiate antitumor immune responses, albeit the role of that transgene in mediating clinical responses has not been proven.

Avoiding immune destruction is one of the hallmarks of cancer and is partly mediated through immunosuppressive cytokine activity [24]. OV's and the inflammatory cytokines they activate, naturally or by design, can awaken antitumor immune responses that are often dampened by the tumor microenvironment [25]. Using a syngeneic murine model of ovarian cancer, Benencia *et al.* showed that HSV1716 can induce NK cell and cytotoxic CD8⁺ T-cell influx into treated tumors [26]. Further, the influx of effector cells was driven in part by type I interferon-dependent chemokine secretion from antigen-presenting cells (APCs) within the tumor [26,27]. Other groups have tried to strengthen the natural inflammatory immune response to OV therapies by engineering OV expression of IL-12 [28,29]. IL-12 is typically secreted by activated APCs in response to toll-like receptor stimulus and promotes NK cell activation and proliferation [30]. Multiple viruses armed with IL-12 showed enhanced CD4⁺, CD8⁺ T cell and NK cell proliferation and trafficking to the tumor and also improved overall survival in tumor-bearing mice [28,29].

The primary mediator of antiviral activity in mammals is the interferon response [21]. Interferon signaling promotes an antiviral state by activating hundreds of interferon-stimulated genes in a paracrine manner [22]. In addition to fostering the antiviral state, cytokines can also be cytotoxic [31]. Another cytokine often associated with viral infection is TNF- α [32]. A study by Shelke *et al.* showed that TNF- α together with IFN- γ causes apoptosis in multiple neuroblastoma cell lines *in vitro*, and patients with increased expression levels of TNF- α and IFN- γ showed a survival advantage compared with counterparts with lower TNF- α and IFN- γ expression [31].

Investigators have also begun arming OV's to secrete linker molecules that serve to bind T cells to tumor cells. So-called bispecific T-cell engagers as proteins are FDA approved for leukemia, but their expression via gene transfer in the context of OV's may bring certain advantages, recently reviewed elsewhere [33].

Another strategy to increase the bystander effect is engineering viruses to secrete molecules that recognize and kill other tumor cells. The strategy is exemplified by the arming of an HSV-2 derived vector with a Her-2 single chain antibody linked to an apoptosis-inducing Fas ligand domain [34].

Extracellular vesicles

A study by Schmidt *et al.* described a fascinating observation that HSV-I activates cell cycle entry and DNA synthesis in uninfected cells near developing viral plaques [35]. Once the virus advanced and gained entry to the surrounding host cells, the cell machinery for DNA replication was subsequently commandeered by HSV-I to promote virus replication [35]. However, the soluble factor initiating DNA synthesis was not identified. One possibility is that such paracrine signaling activity may be driven by extracellular vesicles secreted from virus-infected cells into the periphery.

Exosomes are 30–100 nm vesicles secreted from cells that carry a variety of cellular contents [36–43]. Once thought to be a means of eliminating cellular waste, exosomes and their larger counterpart microvesicles (100–1000 nm) have been shown to play a role in cellular communication through shuttling protein, RNA, DNA and miRNA, and other cellular components from cell to cell [38]. Multiple viruses and host cells utilize extracellular vesicles as a means to modulate the milieu of infection; sometimes setting up a favorable environment for infection while other times promoting antiviral immunity [44]. For example, work by Temme *et al.* revealed a role for HSV glycoprotein B in diverting MHC class II from the cell surface to instead undergo release from infected cells via the exosomal secretion pathway, thereby limiting MHC-II antigen presentation [45]. A study by Kalamvoki *et al.* found that HSV-1 infected cells secrete exosomes containing stimulator of interferon genes (STING) along with viral miRNAs [46]. STING is an antiviral factor that detects cytosolic DNA, thus setting in action a series of steps that ultimately activates

the type I interferon response and promotes antiviral innate immunity [46,47]. In the context of HIV infection, exosomes from virus-infected T cells deliver the viral accessory protein negative regulatory factor thereby causing apoptosis in nearby CD4⁺ cells in a CXCR4-dependent manner [48,49].

In addition to STING, HSV-1 infected cells release extracellular vesicles containing viral miRNAs [46,50]. Although many viruses naturally encode miRNAs [50], herpes viruses have been well studied for their relatively large number of functional miRNAs encoded in the viral genome [51–58]; human herpes viruses encode over 80 miRNAs [59]. For example, Duan *et al.* showed that the miR-H6 helps maintain HSV-1 latency and prevents lytic infection by inhibiting expression of the immediate early gene, infected cell protein 4 (ICP4), in human cornea epithelial cells latently infected with HSV-1 [60]. Several other HSV-1 miRNAs such as hsv1-miR-H2 and hsv1-miR-H3 influence lytic infection by inhibiting expression of the immediate early gene ICP0, and late gene ICP34.5, respectively [52,61]. Overall, extracellular vesicles can transmit many cellular factors in the vicinity of virus infection, which can in turn alter the biology of uninfected cells nearby.

Inflammatory cell death

Interferon-stimulated genes are not the only byproduct of interferon signaling. Interferon leads to the activation of the JAK/STAT1 signaling pathway, which alters the localization of key DNA sensors involved in the antiviral response. One such sensor is the damage-associated molecular pattern HMGB1 [62]. At steady state, HMGB1 is present in the nucleus where it binds to chromatin and DNA. However, once a cell becomes infected by a virus, HMGB1 relocates to the cytoplasm, where HMGB1 acts as a sensor for nucleic acids [63].

An example of the role HMGB1 plays in nucleic acid sensing was reported by Tian *et al.*, where they showed HMGB1 protein binds with high affinity to the TLR9 agonist, CpG. Further, dendritic cells secreted approximately threefold more IFN- α and TNF following stimulation with HMGB1-CpG complexes compared with either HMGB1 or CpG DNA alone [64]. Similar HMGB1-mediated augmentation of TLR signaling was reported by Zou *et al.*, where they found HMGB1 acting in concert with the TLR3 agonist poly-IC enhanced IFN- β secretion in dendritic cells [65]. Taken together, these reports suggest an adjuvant role for HMGB1 in nucleic acid sensing during virus infection.

When a virus infected cell succumbs to lytic viral infection, HMGB1 is often released in high abundance [66–68]. HMGB1 acts both as a chemoattractant and activator of inflammation [69]. The release of inflammatory molecules such as HMGB1 and ATP during viral lysis led to the designation of this cell death process as ‘inflammatory cell death’, or ICD [70,71]. ICD fundamentally differs from the more common immunologically silent mechanism of cell death known as apoptosis. Using an ICP0 null oHSV (dICP0), Workenhe *et al.* reported that oHSV causes ICD in tumor cells marked by increased HMGB1 serum levels, which ultimately increases the abundance of APCs within tumors. The increase of APCs correlated with enhanced incidence and absolute number of antitumor CD8⁺ T-cell responses in tumor-bearing mice treated with dICP0 oHSV [72]. In another study, Workenhe *et al.* also showed that the ICD-inducing drug mitoxantrone in combination with oHSV could enhance the therapeutic potential of oHSVs in cancer models with low immunogenicity, further suggesting that ICD is a key feature of successful OV therapy [73].

The role of HMGB1 is not limited to DNA sensing and inducing inflammatory cell responses. Multiple studies have reported that HMGB1 is cytotoxic and may restrict OV replication [68,74,75]. Work by Gdynia *et al.* found that recombinant HMGB1 is cytotoxic to a variety of human cancer cell lines, including U251MG glioblastoma cells, HCT116 colon cancer cells, HeLa cervical cancer cells and MCF-7 breast cancer cells. Interestingly, they reported that HMGB1 had minimal cytotoxicity on normal human astrocytes, suggesting the cytotoxicity may be limited to malignant cells [74]. Another study by Tang *et al.* found that oxidized HMGB1, but not the reduced form, induces apoptosis and autophagy in the human pancreatic cancer cell line Panc2.03 and human colorectal cancer cell line HCT116 [75]. Together, these studies demonstrate the direct cytotoxic potential of HMGB1 release following OV infection, and one could imagine this process might play a role in the host cell’s ability to limit infection during an ongoing lytic infection in a paracrine manner.

Syncytia formation

Some viruses or specific virus strains are naturally fusogenic, meaning the viral glycoproteins expressed on the infected cell membrane causes fusion of the cell to adjacent cells, resulting in multinucleated giant cell syncytia. In such cases, the surrounding uninfected cells become incorporated into the infected cell and die [76]. The

incorporation of fusion-inducing genes is thus a strategy some have used to increase the bystander effect of oncoytic viruses, so that infection of one cell can destroy many surrounding cells [77].

In situ antitumor vaccination

The consequences of OV infection (lytic infection, cytokine release, chemokine release, inflammation, damage-associated molecular pattern release) culminates with an adaptive immune response targeting both the OV and tumor antigens [78]. For example, lytic infection leads to tumor cell death and local inflammation, which in turn enhances antigen uptake and presentation by APCs [26]. With an increased swath of tumor antigens within the tumor microenvironment and heightened antigen presentation, a robust T-cell repertoire can develop; an occurrence known as antigen spread [78,79]. This aspect of OV therapy ultimately allows an immunocompetent subject to generate an *in situ* antitumor vaccine [80]. However, tumors often counteract adaptive immunity by expressing a variety of immunosuppressive molecules, such as TGF β and PD-L1, that lead to T-cell anergy [24,81–83]. Indeed, current strategies to improve OV efficacy via checkpoint blockade work by counteracting T-cell anergy during the adaptive immune response [84–86]. The bystander effects of OVs indirectly impacts the formation of antitumor immune responses and is likely to be essential for the overall efficacy of OV therapy.

Conclusion

OVs are promising anticancer therapeutics, primarily based on their ability to directly kill infected cells and induce antitumor immunity. An underappreciated aspect of their biology, however, is that virus infection of a cell can indirectly affect surrounding neighbor cells in a bystander effect either through direct cytotoxicity or by enhancing antitumor immunity. Some bystander effects are due to natural consequences of the biology of infection while others can be engineered into the virus via transgenes. As we better understand various types of bystander effects, we should be able to leverage and enhance them to increase the effectiveness of OVs as cancer therapeutics.

Future perspective

In summary, how an OV kills a target cell is an important detail to OV therapy. It is critical for an OV to infect a target cell, and subsequently produce a lytic virus infection. However, a cell does not have to be directly infected to be affected by OV therapy. The mechanisms underlying diverse processes such as inflammation, innate immunity, antigen presentation, adaptive immunity and cell death are influenced by many cytokines and molecules such as HMGB1. OVs can be modified, or ‘armed’, to enhance a multitude of these aspects, and drugs can be used to alter the tumor microenvironment. Whether a single primary OV infection alone will trigger these events and lead to antitumor immunity, or if repeated dosing of OV will improve outcomes remains to be determined and will likely vary between OV platforms. Together, these bystander effects shape the antitumor immune response. It is important to consider all aspects of how OVs mediate their effects, and strategies to maximize certain aspects of oncolytic virotherapy will certainly be relevant in future clinical trials.

Executive summary

- Oncolytic virus (OV) infection causes cell death in both infected and uninfected cells.
- OVs enhance the efficacy of standard chemotherapy.
- OV–chemotherapy combinations are the subject of multiple clinical trials.
- OV infection leads to inflammatory cytokine and chemokine secretion.
- OV infection leads to biologically active extracellular vesicle secretion.
- OV infection promotes an *in situ* antitumor immune response.

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Author's contributions

L Sprague wrote the review; TP Cripe, KA Cassady, J Conner, L Braidwood and F Benencia edited and contributed to the content.

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