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HARNESSING ONCOLYTIC VIRUS-MEDIATED ANTITUMOR IMMUNITY

Topic Editors
Philippe Fournier and Volker Schirrmacher

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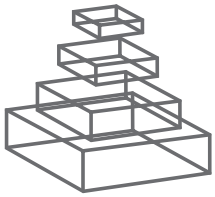

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HARNESSING ONCOLYTIC VIRUS-MEDIATED ANTITUMOR IMMUNITY

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Oncolytic viruses (OVs) have emerged as a promising anticancer treatment. OVs selectively infect, replicate in, and kill tumor cells. Oncolytic viral therapy occurs in two phases: an initial phase where the virus mediates direct oncolysis of tumor cells, and a second phase where an induced post-oncolytic immune response continues to mediate tumor destruction and retards progression of the disease. For a long time, the therapeutic efficacy was thought to depend mainly on the direct viral oncolysis based on their tumor selective replication and killing activities. But the post-oncolytic anti-tumor activity induced by the OV therapy is also a key factor for an efficient therapeutic activity. The topic adresses various strategies how to optimize OVs anti-tumor activity.

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EDITORIAL
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Harnessing oncolytic virus-mediated anti-tumor immunity

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Keywords: oncolytic virus, anti-tumor activity, tumor-associated antigen, oncolytic virotherapy, immunovirotherapy, immunotherapeutic approaches, anti-viral response

Oncolytic viruses (OVs) selectively infect, replicate in, and kill tumor cells. For a long time, the therapeutic efficacy of OVs was thought to depend mainly on this mechanism of direct viral oncolysis. Nowadays, however, the post-oncolytic anti-tumor activity induced by the OV therapy is considered a key factor for an efficient therapeutic activity. The research topic addresses these issues and discusses future strategies how to further optimize OVs anti-tumor activity.

The first two articles deal with viral oncolysis and the immune response. Guo et al. (1) from the University of Pittsburgh Cancer Institute (USA) point out that dying the right way is a key to eliciting potent anti-tumor immunity. They describe that OVs induce mostly immunogenic cancer cell death (ICD) including immunogenic apoptosis, necrosis/necroptosis, pyroptosis, and autophagic cell death. A review of recent advances in our understanding of danger signals is followed by a discussion of potential combination strategies to target cells into specific modes of ICD. Thorne (2) from the same Institution argues in his perspective article that the immune response raised by an OV can also hinder optimal therapeutic activity and repeat dosing. Using oncolytic vaccinia virus (VV) as an example, Thorne summarizes approaches to enhance the anti-tumor immune response by the introduction of immune stimulatory transgenes. His article points our attention also toward interesting new alternative strategies.

The next four articles review and discuss in more detail post-oncolytic anti-tumor immune responses. Gujar and Lee (3) from the Dalhousie University of Halifax (Canada) discuss how OV-induced immunological events override tumor-associated antigen (TAA) presentation impairment and promote appropriate T cell interaction with antigen-presenting cells (APC). Woller et al. (4) from the Medical School in Hannover (Germany) review the role of viral oncolysis for induction of ICD including autophagy, DAMPs and PAMPs, and the ER–stress response. Finally, they highlight developments for exploiting the vaccinative potential of oncolytic virotherapy. Moehler et al. (5) from the University Medical Center in Mainz (Germany) together with Jean Rommelaere from the DKFZ, Heidelberg (Germany) draw our attention to oncolytic parvoviruses and review their evidence that these can trigger maturation of dendritic cells (DCs) and induce activation of antigen-specific cytotoxic T cells. Finally, they discuss the clinical potential of the immunovirotherapy concept and its combination with new targeted therapies or with immune checkpoint blocking antibodies. Janelle and Lamarre (6) from the INRS-Institut Armand-Frappier in Quebec (Canada) discuss the question of how to assess anti-tumor immunity. They exemplify this by reviewing experimental studies with B16 mouse melanoma, which is treated by vesicular stomatitis virus (VSV) variants.

How can OVs be harnessed or combined with other agents such as antibodies to mediate stronger anti-tumor effects? This question is discussed by the following two manuscripts. Bauzon and Hermiston (7) from the Bayer HealthCare US Innovation Center in San Francisco (USA) propose to merge OVs with immune checkpoint blocking antibodies. Immune checkpoints refer to a number of inhibitory pathways that play crucial roles in maintaining self-tolerance and immune homeostasis. The discovery and targeting of immune checkpoints has opened a new immunotherapeutic avenue generating very promising clinical results. Arguments are put forward to combine this strategy with an OV therapy to create synergies between both approaches. This might result in enhanced safety and efficacy and would be also economically advantageous. Schirrmacher and Fournier (8) from the DKFZ, Heidelberg (Germany) and from the IOZK in Cologne (Germany) put forward in a perspective article a new concept of a multimodal cancer therapy involving oncolytic Newcastle disease virus (NDV), autologous immune cells (activated T cells and/or polarized DC1), and bi-specific antibodies (bsAbs). The bsAbs they created are NDV-specific single-chain (scFv) antibodies fused with anti-CD3 or anti-CD28 T cell activating scFvs. These reagents, upon attachment to NDV infected tumor cells, are reported to have a strong potential to activate cancer patients T cells, including TAA-specific memory T cells and not TAA-specific naïve T cells. Such *ex vivo* activated autologous T cells can be transferred back to the patient. To increase their tumor targeting efficacy, it is suggested to pre-activate the tumor microenvironment by low dose irradiation or by local hyperthermia. Tumor targeting of grafted T cells is suggested to become also improved via cell-bound tri-specific antibodies targeting a tumor introduced viral antigen such as HN of NDV.

Delivery of OVs is another important aspect for achievement of optimal effects. Tai and Auer (9) from the Ottawa Hospital Research Institute, Ottawa (Canada) argue that the optimal time point should be either pre- or post-operative to counteract surgery induced immunosuppression and to attack post-operative metastases. They review their preclinical surgery models, in which pre-operative OVs prevented post-operative NK cell dysfunction and attenuated tumor dissemination. Altomonte and Ebert (10) from the Klinikum rechts der Isar, Munich (Germany) discuss the particular challenges of OV therapy for hepatocellular carcinoma as well as some potential strategies for modulating the

immune system and synergizing it with the hepatic microenvironment. Combination strategies involving the adoptive transfer of immune cells together with OVs are expected as an exciting new approach.

Successful therapy using OVs will ultimately depend on effectively navigating the delicate balance between the anti-viral response and the anti-tumor immune response such as to minimize the former in the short term and maximize the latter in the long term. As outlined by Forbes et al. (11) from the Ottawa Hospital Research Institute, Ottawa (Canada), several approved drugs and novel small molecules can be effective tools to dampen the innate and adaptive anti-viral responses, increase the anti-tumor immune response, or both. Such approaches are discussed to be undoubtedly context dependent (e.g., tumor type and tumor site) and OV-dependent. This topic of combining oncolytic virotherapy with chemotherapy is further discussed by Nguyen et al. (12) from the McMaster University, Hamilton (Canada). With a particular focus on pharmaceutical immunomodulators they discuss how specific therapeutic contexts may alter the effects of these synergistic combinations and their implications for future clinical use.

It is remarkable to what extent experts from Canada, Germany, and the USA are in accord in this e-book by emphasizing the potential importance of OVs on systemic T cell-mediated anti-tumor immunity.

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Oncolytic immunotherapy: dying the right way is a key to eliciting potent antitumor immunity

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Oncolytic viruses (OVs) are novel immunotherapeutic agents whose anticancer effects come from both oncolysis and elicited antitumor immunity. OVs induce mostly immunogenic cancer cell death (ICD), including immunogenic apoptosis, necrosis/necroptosis, pyroptosis, and autophagic cell death, leading to exposure of calreticulin and heat-shock proteins to the cell surface, and/or released ATP, high-mobility group box 1, uric acid, and other damage-associated molecular patterns as well as pathogen-associated molecular patterns as danger signals, along with tumor-associated antigens, to activate dendritic cells and elicit adaptive antitumor immunity. Dying the right way may greatly potentiate adaptive antitumor immunity. The mode of cancer cell death may be modulated by individual OVs and cancer cells as they often encode and express genes that inhibit/promote apoptosis, necroptosis, or autophagic cell death. We can genetically engineer OVs with death-pathway-modulating genes and thus skew the infected cancer cells toward certain death pathways for the enhanced immunogenicity. Strategies combining with some standard therapeutic regimens may also change the immunological consequence of cancer cell death. In this review, we discuss recent advances in our understanding of danger signals, modes of cancer cell death induced by OVs, the induced danger signals and functions in eliciting subsequent antitumor immunity. We also discuss potential combination strategies to target cells into specific modes of ICD and enhance cancer immunogenicity, including blockade of immune checkpoints, in order to break immune tolerance, improve antitumor immunity, and thus the overall therapeutic efficacy.

Keywords: immunogenic cancer cell death, DAMPs, PAMP, autophagy, tumor-associated antigen, cross-presentation, immune tolerance, antitumor immunity

INTRODUCTION

Oncolytic viruses (OVs) have been shown to be effective in treating cancer in preclinical models and promising clinical responses in human cancer patients (1–3). OV-mediated cancer therapeutic includes three major mechanisms. The first is the direct infection of cancer and endothelial cells in the tumor tissue leading to direct oncolysis of these cells. The second is necrotic/apoptotic death of uninfected cells induced by anti-angiogenesis and vasculature targeting of the OVs as shown in both animal models and human cancer patients (4–6). The last is the activated innate and adaptive tumor-specific immunity, which exert cytotoxicity to surviving cancer and stromal cells. A number of recent studies have demonstrated that the antitumor immunity has played an important role in the overall efficacy of oncolytic virotherapy, which has been shown to contribute to the efficacy of oncolytic virotherapy (7–14). In the case of oncolytic vesicular stomatitis virus (VSV), reovirus, and herpes simplex virus (HSV), the antitumor immune response is very critical to the overall efficacy of oncolytic virotherapy, sometimes even more important than that of direct oncolysis (7, 9, 11, 14).

Oncolytic viruses provide a number of potential advantages over conventional cancer therapies. First, OVs are tumor-selective antitumor agent, thus providing higher cancer specificity and better safety margin. Second, OV-mediated oncolysis not only leads to regression of tumor size, but this process provides key signals to dendritic cells (DCs) and other antigen presenting cells to initiate a potentially potent antitumor immune response. The immunogenic types of cell death induced by OVs provide danger signal (signal 0) and a natural repertoire of tumor-associated antigens (TAAs) to DCs, both required to trigger an adaptive immunity against cancer (15–17). The danger signals include damage-associated molecular pattern (DAMP) and pathogen-associated molecular pattern (PAMP) molecules derived from the OVs. Therefore, this process could provide a highly favorable immunological backdrop for the host to respond and generate potent adaptive antitumor immunity. However, just like other immunotherapeutic regimens for cancer, a number of challenges remain for OVs-mediated immunotherapy. One is that relative inefficiency of delivering OVs to tumor nodules, viral replication within tumor mass, and spread to distant metastases dampens its overall efficacy. Second, most TAAs are self-antigens and thus weakly immunogenic. As we will discuss below, OVs may enhance tumor immunogenicity in many cases. Yet, this low immunogenicity still is a problem due to the highly immunosuppressive tumor microenvironment (TME). Third, a highly immunosuppressive TME in late stages of cancer often suppresses the activities of tumor-infiltrated lymphocytes (TILs) generated either spontaneously or by an immunotherapeutic regimen (18).

In this review, we will discuss different modes of cell death induced by various OVs, their potential effects on the subsequent antitumor immunity. Then we discuss rationales and strategies of inducing ideal types of cancer cell death by either genetic modification on OVs or by combination with specific antitumor agents that lead to specific mode of immunogenic cancer cell death (ICD). Finally, we provide some perspective on future combination strategies to improve antitumor immunity for enhanced overall efficacy of virotherapy.

OV: TUMOR SELECTIVITY AND RELEVANCE OF ANIMAL MODEL

Ideally, OVs selectively infect and replicate in cancer cells and cancer-associated endothelial cells, leading to direct oncolysis and subsequent antitumor activities without harming normal tissue (1–3). Some OVs display intrinsic tumor tropism (naturally occurring OVs), while others obtain their tumor selectivity through natural evolution or genetic engineering. The mechanisms underlying the tumor selectivity may include altered signaling pathways of ataxia telangiectasia mutated (ATM), epidermal growth factor receptor (EGFR), p53, PKR, Ras, RB/E2F/p16, Wnt, anti-apoptosis, or defects in cellular innate immune signaling pathways or hypoxia conditions in the TME (1, 3, 19, 20).

Viruses display strict viral tropism, specific for a cell type, tissue, or species. However, OVs often broaden their tropism to cancer cells from non-permissive species to various degrees. As an example, human adenovirus (Ad) does not infect normal murine cells, yet infect murine cancer cells even though the production of infectious virus progeny is often limited. A recent study may provide some answer to this phenomenon. McNeish et al. have found that murine cancer cells support viral gene transcription, mRNA processing, and genome replication of human Ad, but there is a profound failure of viral protein synthesis, especially late structural proteins with reduced loading of late mRNA onto ribosomes. Interestingly, *in trans* expression of the non-structural late protein L4-100K increases both viral mRNA loading on ribosomes and late protein synthesis, accompanied by reduced phosphorylation of eIF2α and improved anticancer efficacy (21). The key point is that some OVs display aberrant, non-productive infection in non-native hosts such as mouse cells, leading to mode of cancer cell death different from the mode of cell death in native host. As we will discuss extensively later, the mode of cancer cell death dictates to a significant degree the subsequent antitumor immunity. As a consequence, the OV-elicited antitumor immunity in tumor models of syngeneic animals might not be relevant to the situation in human cancer patients. This is an often overlooked issue when tumor models in animals are chosen along with OVs as therapeutic models for human cancer.

SIGNAL 0: DAMPs AND PAMPs

PAMPs: SIGNAL 0s FROM PATHOGENS

In the late 1980s, Charles Janeway proposed that the immune system protects the host against infectious pathogens by presenting the molecules as signal 0s, which is what now called PAMPs, to the antigen presenting cells (22, 23). PAMPs consist of essential components of microorganisms that direct the targeted host cells, key components in the innate immune arm, to distinguish "self" from "non-self," and promote signals associated with innate immunity (24). Major PAMPs are nucleic acids (DNA, double-stranded RNA, single-stranded RNA, and 5′-triphosphate RNA), proteins (lipoproteins and glycoproteins), as well as other components of the cell surface and membrane (17, 25). Interestingly, defective viral genomes arising *in vivo* are a critical danger signal for triggering antiviral immunity in the lung (26).

This concept of PAMPs has been strongly supported by the discovery of several classes of pattern-recognition receptors (PRRs). These PRRs include the toll-like receptors (TLRs), retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), nucleotide oligodimerization domain (NOD)-like receptors (NLRs), AIM2-like receptors, and the receptor for advanced glycation end products (RAGE) (17, 27). It is now well accepted that both DAMPs and PAMPs stimulate the innate immune system through PRRs. DCs express a wide repertoire of these PRRs. The binding of PAMP to its receptors on the APC activates the DCs (28, 29).

DAMPs: SIGNAL 0s FROM HOST

Matzinger proposed what is known now as the "danger theory" in 1994 (30). In the theory, it proposed that the immune system can distinct self from non-self and dangerous from innocuous signals. In this model, APCs are activated by both PAMPs and DAMPs from distressed or damaged tissues or microbes. The theory has been well accepted in recent years, as we have learned more and more about how dying cells alert immune system to danger (31). Over the years, a number of endogenous danger signals have been discovered. For examples, it was shown that uric acid functions as a principal endogenous danger signal, which is released from injured cells (32).

Damage-associated molecular patterns are molecules derived from normal cells that can initiate and perpetuate immunity in response to cell stress/tissue damage in the absence of pathogenic infection. DAMPs vary greatly depending on the type of cell and injured tissue. They can be proteins, DNA, RNA, or metabolic products. Protein DAMPs include intracellular proteins, such as high-mobility group box 1 (HMGB1), heat-shock proteins (HSPs), and proteins in the intracellular matrix that are generated following injury, such as hyaluronan fragments (33). HMGB1 is one prototypic DAMP (34, 35). The protein DAMPs can be localized within the nucleus, cytoplasm, cell membrane, and in exosomes, the extracellular matrix, or as plasma components (17). Other types of DAMPs may include DNA, ATP, uric acid, and heparin sulfate. It is interesting to note that mitochondria are a rich and unique source of DAMPs, including formyl peptides, the mitochondrial DNA (mtDNA)-binding proteins, transcription factor TFAM, and mtDNA itself (36). Following interactions between DAMPs and PRRs on the target cells, the intracellular signaling cascades triggered by the interactions between DAMPs and PRRs lead to activation of genes encoding inflammatory mediators, which coordinate the elimination of pathogens, damaged, or infected cells (27). In cancer, chronic inflammation and release of DAMPs promotes cancer, while acute inflammation of release/presentation of DAMPs may induce potent antitumor immunity and helps in cancer therapy (35, 37). Based on the work in chemotherapy and radiation therapy, the concept of ICD of cancer cells has been established about 10 years ago (37, 38). As we will discuss below,

this concept leads to development of novel strategies for cancer therapeutics.

OVs INDUCE MOSTLY MULTIMODALITY ICD AND RELEASE/PRESENT DANGER SIGNAL MOLECULES

Investigators have long been interested in what defines the immunogenicity of cancer cells and how we can enhance the immunogenicity for the purpose of immunotherapy. Pioneering work by Lindenmann and Klein almost half a century ago demonstrated that viral oncolysis of cancer cells by influenza virus increases immunogenicity of tumor cell antigens (39). However, it was not clear how this immunogenicity was enhanced at the time. Over a decade ago, it was found that tumor immunogenicity is enhanced by cell death via induced expression of HSPs (40). A few years ago, investigators working on chemotherapy and radiation for cancer therapy have led to this new concept as they classify the types of cancer cell death by the immunological consequence, into "immunogenic cancer cell death" (ICD) and "non-immunogenic cancer cell death" (NICD) (41–43). The original concept of ICD includes only "immunogenic apoptosis." We and others have recently proposed that ICD includes not only immunogenic apoptosis, but also necroptosis, necrosis, autophagic cell death, and pyroptosis of cancer cells (**Figure 1**) (44, 45). Basically, cancer cells dying via ICD have the following common features as summarized by Tesniere, Zitvogel, Kroemer, and their colleagues (46). They stated that, "some characteristics of the plasma membrane, acquired at pre-apoptotic stage, can alarm immune effectors to recognize and then attack these pre-apoptotic tumor cells. The signals that mediate the immunogenicity of tumor cells involve elements of the DNA damage response, elements of the endoplasmic reticulum stress response, as well as elements of the apoptotic response" (46). For cells undergoing pre-apoptotic phase, they may express "danger" and "eat-me" signals on the cell surface (calreticulin and HSPs) or can secrete/release immunostimulatory factors (cytokines, ATP, and HMGB1) to stimulate innate immune effectors (46). For other types of ICD, extracellular ATP, HMGB1, uric acid, other DAMPs, and PAMPs released in the mid or late phases functions as potent danger signals, thus making it highly immunogenic.

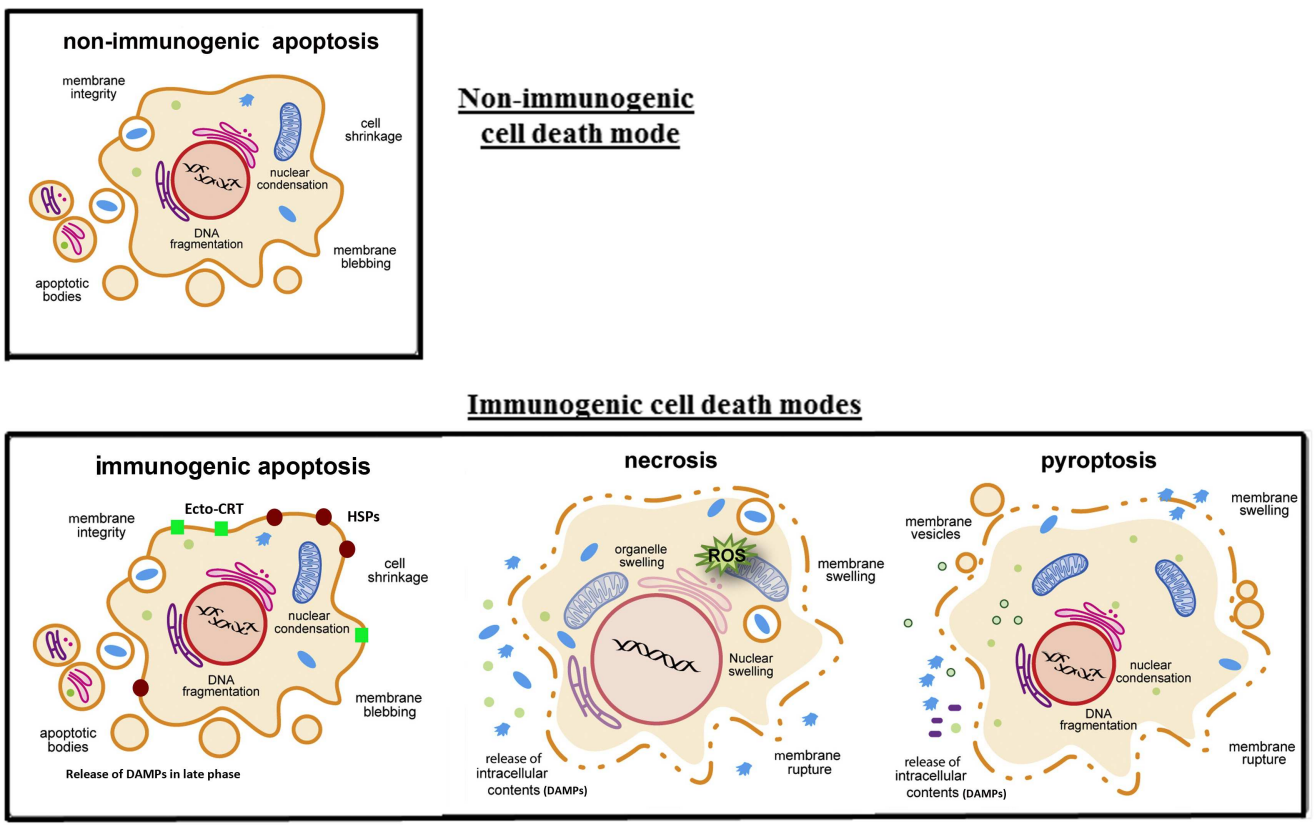

FIGURE 1 | Four key modes of cancer cell death and their immunogenicity. In classic apoptosis, the retention of plasma membrane integrity and the formation of apoptotic bodies render it an immunologically silent death mode, or non-immunogenic cell death. However, recent studies have shown that cancer cells treated with certain cytotoxic agents (some chemotherapeutic agents and oncolytic viruses) lead to the cell surface exposure of calreticulin (ecto-CRT) and heat-shock proteins (HSPs) prior to apoptosis, and other DAMPs released in the later phase of apoptosis, danger signals to DCs. This is immunogenic apoptosis. Cancer cells dying by necrosis/necroptosis or pyroptosis secrete pro-inflammatory cytokines and release their cytoplasmic content, including DAMPs (ATP, HMGB1, and uric acid, etc.), into the extracellular space. Some DAMPs (such as HMGB1) can be secreted through non-classical pathways (25). These later modes of cancer cell death are ICD. Drawings are modified and reprinted from Lamkanfi and Dixit (47), copyright 2010, with permission from Elsevier.

Oncolytic viruses kill cancer and associated endothelial cell through a variety of types of cell death as classically defined by the morphological and ultrastructural changes of dying cells. These include apoptosis, necrosis, necroptosis, pyroptosis, and autophagic cell death, often with one as the predominant form of death for a particular OV. By the new definition, cancer cell death induced by OVs is mostly immunogenic (**Table 1**). Probably all oncolytic Ads induced autophagic cell death in cancer cells (48–51). Coxsackievirus B3 (CVB3) induces immunogenic apoptosis in human non-small cell lung cancer cells (52). Measles virus (MV) causes ICD in human melanoma cells, because inflammatory cytokines and HMGB1 are released, and DCs are activated by MV-infected cancer cells (53). HMGB1 release often happens in late stage of apoptosis, during autophagy process and in necrotic cells infected with OVs. We first reported in 2005 that human cancer cells infected by an oncolytic poxvirus, led to necrotic/apoptotic death pathways and release of HMGB1 (54). Later studies have confirmed and extended the findings of HMGB1 release in cancer cells infected with Ads (12), CVB3 (52), an MV (53), vaccinia viruses (VVs) (55–57), HSV (14, 58), and parvovirus H-1 (H-1PV) (59). Extracellular ATP is another potent danger signal released from OV-infected cancer cells (12, 52, 56, 60). The third danger

Table 1 | Oncolytic viruses lead to specific mode of immunogenic cell death and exposure/release of DAMPs/PAMPs.

OV	DAMP/PAMP	Receptor	Type of cell death	Immunological functions	Reference
Ad5/3-D24-GM-CSF; CVB3; vvDD	ATP	P2Y2 and P2X7	Necrosis, autophagic cell death, and immunogenic apoptosis	Function as a "find-me" signal, and cause NLRP3-inflammasome-based IL-1β production	(52, 56, 60)
Ad5/3-D24-GM-CSF; CVB3	Ecto-CRT (calreticulin)	CD91	Immunogenic apoptosis (either pre-apoptotic, early or mid apoptotic surface exposure) or secondary necrosis	Function as an "eat-me" signal and it is a potent mediator of tumor immunogenicity crucial for elicidation of antitumor immunity	(52, 60)
Parvovirus H-1 (H-1PV)	HSPs: (HSP90, HSP70, Hsp72)	CD91, TLR2, TLR4, SREC1, and FEEL1	Immunogenic apoptosis (surface exposure) or necrosis (passively released)	Surfaced-exposed HSP90 can mediate adaptive antitumor immunity, while secreted HSP90 can inhibit TGF-β1 activation; Leads to TAA-specific antitumor immunity	(65–67)
? (Not identified)	Histones	TLR9	Apoptosis (cell surface exposure) or accidental necrosis (passively released)	Released histones can cause initiation of TLR9-MyD88-mediated inflammation	(68)
Many OVs: Ad; HSV; MV; VV; H-1PV	HMGB1	TLR2, TLR4, RAGE ,and TIM3	Immunogenic apoptosis; necrosis; autophagic cell death	Activate macrophages and DCs; recruit neutrophils; promote *in vivo* the production of IFN-γ, TNF-α, IL-6, IL-12, and antigen-specific activation of $CD8^+$ T cells	(53, 54, 56, 57, 59, 60)
MV-eGFP	IL-6	IL-6R and GP130	Necroptosis	A cell type-specific endokine DAMP with potent pro-inflammatory activity	(53)
Telomelysin (Ad)	Uric acid	P2Y6	Autophagic cell death	Stimulate the production of inflammatory cytokines such as IL-1, TNF-α, and IL-6 and chemotactic factors for neutrophils such as IL-8/CXCL8 and S100A8/A9	(61, 69)
Newcastle disease virus (NDV)	dsRNA and other PAMPs	TLR3; and by the cytoplasmic receptors MDA-5 and RIG-I	Immunogenic Apoptosis; autophagy	(1) Upregulation of HLA antigens and ICAM-1; (2) induction of type I IFNs and chemokines (CCL5 and CXCL10); (3) activate DCs and T effector cells but also to block Treg cells; (4) local therapy with oncolytic NDV induces inflammatory immune infiltrates in distant tumors, making them susceptible to systemic therapy	(70–74)
Reovirus	The virus itself (PAMP)	Dendritic cells (DCs)	(Cancer cell independent mechanism)	Induce DC maturation and stimulate the production of the pro-inflammatory cytokines IFN-α, TNF-α, IL-12p70, and IL-6. Reovirus directly activates human DC and that reovirus-activated DCs stimulate innate killing by not only NK cells, but also T cells	(75)

signal molecule released from OV-infected cells is uric acid (61). Some OVs may induce cell death partly through pyroptosis, a caspase-1 dependent inflammatory form of cell death (62). Both necrotic cells and pyroptotic cells release ATP more efficiently than apoptotic cells do. Pyroptotic cells, just like apoptotic cells, actively induce phagocytosis by macrophages using "eat-me" and "find-me" signals (63). Cytolytic immune cells, elicited by OVs or other agents, kill additional cancer cells leading to release of DAMPs such as HMGB1 (64). In summary, most OVs induce ICD of cancer cells and present/release a number of potent danger signals, and TAAs to DCs to trigger adaptive immune response (**Table 1**).

Cancer cell death induced by some OVs has not been examined for their direct features of ICD. However, other properties suggest that cancer cells infected by the OV are immunogenic, or the viruses themselves are highly immunogenic. Newcastle disease virus (NDV) is a well-studied virus for its virology and immunostimulatory properties (76). NDV induces cancer cells into apoptosis (70), with autophagy taking place during the process (71). Human cancer cells infected by NDV show upregulation of HLA class I and II antigens, and costimulatory molecule ICAM-1, as well as induction of IFNs, chemokines (IP10 and RANTES) before apoptosis (72). Moreover, the inflammatory conditions and type I IFNs inhibit Treg cells (73). With these potent immunostimulatory properties, local administration of oncolytic NDV overcomes systemic tumor resistance to immunotherapy by blockade of immune checkpoints (74). Another RNA virus, reovirus, also induces cancer cells into apoptosis (77, 78), with autophagy taking place in the process (79–81). Melanoma cells infected with reovirus release a range of inflammatory cytokines and chemokines while IL-10 secretion is abrogated (82). These molecules may provide a useful danger signal to reverse the immunologically suppressive environment of this tumor. Even more interestingly, reovirus can also interact with DCs directly and matured DCs activate NK and T cells (75) (**Table 1**). Those activated NK and T cells exert innate killing of cancer cells. This innate effector mechanism may complement the virus's direct cytotoxicity and thus induced adaptive antitumor immunity, potentially enhancing the efficacy of reovirus as a therapeutic agent (75).

OV-INDUCED AUTOPHAGY IN CANCER CELLS PROMOTES CROSS-PRESENTATION OF TAAs AND ELICITS STRONGER ANTITUMOR IMMUNITY

Autophagy mediates sequestration, degradation, and recycling of cellular organelles and proteins, and intracellular pathogens. It is not too surprising that autophagy plays roles in both innate and adaptive immunity (17, 83). A number of OVs, such as Ad (48–51), encephalomyocarditis virus (84), HSV (62, 85, 86), influenza virus (87), NDV (71), reovirus (79–81), and VSV (84), induce autophagy in infected cancer cells. Evidence shows that autophagy may enhance tumor immunogenicity. One mechanism is that autophagic cells selectively release DAMPs such as ATP (88, 89), HMGB1 (90), and uric acid (61). The other mechanism is that autophagy promotes antigen cross-presentation from cancer cells by DCs to naïve T cells. It stimulates antigen processing for both MHC class II (91), and MHC class I pathways. These have been demonstrated for endogenous viral antigens during HSV-1 infection (85), and for cross-presentation of TAAs from uninfected cancer cells (92), and influenza A virus-infected tumor cells (93). In other words, autophagy within the antigen donor cells facilitates antigen cross-priming to generate TAA-specific or virus-specific $CD8^+$ T cells (92–95). This property has been explored for cancer vaccines (96), and for enhanced OV-mediated antitumor effects in the future (97).

VIRUSES OFTEN ENCODE SPECIFIC GENES TO MODULATE APOPTOSIS, AUTOPHAGY, NECROPTOSIS, AND POSSIBLY OTHER DEATH PATHWAYS

Successful viral replication requires the efficient production and spread of progeny virus, which can be achieved through efficient evasion of host defense mechanisms that limit replication by killing infected cells. Viruses have thus evolved to encode genes whose products function to block or delay certain cell death pathways until sufficient progeny have been produced (47). These gene-encode products target a variety of strategic points in apoptosis, necroptosis, autophagy, or other death pathways. **Table 2** lists some examples of genes encoded by viruses especially OVs that can intervene apoptosis, autophagy, or necroptosis. The presence of these types of viral genes may skew the mode of infected cancer cells from one to another cell death pathway(s). OVs can be engineered genetically with deletion or insertion of such genes so that a desired mode of ICD would happen in the virus-infected cancer cells.

CANCER CELLS OFTEN SHOW DEFECTS IN CERTAIN CELL DEATH PATHWAYS

Every cell in a multicellular organism has the potential to die by apoptosis. However, cancer cells often have faulty apoptotic signaling pathways evolved during carcinogenesis. This property derives from the overexpression of anti-apoptotic genes, deficiency of pro-apoptotic genes, or both (121). These defects not only increase tumor mass, but also render the cancer resistant to therapy.

Evidence has also been accumulating that necroptosis can be impaired in cancer cells. Chronic lymphocytic leukemia cells have defects in signaling pathways involved in necroptosis regulation such as RIP3 and the deubiquitination cylindromatosis (CYLD), an enzyme directly regulating RIP1 ubiquitination (122). Skin cancer cells contain an inactivating CYLD mutation (123). Despite the fact some cancers are resistant to necroptosis due to genetic and epigenetic defects, necroptosis undoubtedly represents an important death pathway induced by many anticancer regimens, particularly important to those cancer resistant to apoptosis. In this case, investigators have found that some compounds can circumvent cancer drug resistance by induction of a necroptotic death (124).

The fact that cancer cells resist certain death pathways will dictate to a degree which types of drugs (including OVs) to be used in therapeutic regimens. As we stated before, a number of OVs, such as VVs, often induces cancer cells into necroptotic cell death (54, 56, 57), while other viruses such as oncolytic Ad often induce cancer cells into autophagic cell death. Appropriate OVs can be picked depending on the sensitivity of the cancer to certain death pathways, and the immunogenic consequence if it is combined for immunotherapy.

Table 2 | Examples of viruses and viral genes modulating apoptosis, autophagy, and necroptosis.

Virus	Gene	Type of action	Mechanism of action	Reference
Ad	E1A	AS	Associate with the pRb/p300 family and induce p53-dependent apoptosis	(98)
	E1B-19K	AI	Sequester pro-apoptotic Bcl-2-like proteins and p53; inhibit apoptosis triggered by numerous stimuli	(99–101)
	E1B-55K	AI	Bind to p53 and functionally inactivates it	(102)
	E3-6.7	AI	Complexes with 10.4 and 14.5 resulting in downregulation of TRAIL receptors	(103)
HSV	ICP34.5	ATI	Inhibit PKR signaling and directly bind to beclin-1	(104)
	ICP34.5	AI	IFN-mediated pathway; decrease eIF-2α phosphorylation by PKR	(105–107)
	Us3	AI	Ser/Thr kinase that prevents virus-induced apoptosis	(108)
	Us5	AI	Cooperates with Us3	(108)
VV	SPI-1		Serpin, inhibit cell-cell fusion	(109)
	SPI-2	AI	Serpin, direct inhibitor of caspases	(110)
	F1L	AI	Interact with the pro-apoptotic protein Bak and inhibit Bak activation	(111)
	N1L	AI	Inhibit multiple pro-apoptotic Bcl-2-like proteins	(112)
MYXV	M11L	AI	Prevent the mitochondria from undergoing a permeability transition; inhibit apoptotic response of macrophages and monocytes	(113, 114)
MCMV	vIRA	NI	Target RIP1, RIP3, TRIF, and DAI; inhibit RIP3-dependent necrosis	(115)
Influenza virus	M2	ATI	Block autophagosome fusion with lysosomes	(116)
	NS1	AI/ATS	Inhibit apoptosis and upregulate autophagy	(117)
Measles virus	H	AS	Induce apoptosis of HeLa cells via both extrinsic and intrinsic pathways	(118)
	Virion	ATS	Binding of virus to CD46 on cell surface induces autophagy	(119)
NDV	V	AI	Inhibit IFN response and apoptosis	(120)

AI, apoptosis inhibitor; AS, apoptosis stimulator; NI, necroptosis inhibitor; ATI, autophagy inhibitor; ATS, autophagy stimulator.

STRATEGIES TO MODULATE THE MODE OF CANCER CELL DEATH FOR ENHANCED IMMUNOGENICITY

We know now that immunogenic apoptosis, necrosis/necroptosis, and autophagic cell death are desired modes of cancer cell death because they are ICD. Is immunogenic apoptosis (the original form of ICD) better than other forms of ICD in the induction of antitumor immunity? We do not know for sure. This question needs to be addressed in the future. What we do know now is that there are strategies that can enhance the ICD and subsequent anti-tumor immunity. They can be classified into, genetic modification of OV vectors, combination with ICD inducers, and combination with specific immunostimulatory regimens.

GENETIC ENGINEERING OF VIRAL VECTORS

Cancer cells have usually accumulated a number of genetic mutations and epigenetic modifications that enable them to resist apoptosis. Based on this property, a number of OVs are built for high tumor selectivity by deleting viral genes encoding anti-apoptotic genes (see **Table 2**). These viruses can replicate in cancer cells but lead to rapid apoptosis in normal cells. For examples, the γ34.5 gene has been deleted in many oncolytic HSVs, including the T-VEC that is going through a successful phase III clinical trial (125). The adenoviral protein E1B-19K is a Bcl-2 homolog that blocks apoptosis induction via the intrinsic and extrinsic pathways, specifically including tumor necrosis factor (TNF)-mediated cell death. Liu et al. have demonstrated that an E1B-19K gene deletion mutant had TNF-enhanced cancer selectivity due to genetic blocks in apoptosis pathways in cancer cells (126). Similarly, a tumor-selective oncolytic vaccinia virus was constructed by deleting two serpin genes, SPI-1 and SPI-2 (54). Due to the deletion of viral anti-apoptosis genes, these mutant OVs display more potent oncolysis through apoptosis pathways when combined with appropriate apoptosis-inducing agents.

We believe that by arming OVs with necrosis and autophagy-promoting genes, it is possible that the desired cell death pathway can be activated in cancer cells when infected with such OVs, leading to more ICD. More future studies with this strategy are warranted.

COMBINATION WITH ICD INDUCER OR AUTOPHAGY INDUCER

In theory, OV in combination with an ICD inducer would provide more potent danger signals to DCs and potentially elicit stronger antitumor immunity. Workenhe et al. demonstrated in a recent study that such a strategy worked well indeed (127). HSV-1 ICP0 null oncolytic vectors possess antitumor activity, but the virus alone is insufficient to break immune tolerance. Thus, the authors hypothesized that combination therapy with an ICD-inducing chemotherapeutic drug might get the job done. Indeed, the combination of HSV-1 ICP0 null oncolytic virus with mitoxantrone, which induces ICD, provided significant survival benefit to the Balb/C mice bearing Her2/neu TUBO-derived mammary tumors. Increased infiltration of neutrophils and tumor

antigen-specific $CD8^+$ T cells into tumor tissues provide the protection, as depletion studies verified that CD8-, CD4-, and Ly6G-expressing cells are essential for the enhanced efficacy. Importantly, the combination therapy broke immune tolerance. In conclusion, this study suggests that such a combination can enhance the tumor immunogenicity, breaking immunologic tolerance established toward the tumor antigens, thus a promising novel strategy for cancer therapy (127).

As we stated earlier, the autophagy in antigen donor cells (cancer cells) promotes the cross-presentation of antigens from DCs to T cells. The autophagy could be induced by some OVs, or its inducer could be provided *in trans*. This strategy works in combination with oncolytic adenoviruses that induce autophagy by themselves (60, 128). However, it may not work with an oncolytic vaccinia virus that does not induce autophagy by itself (our unpublished data).

ARMED VIRUS AND COMBINATION STRATEGIES FOR BREAKING IMMUNE TOLERANCE AND ENHANCING ANTITUMOR IMMUNITY

In order to further enhance the antitumor immunity, OVs have been armed with TAAs, cytokines (e.g., GM-CSF), chemokines (such as CCL5), or other innovative and artificial genes. We have recently reviewed the promising strategies of OVs in combination with other immunotherapeutic regimens (44). As we mentioned, two OVs in the most advanced stages of clinical trials, T-VEC, and Pexa-Vec, are HSV and VV armed with GM-CSF (125, 129). An oncolytic VV expressing the 4-1BBL T cell costimulatory molecule (rV-4-1BBL) showed modest tumor regression in the poorly immunogenic B16 murine melanoma model. However, rV-4-1BBL injection with lymphodepletion promoted viral persistence by reducing antiviral antibody titers, and promoted MHC class I expression, and rescued effector-memory $CD8^+$ T cells. This significantly improved the therapeutic effectiveness of the oncolytic virus (130). Similarly, an unarmed oncolytic virus combined with anti-4-1BB agonist antibody elicits strong antitumor immunity against established cancer (56). We have also shown that the chemokine CCL5-expressing oncolytic VV in combination with a cancer vaccine or activated T cells resulted in better therapeutic effect in a MC38 colon cancer model (131). Recently, our collaborators have made an oncolytic VV encoding a secretory bispecific T cell engager consisting of two single-chain variable fragments specific for CD3 and the tumor cell surface antigen EphA2 [EphA2-T cell engager-armed VV (EphA2-TEA-VV)] (132). This virus retains its normal oncolytic potency and the secreted molecule also activates T cells. The virus plus T cells had potent antitumor activity in a lung cancer xenograft model. Thus, arming oncolytic VVs with T cell engagers may represent a promising approach to improve oncolytic virotherapy. In the context of OV-mediated cancer immunotherapy, it is interesting to observe the dual effects of antiviral immunity on cancer therapy. On one hand, the antiviral immunity may attenuate the replication of an OV and thus diminish the effect of direct oncolysis; on the other hand, antiviral immunity plays a key role for the therapeutic success of oncolytic virotherapy in some cases (11, 133).

The tumor-associated immune tolerance is a big obstacle in cancer immunotherapy. Some armed OVs (such as a GM-CSF-armed oncolytic Ad) can break immune tolerance and generated antitumor immunity in at least some human cancer patients (134). In other cases, an OV alone is not enough to break the immune tolerance in highly immunosuppressive TME (127). In these cases, a combination with an ICD-inducing chemotherapeutic drug may break the immune tolerance (127). Alternatively, an OV can be combined with an immune checkpoint inhibitor to achieve the same effect. During the preparation of this review, a study has just been published on such a strategy with oncolytic NDV and systemic CTLA-4 blockade. This combination led to rejection of pre-established distant tumors and protection from tumor rechallenge in poorly immunogenic tumor models (74). It showcases the promise of such a combination strategy.

CONCLUSION AND PERSPECTIVES

The TME in the advanced stage of disease is highly immunosuppressive (18). This immunological property is a double-edged sword for OV-mediated cancer therapy: good for viral replication but bad for the antitumor immunity. The evidence is accumulating that OVs not only kill infected cancer cells and associated endothelial cells by direct and indirect oncolysis, but also release/present danger signals to DCs and other professional APCs to elicit both antiviral and antitumor immunity. It has been demonstrated for a number of OVs, that the virus-elicited antitumor immunity plays a critical role in the overall efficacy of oncolytic virotherapy. As we and other colleagues have realized, ICD is important to elicit antitumor immunity (44, 45, 135).

In order to improve the potency of antitumor immunity, one key step is the initial presentation of danger signal (signal 0) and cross-presentation of TAAs (signal 1). Recent studies demonstrated that ICD of cancer cells leads to potent danger signals, and autophagy in antigen donor cells, in this case cancer cells and associated endothelial cells, enhance the cross-presentation of TAAs to naïve T cells by DCs. Genetic engineering and combination strategies can skew the cancer cell death into modes of ICD and autophagy, leading to potent and sustained antitumor immunity and thus enhancing the efficacy of oncolytic immunotherapy. Which mode of ICD in the context of OVs is the most potent way to elicit antitumor immunity needs careful investigation in the near future. It is also important to keep in mind that oncolytic viruses modulate cancer immunogenicity through multiple mechanisms (136). Other than the induced danger signals, they are out of the scope of this review article and thus have not been discussed. Finally, we and others believe that it is important to further test the idea that combination of OV with blockade of immune checkpoints for potent and sustained antitumor immunity would enhance this novel form of immunotherapy for cancer. We look forward to more exciting development of both preclinical and clinical studies with OVs as tools for cancer immunotherapy.

AUTHOR CONTRIBUTIONS

Zong Sheng Guo collected and read relevant papers; designed and drafted the manuscript. David L. Bartlett and Zuqiang Liu have made suggestions to the manuscript. All authors have read and approved the final manuscript.

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Immunotherapeutic potential of oncolytic vaccinia virus

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The concept of oncolytic viral therapy was based on the hypothesis that engineering tumor-selectivity into the replication potential of viruses would permit direct destruction of tumor cells as a result of viral-mediated lysis, resulting in amplification of the therapy exclusively within the tumor environment. The immune response raised by the virus was not only considered to be necessary for the safety of the approach, but also something of a hindrance to optimal therapeutic activity and repeat dosing. However, the pre-clinical and subsequent clinical success of several oncolytic viruses expressing selected cytokines has demonstrated the potential for harnessing the immune response as an additional and beneficial mechanism of therapeutic activity within the platform. Over the last few years, a variety of novel approaches have been incorporated to try to enhance this immunotherapeutic activity. Several innovative and subtle approaches have moved far beyond the expression of a single cytokine transgene, with the hope of optimizing anti-tumor immunity while having minimal detrimental impact on viral oncolytic activity.

Keywords: oncolytic virus, cytokine, chemokine, vaccine, MDSC

BACKGROUND

Viral infections and cancer bear a variety of striking similarities, as seen with the fact that several cancers are caused as a result of chronic viral infection (1, 2) and the fact that the first oncogenes were identified through their homology to viral genes (3, 4). Indeed, many of the hallmarks of cancer strongly resemble the adaptations a virus induces in a susceptible cell during its replication cycle (5). It is therefore unsurprising that some viral virulence genes are redundant for replication in malignant cells or the tumor microenvironment, meaning that their deletion results in the production of vectors whose replication is attenuated in normal, but not cancer cells (6). This finding was the basis for the design and construction of the first oncolytic viral therapies (7, 8). As the name "Oncolytic" suggests, these were hypothesized to function through the direct destruction of cancer cells, primarily as a result of viral replication in infected cells, but also as a result of immune recognition of these cells. Initial clinical testing of this approach centered around strains of adenovirus (8–12), perhaps more due to the historical use of non-replicating adenoviruses as gene therapy vectors than because of any special attributes of this backbone particularly appropriate for an oncolytic platform. However, importantly these early clinical studies demonstrated both safety and therapeutic responses (13–16). The observation that the viral infection occurring primarily within the tumor was cleared, leading to induction of anti-viral immunity, and implied the agents were capable of at least transiently overcoming localized immune suppression within the tumor.

The slow replication and limited systemic spread of the Ad5-based vectors proved to be especial limitations (17, 18), however, the excellent safety profile and indications of responses led investigators to examine other adenoviral serotypes and more potent viral backbones as the basis for next generation oncolytics. In some cases, combination with immunosuppression was investigated as a means to enhance oncolytic activity through delaying clearance of the therapy (19, 20). However, in general pre-clinical and clinical testing of different viral backbones in combination with expression of different therapeutic transgenes led to the observation that the most effective approaches frequently incorporated rapidly replicating viral backbones expressing cytokines as transgenes, notably GM-CSF (2, 21–23). In addition, when immunocompetent pre-clinical tumor models were available, it was frequently seen that complete responses after viral therapy was coupled with induction of anti-tumor immunity and the capacity to reject re-challenge with the same tumor cell lines (24). As such considerable focus has turned to development of approaches to enhance or optimize this immunotherapeutic effect. However, there is clearly a fine balance to be considered as robust induction of the immune response can lead to premature clearance of the therapy, meaning that the oncolytic effects are lost and adaptive immunity is targeted against the viral component only, with little or no cross-presentation of tumor antigens.

One viral strain that has been developed as an alternative to adenovirus in forming the backbone of many oncolytic viral strains is vaccinia virus (25). This enveloped DNA virus has a large genome, with many virulence genes that target host cell cycle, apoptotic pathways, or immune response, and whose deletion leads to viral strains with demonstrated tumor-selective replication (26–28). In addition, the use of this virus during the eradication of smallpox means that its immune activating capacity is well understood. The clinical use of a GM-CSF expressing oncolytic vaccinia virus has also been instrumental in demonstrating the potential for enhancing the immune response induced by oncolytic viruses as a means to enhance therapeutic activity (23, 29, 30). Oncolytic vaccinia will therefore be used as the primary example to illustrate the

potential for enhancing immune-mediated mechanisms in this platform throughout the remainder of this review.

APPROACHES TO ENHANCE THE ANTI-TUMOR IMMUNE RESPONSE INDUCED BY ONCOLYTIC VACCINIA

Several standard strategies have been routinely applied to enhance the anti-tumor immune response induced through oncolytic viral therapy, primarily focused on expression of either cytokines or co-stimulatory molecules (31). Although several vaccinia and related vectors expressing single cytokines have clearly demonstrated therapeutic benefits in both animal models and in the clinic, this approach typically suffers from several handicaps, including reduced viral replication due to reduced initial infection of the tumor or early clearance of the virus (32); in addition, the use of a single cytokine means that typically only one step in the complex kinetic process of innate to adaptive immune response can be stimulated. The most clinically successful approach to date has been the expression of GM-CSF (33), both from vaccinia virus and from oncolytic HSV. The choice of GM-CSF was based on early reports that expression of GM-CSF from mouse melanoma cell lines resulted in failure of these cells to form tumors in syngeneic mice (34). It is likely that the reason for the success of vectors expressing this cytokine is primarily based on the fact that it has broad effects on induction of proliferation in many immune cell subtypes, while having little or no direct anti-viral properties. However, more recently the role of GM-CSF expression in increasing proliferation of some suppressive cells [notably monocyte derived suppressor cells (MDSC)] (35) has been elucidated meaning that some caution in the use of this cytokine might be needed.

Several other cytokines, including IL-2, TNF, and IFN have been used in pre-clinical vaccinia-based models but have not been successfully translated into a clinical setting, possibly due to their capacity to also induce more directly anti-viral effects (32, 36, 37). Because of this limited success with cytokines other than GM-CSF alternative immune stimulating strategies has been explored.

For example, the expression of antibodies represents a promising strategy. The relative success of monoclonal antibody therapy and the recent emergence of antibodies targeting immune checkpoint inhibitors or that mimic co-stimulators has demonstrated the potential of this platform. The requirements for expression and assembly of multiple large peptides had traditionally limited the use of antibodies as transgenes, however, more recent development of single peptide antibodies means this is likely to be a fruitful approach moving forward and initial reports of vaccinia strains expressing antibodies are promising (38).

An alternative strategy to enhancing the immune effects of oncolytic viruses is to express chemokines from the vectors that specifically attract T-cells into the tumor (39, 40). This approach appears to have minimal negative impacts on viral replication and oncolytic activity, yet enhances the immunotherapeutic effects. One of the major hurdles found with the use of therapeutic tumor vaccines has been the limited trafficking of tumor-specific T-cells into the tumor itself, so the combination of chemokine expressing oncolytic viruses with vaccination against tumor antigens may be a promising strategy.

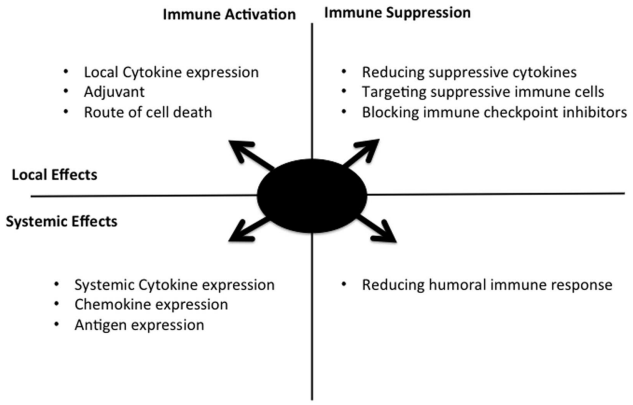

FIGURE 1 | Selective replication of an oncolytic virus within an infected tumor cell might be engineered in multiple ways to optimize the kinetics, type, and level of resultant immune response. The approaches covered in this perspective are summarized here, along with their range of activity (local acting within the tumor, or systemic activity) and whether immune activation or blocking of immune suppression are involved. Ideal combination approaches would be predicted to involve components of different quadrants in the figure.

Several other alternatives to cytokine expression have been explored in different oncolytic backbones as a means to enhance the immunotherapeutic effects (**Figure 1**), including the following: (i) Enhancing immunogenic cell death, it has been proposed that the route of cancer cell death after therapy may be a critical mediator of the immune response. As a result, adjusting how an oncolytic virus destroys the infected cell may promote a more robust anti-tumor immune response (41). (ii) Targeted inhibition of specific components of the immune response: as an alternative to specifically activating key pathways, key mediators of less desirable immune response pathways may be targeted for removal or depletion. One example is the use of TGFb decoy receptors to remove this cytokine that has been implicated in metastasis and tumor growth and angiogenesis (42). Alternatively, direct targeting of B-cells or other components of the humoral response may limit production of anti-viral neutralizing antibody, a key limiting factor in repeat dosing with the same therapeutic virus (43). (iii) Role of adjuvant: the field of vaccine development has helped define the importance of adjuvant in eliciting a robust immune response. Although the expectation is that the viral vectors themselves will provide a source of potent adjuvant, this can and has been enhanced through expression or manipulation of the virus, such as through the incorporation of CpG rich motifs into the DNA sequence (44), or through combination with CpG (45).

ALTERNATIVE STRATEGIES

In addition to the expression of immune stimulatory (or inhibitory) transgenes from the viral vectors, a variety of other options exist that can also have direct impact on the immune responses induced by the viral vectors.

(i) *Viral mutation*: large DNA viruses such as vaccinia or HSV encode multiple virulence genes whose role is to antagonize or

deplete specific cytokines or to disrupt steps in the immune response. Selective deletion of specific virulence genes can therefore have two effects; deletion of these genes often results in tumor targeting (through attenuation of viral replication in non-tumor tissues) while also allowing additional induction of specific components of the immune response to be activated (28). In this way, the immune response can be manipulated without seriously depleting viral oncolytic activity.

(ii) *Timing of immune activation*: the therapeutic activity of oncolytic viral therapy can be considered to function in two steps, with an initial directly oncolytic phase mediated by viral lysis of tumor cells followed by an immunotherapeutic phase, where the host immune response clears additional infected tumor cells and ideally results in induction of an adaptive immune response against tumor antigens (as a result of the release of the tumor antigens along with viral PAMPs and host cell DAMPs). It is therefore apparent that the expression of immune activating transgenes would be most effective if limited to this second phase. One approach to achieve this is to apply exogenous regulation of transgene expression or to control the function of the protein after it is translated (32, 37). In this way, it may be possible to achieve unhindered viral replication and so full oncolytic potential during the initial period after viral delivery and tumor infection, while subsequent activation of immune stimulatory transgene activity would enhance the level and type of immune response produced at later times.

(iii) *Targeting immune suppression*: in addition to activating the immune response, oncolytic viral vectors can also transiently overcome immune suppression within the tumor. However, this effect is likely only temporary or limited and some evidence exists that additional targeting of these suppressive cells may further enhance oncolytic viral activity (46). Because multiple suppressive immune cell types (including MDSC, M2 macrophages, and regulatory T-cells) often exist within the tumor, it may be necessary to target all these in a concerted fashion to ensure that a robust adaptive immune response is produced.

(iv) *Combination therapies*: in addition, the development of multiple novel and effective immunotherapies means that the scope for combining oncolytic viral therapies with these other therapies continues to increase. There are a variety of approaches (such as combination with alternative adjuvants or anti-immune checkpoint inhibitors) that would be expected to produce significant synergistic benefit, and promising initial pre-clinical data means that these will be explored in more detail in the future.

(v) *Oncolytic viral vaccines*: the fact that oncolytic viral therapies are capable of inducing an adaptive immune response against tumor antigens is likely to be hugely beneficial in the clearance of residual disease and metastases as well as in long term immune surveillance to prevent relapse. However, it is also likely that any adaptive immune response that is produced after viral therapy will primarily target antigens expressed on the bulk tumor cells. Because the cells that mediate relapse or metastasis often express distinct antigens to those on the bulk tumor cells within the primary tumor, it may be necessary to induce additional immunity against antigens on these cells. It has been demonstrated that the expression of these antigens as peptides or whole proteins from the oncolytic virus can permit additional protection against subsequent relapse (47). It is therefore possible that expression of antigens from the virus may be further used to target other stromal cells within the tumor or to boost the immune response against tumor antigens and away from viral antigens.

PERSPECTIVE

Although never becoming an approved therapy outside the Chinese market the ONYX-015 (Sunway H-101) virus, the first oncolytic virus to undergo extensive clinical testing, clearly demonstrated therapeutic responses in at least a subset of patients treated. Researchers in the field have spent the last 15 years trying to enhance the activity and deliverability of these vectors so as to achieve more reliable and significant responses in the clinic. Although approaches to enhance the delivery of the vectors have met only limited success, recent clinical results with T-Vec and Pexa-Vec clearly show that significant improvements have been made in the anti-tumor activity of the vectors, especially when intratumoral delivery is employed. This has apparently been achieved through a combination of use of a faster replicating backbone and expression of an immune stimulating transgene. It is felt to be unlikely that significant additional advantage will be achieved through further enhancing replication potential without safety concerns being raised. The main avenue for further enhancing therapeutic potential may therefore be through careful enhancement of the interaction of the vectors with the host immune response. In this respect, it may be possible to learn from the advances made recently in the fields of vaccine development and cancer immunotherapy. However, it is also clear that simply activating immune stimulation will be unlikely to result in improved therapeutic activity, instead leading to reduced oncolytic activity through rapid clearance of the virus, possible with reduced induction of anti-tumor immunity. Instead, the most promising approaches look to redirect or subtly manipulate the immune response. This goal is complex and relies on inducing an increased recognition of weak tumor antigens with less targeting of typically much stronger viral antigens; increased CTL induction, with reduced humoral response; all while having minimal effects on viral oncolytic activity. However, recent pre-clinical data indicate that some major advances have been made in achieving these goals and there is renewed hope that next generation clinical vectors will significantly improve responses in a variety of cancer patients.

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Oncolytic virus-mediated reversal of impaired tumor antigen presentation

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Anti-tumor immunity can eliminate existing cancer cells and also maintain a constant surveillance against possible relapse. Such an antigen-specific adaptive response begins when tumor-specific T cells become activated. T-cell activation requires two signals on antigen presenting cells (APCs): antigen presentation through major histocombatibility complex (MHC) molecules and co-stimulation. In the absence of one or both these signals, T cells remain inactivated or can even become tolerized. Cancer cells and their associated microenvironment strategically hinder the processing and presentation of tumor antigens and consequently prevent the development of anti-tumor immunity. Many studies, however, demonstrate that interventions that over-turn tumor-associated immune evasion mechanisms can establish anti-tumor immune responses of therapeutic potential. One such intervention is oncolytic virus (OV)-based anti-cancer therapy. Here, we discuss how OV-induced immunological events override tumor-associated antigen presentation impairment and promote appropriate T cell–APC interaction. Detailed understanding of this phenomenon is pivotal for devising the strategies that will enhance the efficacy of OV-based anti-cancer therapy by complementing its inherent oncolytic activities with desired anti-tumor immune responses.

Keywords: reovirus, oncolytic virus, immunotherapy, antigen presentation, anti-tumor immunity

INTRODUCTION

Anti-tumor immune response of appropriate magnitude and specificity has become a valid indicator of good prognosis of cancer and associated disease pathology (1–6). As such, many therapeutic options are being investigated for their capacity to promote anti-tumor immune responses. These immunotherapies, which are based on exploiting the functions of immune cells [e.g., T cells, dendritic cells (DCs)] or immune mediators (e.g., antibodies, cytokines), represent a highly promising group of interventions and have the potential to target a multitude of cancers. Considering the fact that the presence of tumor-specific CD8 T-cell responses almost always correlate with positive patient outcomes (3), the ultimate goal of most of these immunotherapies primarily focuses on establishing anti-tumor T-cell immunity (3, 4, 7). Fully functional tumor-specific T cells can not only eliminate existing cancer cells but also establish an active, ongoing, and long-term surveillance against possibly relapsing cancer cells. Indeed, the immunotherapy-promoted anti-tumor T-cell responses have shown to delay the onset of pathology, reduce the severity of disease, and prolong the survival of cancer-bearing hosts in animal experiments and in clinical settings (1–7).

Oncolytic viruses (OVs), in their naturally unmodified or genetically engineered form, preferentially infect and lyse transformed or cancerous cells in a process called oncolysis. Some of the more prominent examples of these OVs include adenoviruses, reovirus, herpes simplex virus (HSV), vaccinia, vesicular stomatitis virus (VSV), measles, maraba, and so on. In addition, every year new candidate viruses are being proposed and investigated for their potential oncolytic abilities (8). Thus far, OVs have been shown to target cancers of almost every possible tissue origin including breast, ovarian, prostate, brain, colorectal, kidney, etc. both *in vitro* and *in vivo*. Considering the capacity of OVs to target cancer cells preferentially, many of these OVs are employed as anti-cancer agents to target various cancers and are currently under phase I, II, and III clinical trials internationally (8–12).

The primary mode of action for OVs is direct oncolysis. In recent years, however, another aspect of OV-based oncotherapy has become evident. Many reports have shown that, in addition to their direct oncolytic activities, OVs aid in the development of tumor-specific T-cell responses (13–20). Thus, if appropriately managed, OV-based oncotherapies can target cancers through two distinct mechanisms: direct oncolysis and anti-tumor immune responses.

The induction of antigen-specific T-cell response begins when antigen presenting cell (APC) presents an antigenic peptide to a naïve T cell. In the absence of a successful antigen presentation event, T cells either remain inactivated or become dysfunctional. Hence, the process of antigen presentation is a critical step during the initiation of T-cell response. Here, we first explain how the components of the APC–T-cell interaction work, then discuss how cancer cells avoid the presentation of tumor antigens, and finally elucidate how the OV-driven immunological events influence the tumor antigen presentation. We believe that the comprehensive understanding on this aspect of OV-based oncotherapy will

advocate the development of a clinically meaningful anti-tumor immunity and consequently promote better cancer outcomes.

COMPONENTS OF THE NORMAL ANTIGEN PRESENTATION PROCESS

As illustrated in **Figure 1**, the priming of antigen-specific T cell occurs in lymphoid tissues and requires three signals on APCs: antigen, co-stimulation, and cytokines. Antigenic peptides are presented through major histocombatibility complex (MHC) molecules, co-stimulation is carried out by co-stimulatory molecules such as B7 family member proteins, and cytokines such as interferon (IFN)-α/β, interleukin (IL)-12, and IL-1 constitute the third signal. Both CD8 and CD4 cells bear distinct receptors (called T-cell receptors; TCRs) that interact with MHC class I or II molecules, respectively (22–26). Class I and II MHC molecules have distinct pathways through which proteins are processed and ultimately presented to T cells. For MHC class I pathway, cytosolic proteins go through the antigen processing and presentation machinery (APM), which is made up of peptide transporters, chaperone proteins, and the Golgi complex. First, proteasomes break down designated ubiquitinated proteins into peptides of 2–25 amino acids in length. These peptides are transported with the help of peptide transporters (TAP1/TAP2) into the endoplasmic reticulum (ER), where they are further trimmed to 8–10 amino acid length to fit within the MHC groove (27–30). Next, chaperones such as calnexin, calreticulin, ERp57, and tapasin aid the loading of the trimmed peptide into the MHC groove. These MHC–peptide complexes then migrate to the cell surface and become available for the recognition by CD8 T cells (21, 30).

Apart from this classical pathway, extracellular antigens can also be presented through MHC class I pathway using a specialized pathway called cross-presentation (21, 31). *In vitro*, various APCs have shown to bear a capacity to cross-present extracellular antigens; however, *in vivo*, the main mediators of cross-presentation are DCs (32). There are two main pathways through

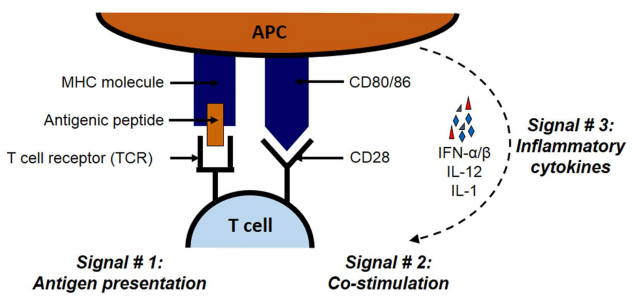

FIGURE 1 | The three signals necessary for the stimulation of antigen-specific T cell. The priming of antigen-specific T cell requires three signals: antigen, co-stimulation, and cytokines. Antigenic proteins undergo antigen processing and then the peptides are presented through MHC class I or II molecules for CD8 and CD4 T cells, respectively. The second signal in the form of co-stimulation is provided by molecules such as B7 family member proteins such as B7.1 (CD86) and B7.2 (CD80) expressed on APCs. These B7 proteins interact with their receptors such as CD28 on interacting T cells. Inflammatory cytokines such as IFN-α/β, IL-12, and IL-1 constitute the third signal.

which cross-presentation can happen: cytosolic and vacuolar. In the cytosolic pathway, first antigen processing occurs in cytosol and then proteasome-generated peptides are fed in MHC class I molecules. On the other hand, for vacuolar pathway, lysosomal proteolysis contributes toward peptide generation, and antigen processing and peptide loading occurs in endocytic compartments. Together, both these pathways facilitate the presentation of extracellular antigens, e.g., antigens from the pathogens that do not infect DCs or self-antigens, to CD8 T cells (33–35).

The expression of MHC class II is more tightly regulated than MHC class I and is primarily found on the surface of professional APCs, such as DCs and macrophages (21). MHC class II antigen processing primarily uses a lysosomal pathway that degrades proteins taken up by endocytosis (extracellular antigens) or autophagy (intracellular antigens). The newly synthesized MHC class II molecules assemble with a protein known as an invariant chain (Ii). The Ii protein prevents the premature binding of endogenous peptides or misfolded proteins in the MHC class II groove, and also directs delivery of MHC molecules to endosomal vesicles where the loading of the appropriate peptide happens. Once inside the endosomal vesicle, the Ii is cleaved off, leaving a short class II-associated invariant chain peptide (CLIP) fragment still bound in the MHC groove. Finally, the release of the CLIP fragment and the loading of the appropriate peptide are facilitated by HLA-DM (H-2M in mouse) molecules (36). The MHC class II molecule displays the appropriate peptide and then travels to the surface to be available for CD4 T-cell recognition (21, 34, 37, 38).

The second signal in the form of co-stimulation is induced when molecules such as B7.1 (CD80) or B7.2 (CD86) expressed on the same MHC–peptide bearing APC interact with its cognate receptor such as CD28 on the interacting T cell (39–42). Other similar co-stimulatory molecule–receptor interactions include the dialogs between CD40L and 4-1BB (CD137) on T cells and CD40 and 4-1BB ligand (4-1BBL) on APCs, respectively. On the other hand, molecules like CTLA-4 on T cells can also bind to B7 molecules and induce inhibitory signals that are especially important in preventing unchecked, sustained proliferation following the initiation of T-cell response. Indeed, mice lacking *CTLA-4* gene display massive proliferation of lymphocytes which becomes fatal overtime (41). Together, the balanced actions of these co-stimulatory and co-inhibitory molecules dictate the fate of T-cell activation.

In recent times, the third signal in the form of inflammatory cytokines has been recognized for the activation of both CD4 and CD8 T cells (43, 44). Cumulative evidence demonstrates that IFN-α/β and IL-12 are required as the third signal for the functional activation of CD8 T cells (43, 45, 46), and that the absence of these cytokines results in the development of defective CD8 T primary and memory responses (47). For CD4 T cells, this third signal is provided by IL-1 (43, 48).

When naïve CD8 or CD4 T cells interact with APCs expressing both the necessary signals, they undergo clonal expansion and differentiate into effector cells. Activated CD8 cells can kill target cells through perforin, granzyme, or FasL-mediated mechanisms or can produce cytotoxic cytokines such as IFN-γ or tumor necrosis factor alpha (TNF-α). On the other hand, activated CD4 cells can also kill target cells or further provide "help" for the activation of other immune cells including macrophages and (T and B) lymphocytes

through the action of cytokines such as TNF-α, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), CD40L, IL-4, IL-5, IL-10, and transforming growth factor beta (TGF-β). Most importantly, a fraction of primed T cells further evolves into a memory phenotype that establishes protection against the same immunogen in the future (23, 26, 49, 50).

TUMOR-ASSOCIATED IMPAIRMENT OF ANTIGEN PRESENTATION

Tumors have developed various immune evasion mechanisms that specifically target different aspects of signal 1, 2 or 3, and thus prevent the initiation of functional tumor-specific T-cell response (51, 52). More importantly, such defects in antigen presentation and co-stimulation processes, alone or in combination with each other, have been correlated with poor cancer outcomes (17, 30, 37). These defects, which can occur on tumors themselves or on the tumor-associated APCs, have been observed at the transcriptional and/or post-transcriptional levels, and are affected by genetic and environmental factors. For example, completely absent or aberrant expression of MHC class I as well as its constituent protein β2 microglobulin (β2M) has been reported in patients with breast, ovarian, cervical, skin, esophageal, and colorectal cancers (30, 52, 53). Furthermore, other components of the APM such as transporter proteins TAP1 and TAP2, ER enzymes (ERAP1 and ERAP2), proteasome subunits (LMP2, LMP7, and LMP10), and chaperone proteins have been found to be defective in various cancers (4, 5, 30, 51, 54). Unlike MHC class I, the clinical significance of MHC class II expression on tumor cells is still not clear (36). Many tumor cells display constitutive or inducible levels of MHC class II (3, 4, 38). Breast and colorectal carcinomas express MHC class II molecules on the surface; however, they often display the defects in the expression of MHC class II pathway-associated components (55). In contrast to healthy cells, melanoma cells do not upregulate the expression of MHC class II following IFN-γ stimulation. Recently, defects in MHC class II transactivator (CIITA) synthesis was associated with impaired MHC class II expression in head and neck cancer cells and some lymphomas (55–58). Similarly, the impaired levels and functional attributes of HLA-DM and HLA-DO are known to influence the presentation of tumor antigens through MHC class II pathway (36, 55). In the context of such aberrant MHC expression, both CD4 and CD8 cells cannot identify tumors as targets.

Tumor-associated APCs also demonstrate defects in their antigen presentation capacities and could directly contribute toward the establishment of dysfunctional anti-tumor immune response (52). Of note, tumor cells as well as their microenvironment promote an immunosuppressive environment that prohibits the generation of one or more of the three signals of antigen presentation on APCs (52, 54). For example, intra-tumoral DCs obtained from cancer patients or cancer-bearing experimental animals display lower expression of MHC class I and II as well as CD80 and CD86 molecules (51, 52, 54, 59). Similar aberrant expression of MHC and co-stimulatory molecules can be induced on the DCs isolated from healthy, non-cancer-bearing hosts when incubated in the presence of cancer cells and supernatant from cancer cell cultures (17). Additionally, tumor-associated DCs also express various inhibitory molecules, such as programed death ligand-1 (PDL-1) and CTLA-4, which further contribute toward the silencing of anti-tumor T-cell response (41, 42). Finally, tumor microenvironment also recruits many suppressive cells [e.g., regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)] and cytokines (e.g., TGF-β, PGE-2) which further affect the antigen presentation function of APCs (51, 52).

CONTRIBUTION OF VIRUS-DRIVEN IMMUNE RESPONSE IN THE ANTIGEN PRESENTATION PROCESS

Viruses are strong immunogens, and bear a capacity to induce all three signals, i.e., antigen, co-stimulation and inflammatory cytokines, necessary for the activation of antigen-specific T-cell response (60). Following exposure to a virus, the immune system recognize the virus as a "foreign" entity through conserved receptors of the innate immune system known as pattern recognition receptors (PRRs, e.g., toll-like receptors, TLRs). These receptors on APCs can identify molecular motifs known as pathogen-associated molecular patterns (PAMPs) and virus-associated DNA and single- or double-stranded RNA of genomic or replicative intermediate origin. Additionally, replicating viruses are also recognized through intracellular helicases (60, 61). The recognition of viral PAMPs through PRRs drives the immediate innate immune response that constitutes the production of type I interferons, including multiple forms of IFN-α and -β (62–64). These Type I interferons enhance the expression of MHC class I and II, CD40, CD80, CD83, and CD86 on the surface of DCs (46, 65, 66). Such IFN-α/β response further stimulates the production of cytokines (e.g., IL-1β, IL-6, IL-12, TNF-α) and chemokines [e.g., IL-8, monocyte chemotactic protein-1 (MCP-1)], and amplifies the initial innate response when these cytokines act through autocrine and paracrine fashion (67). This cytokine-driven pro-inflammatory response is critical in driving the expression of MHC as well as co-stimulatory molecules involved in antigen presentation. Of note, IFN-α has been shown to enhance the proliferative capacity of naïve CD8 T cells, and thus is considered as a "signal 3" necessary for successful T-cell activation (44). Additionally, this innate response is also known to promote the cross-presentation of antigens (3, 68). The APCs primed in this fashion travel to the lymphoid organs wherein they interact with naïve T cells and prime an antigen-specific adaptive immune response (34).

OV-MEDIATED REVERSAL OF TUMOR-ASSOCIATED IMPAIRED ANTIGEN PRESENTATION

The immune responses that accompany oncolytic virotherapy warrant a special consideration as the circumstances under which these responses occur are very unique to this system. It should always be remembered that OV-driven immune responses are strong, whereas cancers usually persist in suppressive environments. The combination of these two contrasting entities most likely produces the immunological consequences that are uncharacteristic of either the tumor- or virus-driven immune response on their own (14). Interestingly, OVs preferentially target cancer cells for their replication, and hence attract the anti-viral immune response in a cancer microenvironment (14, 69, 70).

The strong immune responses initiated by viruses have the potential to over-turn the suppressive effects of tumor-associated immune evasion mechanisms (**Figure 2**), including those involved

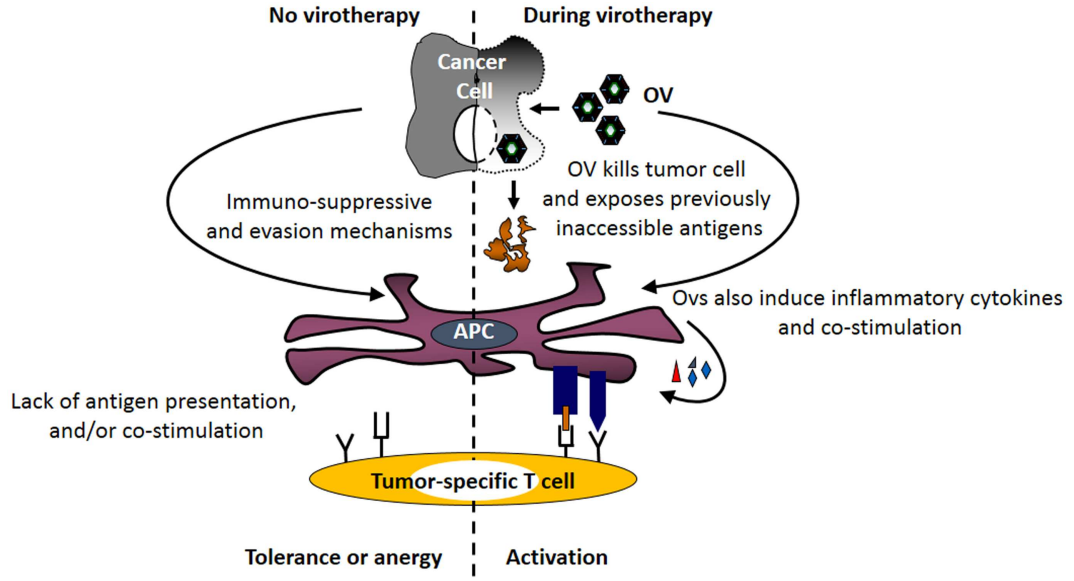

FIGURE 2 | Oncolytic viruses facilitate the tumor antigen presentation preceding the initiation of anti-tumor immunity. Following its therapeutic administration, OVs enhance the expression of MHC molecules on tumor and immune cells. At the same time, OV-mediated direct oncolysis of tumor cells exposes tumor-associated antigens (TAAs) for the processing by APCs. Through the combined actions of these immunological events, OVs facilitate the display of otherwise inaccessible tumor-specific immunogenic peptides on the surface of APCs (*generation of signal no. 1*). Additionally, OV-induced inflammatory response promotes the expression of co-stimulatory molecules on APCs (*generation of signal no. 2*) and production of inflammatory cytokines (*generation of signal no. 3*). Together, OV-driven immunological events over-turn tumor-associated antigen presentation impairments, and initiate anti-tumor immunity.

in antigen processing and presentation pathway (71–74). Exposure of immune as well as cancer cells to OVs induces the expression of type I interferons (75). Similarly, animals injected with the OV gain elevated IFN-α mRNA and protein levels immediately following the administration of the virus. Furthermore, DCs cultured in the presence of reovirus produce IL-1α, IL-1β, IL-6, IL-12p40/70, IL-17, CD30L, eotaxin, GM-CSF, MCP-1, MCP-2, MCP-5, macrophage colony-stimulating factor (M-CSF), monokine induced by gamma interferon (MIG), macrophage inflammatory protein-1 alpha (MIP-1α), RANTES, TNF-α, vascular cell adhesion protein-1 (VCAM-1), etc., and show enhanced expression of CD80, CD86, and CD40 (71). Similar phenotype is also observed in DCs exposed to other OVs including HSV, vaccinia, and measles (72, 76–78). Most importantly, APCs isolated from the spleens of the tumor-bearing mice injected with a therapeutic regimen of OVs also display higher expression of co-stimulatory molecules as compared with those isolated from the untreated or PBS-injected tumor-bearing animals (71, 79). It should be noted that DCs isolated from tumor-bearing mice have lower expression of co-stimulatory molecules as compared with their healthy counterparts. However, this lowered expression is over-turned following OV administration (17, 71).

Most OVs are potent inducers of MHC class I pathway-related molecules (13, 14, 18, 19, 80). Exposure of tumor cells to OVs *in vitro* enhances the expression of MHC class I molecules as compared with that observed in untreated cells (17). For example, when mouse ovarian tumor cells (ID8), which show complete absence of MHC class I protein on its surface under native conditions, manifest significantly higher MHC class I expression upon exposure to reovirus for 24 h in vitro (17). Furthermore, ID8 tumors collected from reovirus-treated C57BL/6 immunocompetent mice also displayed significantly higher expression of mRNA transcripts encoding MHC class I, β2M and TAP1/TAP2, molecules as compared with that of tumors from untreated animals (17).

From a functional point of view, OVs are known to directly enhance the antigen presentation capacity of DCs (71). When DCs are incubated in the presence of OV-infected ova-expressing tumor cells, they can efficiently process and present a tumor-associated antigen (TAA) to antigen-specific CD8 T cells. This was shown in a cancer model wherein an ovalbumin (ova) is employed as a surrogate tumor antigen. In this model, when bone marrow-derived dendritic cells (BMDCs) are incubated with reovirus-infected ova-expressing mouse melanoma (B16-ova) or lung carcinoma (Lewis lung carcinoma, LLC-ova) cells, they display the ova-specific immune-dominant epitope in the context of MHC class I molecules on their surface. Such display of surrogate TAA is non-existent when BMDCs are incubated with B16-ova or LLC-ova in the presence of inactivated virus or medium alone. Most importantly, OV-induced TAA presentation on the BMDC surface further stimulates the activation of TAA-specific CD8 T cells (71). These observations conclusively demonstrate that OVs can (1) promote the antigen presentation of TAAs on APCs and (2) endow APCs with a functional capacity to stimulate TAA-specific CD8 T cells. Of note, the use of ova as a surrogate TAA should be cautiously considered as it could potentially undergo

differential antigen processing and presentation than that for endogenous TAA.

The over-turning of the tumor-associated impaired antigen presentation, however, is only observed following exposure to live, but not inactivated, OVs (71, 72, 81), and is thought to be directly associated with the process of oncolysis. It is believed that OVs expose otherwise inaccessible tumor antigens through oncolysis and make them available to APCs. Simultaneously, OV-driven inflammatory response also promotes the expression of co-stimulatory signals on these APCs that are now armed with tumor antigen. Thus, oncolytic activities of OV coupled with virus-driven immunological events induce the signals necessary for the activation of tumor-specific T cells and aid in the development of anti-tumor adaptive immunity.

Nevertheless, not all OVs aid in the antigen presentation process. Thus far, VSV has been shown to downregulate the co-stimulatory and antigen presentation functions, along with the survival of DCs (82). This observation bears special significance especially in the context of the capacity of various other viruses to subvert and manipulate antigen presentation pathways (53, 68, 83, 84). Hence, it is imperative that candidate OVs be tested extensively for their respective beneficial or detrimental immunological capacities related to the process of tumor antigen presentation.

FUTURE DIRECTIONS

As outlined in this perspective, OVs bear a comprehensive capacity to over-turn TAA presentation evasion mechanisms and to promote a functional anti-tumor T-cell response. However, available information on this phenomenon is still limited and warrants a detailed exploration on various molecular and functional aspects of OV-driven antigen presentation. Especially, the effect of OVs on the processing and presentation of endogenous tumor antigens in the context of various molecular components of MHC class I and II pathway, and in relation with resultant anti-tumor immune response, must be thoroughly explored. It should also be noted that OV-induced antigen presentation also promotes the development of the anti-viral adaptive immune response that is known to prematurely curtail the spread of OV in cancer cells. Only in recent years, the importance of OV-driven immunological events has been acknowledged and given appropriate attention. However, one thing is now clear: OV-induced immune response dictates the efficacy of OV-based oncotherapy. In the future, appropriate immune interventions that promote a fine balance between anti-tumor and anti-viral immune responses will ensure the maximum anti-cancer benefits of OV-based oncotherapies.

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Oncolytic viruses as anticancer vaccines

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Oncolytic virotherapy has shown impressive results in preclinical studies and first promising therapeutic outcomes in clinical trials as well. Since viruses are known for a long time as excellent vaccination agents, oncolytic viruses are now designed as novel anticancer agents combining the aspect of lysis-dependent cytoreductive activity with concomitant induction of antitumoral immune responses. Antitumoral immune activation by oncolytic virus infection of tumor tissue comprises both, immediate effects of innate immunity and also adaptive responses for long lasting antitumoral activity, which is regarded as the most prominent challenge in clinical oncology. To date, the complex effects of a viral tumor infection on the tumor microenvironment and the consequences for the tumor-infiltrating immune cell compartment are poorly understood. However, there is more and more evidence that a tumor infection by an oncolytic virus opens up a number of options for further immunomodulating interventions such as systemic chemotherapy, generic immunostimulating strategies, dendritic cell-based vaccines, and antigenic libraries to further support clinical efficacy of oncolytic virotherapy.

Keywords: oncolytic virotherapy, oncolytic virus, antitumor immunity, antitumor immune response, oncolytic agents

INTRODUCTION

Oncolytic viruses are novel antitumor agents with the ability to selectively replicate and lyse tumor cells while sparing healthy tissue. This intriguing characteristic is either an inherent feature of certain virus species or a result of targeted genetic engineering, which harnesses tumor-specific molecular alterations for virus replication and tumor cell lysis (1). The ideal and intriguing concept has been that the oncolytic virus infection proceeds throughout the whole tumor, thereby leading to effective tumor cell lysis until the rim of malignant tissue is being reached and further infection is kept in check. Although numerous oncolytic viruses have been generated according to this concept, first clinical trials did not meet the high expectations that have been raised by promising preclinical developments (2). Though clinical benefit by these first wave oncolytic agents, such as the mutated Adenovirus (Ad) Onyx-015 has been rather modest, these studies confirmed that oncolytic viruses can be safely administered in human patients and may also work synergistically with systemic radio- or chemotherapy (3). H101, a direct derivative of the E1B55k-deleted Onyx-015, was approved in China in 2006 being the first clinically applicable oncolytic virus (4). At the same time, many factors have been recognized, which severely impair therapeutic efficacy of oncolytic viruses such as virus neutralization by blood components, ineffective transduction of tumor tissue, intratumoral stromal barriers that inhibit virus spread, hypoxic conditions, interstitial pressure, and finally, the rapid immune-mediated elimination of the virus from the tumor tissue (5).

Apart from the cytoreductive aspect, oncolytic viruses have been initially developed for, it has become increasingly clear during the recent years that virotherapy exerts multiple antitumoral activities. These include direct effects by cytotoxic cytokines released upon infection by tumor-resident or infiltrating immune cells (6, 7). Also, effects on the tumor vasculature have been demonstrated (8, 9). In contrast to the notion that the host's immune system limits the efficacy of virotherapy by rapid clearance of infection, it has been perceived that collateral induction of innate and adaptive immune responses against the tumor essentially contributes to therapeutic efficacy of virotherapy (10). Oncolytic virus-mediated destruction of tumor tissue activates innate immune receptors once the immunogenic cell debris is taken up and cross-presented by antigen-presenting cells. Antigen-presenting cells are additionally activated by signals coming from innate cells and the damaged tissue. The local inflammation of tumor tissue during oncolytic virus infection therefore provides suitable conditions for the triggering of tumor-directed immune responses (11, 12). Oncolytic viruses that are currently most advanced in clinical development have been designed to amplify the *in situ* vaccinative and immunostimulatory effect of virus infection. The GM-CSF-expressing oncolytic vaccinia virus JX-594 has shown promising results in phase I/II clinical studies in hepatocellular carcinoma (13). In advanced melanoma, the GM-CSF-expressing herpes virus T-Vec led to a significant number of durable responses and improved survival in a phase III trial in human patients, thus demonstrating clinical efficacy of virotherapy in human cancer patients (14).

There has been evidence that virotherapy may profit from general immunosuppression by increased intratumoral virus spread and by delayed virus clearance (15). Apart from safety aspects, the increased immediate tumor response due to oncolysis would be in this case achieved at the cost of losing effective tumor-antigen cross priming and the perspective of long-term antitumoral efficacy. In this review, we want to deliver a closer look on

how oncolytic viruses induce and shape tumor-antigen directed immune responses. First, we want to address the origin of antitumoral immune responses on the level of the infected tumor cell by discussing the role of viral oncolysis for induction of immunogenic cell death (ICD). The aspect of ICD has also been recently reviewed in depth by Bartlett et al. providing complementary information on how armed viruses and combination strategies work to enhance antitumor immunity (16). In the second part of our review, we want to shed light on the role of several immune cells populations that contribute to the tumor microenvironment. Finally, we want to highlight some current trends and developments exploiting the immunostimulatory and vaccinative potential of oncolytic virotherapy to raise T cell responses against the tumor mutanome.

ONCOLYTIC VIRUS-MEDIATED CELL DEATH MECHANISMS

Viruses, mainly DNA viruses, need time after cell entry to complete the viral life cycle and have consequently developed elaborate strategies to hide from being detected by the host's immune system (17). The requirement of effective "stealth" mechanisms illustrates that virus-mediated cell killing can be a highly immunogenic way for cells to die. This perception has been exploited in vaccinations for a long time since vaccines can be more potent when delivered and expressed by viral vectors (18). Due to the fundamental relevance in multiple physiological processes, enormous efforts have been made to understand the immunological consequences of different kinds of cell death, which have been classified into three major kinds: apoptosis, necrosis, and autophagy (19). Apoptosis is mainly characterized by defined morphological changes such as formation of apoptotic bodies and biochemical signaling such as caspase activation and loss of mitochondrial membrane integrity. Flipping of phosphatidylserines to the outer membrane surface during apoptosis facilitates silent removal of apoptotic bodies by phagocytes. This process is usually accompanied by release of anti-inflammatory cytokines to minimize immune-mediated collateral damage (20). The coordinated cell demise by apoptosis is essential for normal development and tissue homeostasis and has been therefore regarded for long time as a non-immunogenic or even a tolerogenic event. A second cell death type, necrosis, appears to be a less coordinated process and the biochemical pathways have been much less intensively studied. Necrosis is characterized by swelling of organelles and cytoplasm followed by rupture of the plasma membrane with release of cytoplasmic contents. Since necrosis is frequently accompanied by release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) (21), and other immune activating mediators, necrosis has been more or less regarded as being immunogenic. However, the traditional perspective of non-immunogenic/tolerogenic apoptosis and immunogenic necrosis has been challenged by the finding of "immunogenic" apoptosis in tumor cells, which can be induced by specific chemotherapies such as anthracyclines and oxaliplatin (22, 23). When mice were treated with tumor cells that have been killed by these "ICD" inducers, long-term immunity against a challenge with the same tumor could be observed whereas other chemotherapeutic agents failed to induce antitumoral immunity. Since then, several other systemically applicable ICD inducers have been described (24).

Oncolytic virus-mediated cell death does not exactly follow the classical schemes of apoptosis or necrosis but rather displays specific features of both cell death modalities with some variation between different oncolytic virus types. Accordingly, terms like programed apoptosis, necroptosis, pyroptosis, or necrosis-like programed cell death have been used to describe cell death by different oncolytic virus species, trying to describe the coordinated manner in which cells are rearranged in the course of the viral infection cycle, and the membrane disruptive and inflammatory release of viral progeny and cytoplasmic/nucleic contents during lysis. Necrosis-like programed cell death has been observed using oncolytic Ads (25). Though activity of caspases could be observed, p53 activity and mitochondrial pathways were effectively blocked whereby execution of cell death was essentially independent of caspase activation. Likewise, programed necrosis was also observed in cells infected with an oncolytic vaccinia virus. Though some limited features of apoptosis and autophagy were detectable such as phosphatidylserine exposure and LC3 lipidation, necrotic morphology predominated and the necrotic process was also identified as causative cell death modality (26).

Recently, receptor-interacting protein kinases RIP1 and RIP3 have emerged as a decisive switch from immunologically silent apoptosis to necrotic inflammation (27). Once caspase-8 activity, located in a receptor-associated complex called necrosome, is suppressed, e.g., by a pathogen-encoded inhibitor, RIP1 is stabilized, then attracting and phosphorylating RIP3 (28). RIP3 activation phosphorylates the major downstream target mixed lineage kinase domain-like (MLKL) by phosporylation and trimerization that translocates to the plasma membrane to mediate Ca^{2+} influx and initializing membrane rupture (29). RIP1/RIP3-dependent necroptosis therefore appears to function like a backup mechanism allowing the elimination of pathogen-infected cells that cannot undergo apoptosis (30). Necrotic features of RIP3-dependent cell death are necessary for induction of inflammation, improved antigen presentation and effective defense against the pathogen. It has been demonstrated that the highly specific caspase-8 inhibitor vICA, encoded by cytomegalovirus, predisposes to RIP3-dependent necrosis (31). Additionally, CrmA related apoptosis inhibitors activate TNFR-dependent necroptosis in vaccinia virus infections in mice augmenting clearance of the virus (32). Interestingly, cytomegalovirus also express a RIP3 inhibitor, vIRA, which blocks this "backup" cell death pathway to reduce inflammatory responses (33). A downstream target of the RIP1-RIP3-necrosome in necroptosis is JNK-1 and its substrate c-Jun with a final impact on the production of reactive oxygen species (ROS) (34). We could show that oncolytic Ad infection in human tumor cells strongly induced JNK-1 activation, downstream phosphorylation of c-Jun, and activation of other stress-activated kinases (35). It has further been shown that programed necrosis by oncolytic vaccinia virus infection involved formation of a RIP1/Caspase-8 complex (26). In this study, the relevance of RIP1 in vaccinia virus-induced programed necrosis was demonstrated by pharmacological inhibition of both RIP1 and downstream targets including MLKL, which significantly attenuated necrotic cell death. Using an oncolytic influenza viruses, armed with the antitumoral cytokine IL-24, it has been shown that IL-24 turned cell death, mediated by a TLR3-associated, RIP-1 containing signaling complex, into a pure apoptotic phenotype by unleashing caspase-8 activity (36). Though enhanced tumor cell

killing was observed *in vitro*, the consequences of this approach on immunogenicity and antitumoral immune responses *in vivo* are unclear.

In summary, the RIP1/RIP3 necrosome plays a central role in induction of inflammation and virus-mediated ICD and is therefore an interesting target for more detailed investigations, and targeted modulation in oncolytic virotherapy. Again, it has to be considered that enhanced immunogenicity of oncolytic virus-mediated cell death will probably affect viral spread.

THE ROLE OF AUTOPHAGY IN ONCOLYTIC VIRUS-MEDIATED ICD

Another cell death type, autophagy, is a process that leads to self-digestion of organelles after inclusion in cytosolic lysosomes (autophagolysosomes). Since signs of autophagy also occur as a reversible process in the context of nutrient starvation, it is not completely clear whether autophagy is causative for cell death or is an epiphenomenon of other cell death triggers. However, autophagy plays a definitive role in triggering immune responses. Autophagic mechanisms are involved in the clearance of intracellular microbial or viral pathogens not only by intracellular digestion but also by improved processing of microbial/viral antigens for antigen presentation on MHC I as known for herpes simplex virus infections (37). Autophagy can be a part of a cellular reaction to infection by oncolytic viruses, which has been observed first in glioma treatment with oncolytic Ads (38, 39). Induction of autophagy has also been demonstrated for Newcastle disease virus (NDV) (40). In both cases, investigations using the autophagy inducer rapamycin suggested that autophagy augments viral replication and propagation and may lead to improved antitumor responses (41, 42). An interesting subtype of autophagy, called mitophagy, has been reported recently (43). The authors have shown that attenuated measles viruses of the Edmonton strain exploit selective reduction of mitochondria via SQSTM1/p62-mediated mitophagy for enhanced viral replication. Mitophagy resulted in decreased mitochondrion-bound mitochondrial antiviral signaling protein (MAVS) thus weakening the innate immune response mediated by RIG-I-like receptors. In summary, cell death by oncolytic viruses displays signs of apoptosis, autophagy, and necrosis to a variable extent. What all oncolytic viruses have in common is the immunogenic nature of virus-induced cell death (see also **Figure 1** for an overview). The determinants characterizing ICD are summarized in the next chapter.

INDUCERS AND MEDIATORS OF IMMUNOGENIC CELL DEATH: DAMPs AND PAMPs

Antigen-presenting cells such as dendritic cells (DC) fulfill a central role in triggering effective T cell responses in case of a pathogenic threat. Antigen-presenting cells are activated when encountering pathogen-derived structures, called PAMPs (pathogen-associated molecular patterns), which reflect conserved components of microbes and viruses. Classical PAMPs are microbial DNA with unmethylated CpG, defective viral genomes that occur during viral lysis, double stranded RNA, single stranded RNA, 5′-triphospate RNA, lipoproteins, surface glycoproteins, and bacterial membrane components such as LPS. PAMPs are recognized by pattern recognition receptors (PRRs) present on innate immune cells, antigen-presenting cells, and also on epithelial cells. PRR include toll-like receptors, retinoid acid inducible gene I (RIG-I)-like receptors (RLRs), AIM like receptors (ALRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (44). In 1994, the "danger" hypothesis by Polly Matzinger (45) brought up the idea that, besides the classical feature to distinguish between self and non-self, the immune system must be able to adequately respond to tissue distress, and that this additional competence requires molecular signaling coming from affected tissue. According to this hypothesis, molecular danger signaling immediately alerts innate immune cells and facilitates their attraction to the site where ICD occurred. Furthermore, danger signaling must activate DCs to provide for the stimulation needed to activate antigen-specific T cells. A number of molecular factors called danger-associated molecular patterns or DAMPs have been described functioning as such danger signals to orchestrate attraction of innate immune cells, phagocytosis of immunogenic cell debris, and to activate effective T cell priming. Some DAMPs are immune activating cytokines such as TNFs or type I interferons that can be immediately emitted in response to threat. Other factors are metabolites that create a chemotactic gradient for innate immune cells corresponding to a "find me" signal. Further, DAMPs already reflect signs of structural damage caused by the infection process. Externalized proteins, more or less linked to the membrane of the infected cell can provide an "eat me" signal to attracted phagocytes. When cells undergo immunogenic apoptosis, the release of ATP is a known "find me" signal to promote phagocytic clearance of those cells at a very early time point (46, 47). ATP is released by Pannexin channels and sensed by P2Y (2) purinergic receptors on monocytes to facilitate their attraction to the site of apoptotic cell death. Additionally, ATP acts on P2X (7) purinergic receptors on DCs, thus activating the NLRP3 inflammasome (48). ATP has also been described being released by cells infected by oncolytic viruses (49, 50). In induction of ICD, ATP can also act synergistically with another DAMP, cell surface exposed calreticulin or ecto-CRT (51). Calreticulin is under physiological conditions located in the lumen of the endoplasmic reticulum (ER). However, dying cells externalize and present calreticulin on their surface where it serves as a potent "eat me" signal to phagocytes (52). It has been shown that calreticulin is exposed on the cell surface of lung adenocarcinoma cells after treatment with an oncolytic coxsackievirus B3 (50). Ecto-CRT has also been observed with several oncolytic Ads (49, 53).

When cells succumb to necrosis, they also externalize and release the high mobility group box 1 (HMGB1) protein into the cellular environment, which is known for its proinflammatory properties (54). The relevance of HMGB1, Ecto-CRT, and ATP in characterizing ICD has facilitated reliable high throughput screens for ICD-inducing agents (55). HMGB1 release has been observed with multiple oncolytic viruses, e.g., Ad, Vv, and Mv (26, 53, 56, 57).

Further, important DAMPs are released heat shock proteins, such as HSP70 and HSP90, and uric acid. Heat shock protein release has been demonstrated to play a role in induction of tumor-specific immune responses by the oncolytic parvovirus H1 (58).

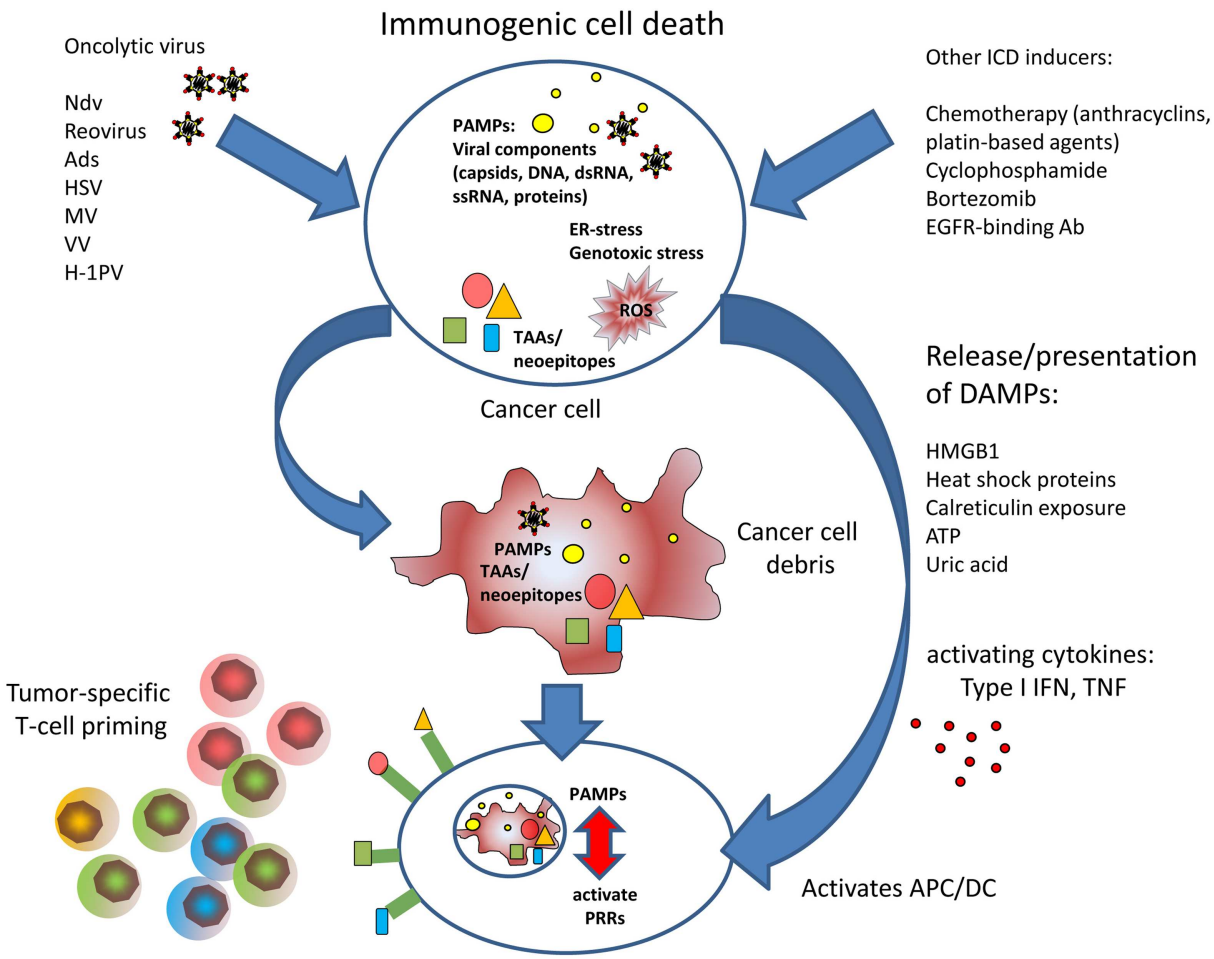

FIGURE 1 | The figure illustrates the improved T cell priming in oncolytic virotherapy. Viral oncolysis of tumor cell induces immunogenic cell death by accumulation of PAMPs and accompanied by release of DAMPs. PAMPs and DAMPs activate antigen-presenting dendritic cells that can induce cytotoxic T cell responses against tumor-associated antigens or neoepitopes, respectively.

Uric acid is a product of nucleic acid catabolism and constitutively present in the cytosol of normal cells in high concentrations that can even rise in stress situations due to increased DNA/RNA degradation. Even the debris of dead cells is able to continue production and release of uric acid providing a sustained danger signal (59). It is believed that a chemical phase change to urate microcrystals at supersaturated loci is the actual immune activating event. Using the oncolytic Ad Telomelysin, it has been shown that infected tumor cells produced uric acid, which in turn stimulated IFN-γ and IL-12 secretion by DC and supported the induction of cytotoxic T cells (60). The DAMPs described so far represent potent immune activators in case of immunogenic apoptosis or necrosis. However, also cell-intrinsic inhibitors of DAMPs exist. Recent results showed that the cellular peptidases dipeptidylpeptidase 3 (DPP-3) and thimet oligopeptidase 1 (TOP-1) present in and released by necrotic cells were able to provide a non-immunogenic signal and inhib antigen cross presentation (61). Since inhibition of the peptidases restored immunogenicity and antigen-specific Tcell priming, interfering with these mechanisms in oncolytic virus-mediated cell death could be a promising option to enhance immunogenicity.

THE ROLE OF ER-STRESS IN ONCOLYTIC VIRUS-MEDIATED ICD

A further important mechanism that provides dying cells with an immunogenic signature is ER-stress. The ER is a central production site for proteins and membrane components involved in the secretory pathway. The ER is also an important sensor for ER-stress, a physiological reaction to dysbalanced protein synthesis, e.g., in the context of viral infections. Under homeostatic conditions, the luminal ER-stress sensors IRE1α, ATF6, and PERK are bound and silenced by the molecular chaperone Grp78/BiP. Once unfolded proteins accumulate in the ER due to an unphysiologic increase in protein synthesis, Grp78/BiP is competitively displaced from the ER-stress sensors leading to their subsequent activation for downstream induction of an unfolded protein response (UPR) (55). Whereas activation of IRE1α and ATF6 leads to expression of compensatory acting genes, PERK/ATF activation

facilitates phosphorylation of eIF2α to induce a general stop of translation until ER-stress has been released. eIF2α-dependent shutdown of translation is also an intrinsic defense reaction to prevent that intracellular pathogens from occupying the protein synthesis machinery for their own purposes. Consistent with this function, ER-stress can confer a significant immunogenic signal to dying cells, which has been demonstrated using chemotherapeutics that are able to directly induce ER-stress (55). According to the relevance of ER-stress as pathogen sensor, many viruses have evolved elaborate ways to circumvent or to adopt ER-stress pathways to their benefit and interfere with ER-stress pathways and UPR (17). ER-stress pathways are also an interesting target to modulate the outcome of oncolytic virutherapy and to increase ICD. Genome-wide RNAi-screens for host factors that modulate viral oncolysis showed that ER-stress and UPR are highly important modulators of viral oncolysis by rhabdovirus (62). To confirm the screening results, the authors showed that inhibition of IRE1α dramatically improved rhabdovirus-mediated oncolysis. Accordingly, ER-stress has been a promising mechanism for pharmacological interference to support viral oncolysis. Bortezomib is a clinically approved inhibitor of the 26S proteasome and leads to collateral ER-stress and ICD with both apoptotic and necrotic features. We showed that low-dose bortezomib enhanced immunogenic tumor cell killing and antitumoral T cell responses in hepatocellular carcinoma models in mice (35). Another study showed that Reovirus and bortezomib synergistically induced apoptosis in multiple myeloma (63). In case of oncolytic herpes simplex virus (oHSV), it could be recently demonstrated that bortezomib-induced UPR even increased virus replication thus leading to enhanced, synergistic tumor effects (64).

ONCOLYTIC VIRUS INFECTION DISRUPTS THE TUMOR MICROENVIRONMENT

Immunogenic cell death is basically the first aspect in innate and adaptive immune effects that have been recognized as a central mode of action in virotherapy (65). The tumor microenvironment also essentially contributes to the triggering of antitumoral immunity. Tumors not only consist of tumor cells but also of stromal fibroblasts, endothelial cells and resistant leukocytes which together with the extracellular matrix constitute the tumor microenvironment. Intratumoral infection by an oncolytic virus is not only a dramatic impact for tumor cells but is also disruptive for tissue architecture and immune homeostasis within the tumor microenvironment. The effect of the tumor stroma to oncolysis is a most enigmatic and barely understood phenomenon since fibroblasts are relatively resistant to virus infection and generate important intratumoral barriers that inhibit virus distribution. To address these barriers, it has been tried to interfere with stroma integrity by oncolytic viruses expressing collagenase and matrix-modifying enzymes (66, 67). The activation of the innate immune system following intratumoral virus infection represents the first defense wave of the host reaction to tumor lysis. Tumor-resident innate immune cells become modulated by inflammatory cytokines that are immediately released upon contact of macrophages with viral structures (68, 69). Further innate immune cells invade the damaged tumor tissue and induce an acute inflammation to fight the viral infection. Neutrophils invade the oncolytic tumor and contribute to immediate antitumoral cytotoxic effects (9, 70). Additional neutrophil-activating signals have been used to increase this effect of oncolytic virotherapy (71). Interestingly, in case of measles virus, it has been shown that attenuated, oncolytic viruses can be even better neutrophil activators compared to their wild-type counterparts (72). Results of several studies suggested that the innate immune response should be suppressed to enhance oncolytic virus propagation and intratumoral spread (73–76). It has also been shown with measles virus that innate immune cytokines can confer resistance to tumor cells against virus-mediated lysis (77). However, the innate immune response is an essential interface for triggering of adaptive immune response including long-term antitumoral T cell responses. It could be rather promising to selectively address suppressive innate immune cell subpopulations in oncolytic virotherapy (6). Since the oncolysis-mediated modulation of the tumor microenvironment decisively governs the priming of adaptive immune responses, the individual immune cell types that contribute to the tumor microenvironment and the immediate reaction to viral oncolysis need a more detailed description.

MYELOID CELLS

Aside of neutrophils, macrophages and monocytes belong to the initial defense response by the innate immunity against pathogens. These populations are highly activated after viral infections, are capable of phagocytosis, support the professional antigen-presenting cells, and contribute to adaptive immunity. Within an intact tumor, secretion of immunosuppressive cytokines determines the phenotypic differentiation of these innate immune cells to adopt an immunosuppressive status to promote tumor progression and metastases (78). Consequently, the immunosuppressive phenotype of these cells can interfere with therapeutic antitumor immune activities. Macrophages residing in the tumor microenvironment have been designated as tumor-associated macrophages (TAMs) and can be divided into two groups, one showing an inflammatory M1 phenotype and the other showing, an immune suppressive M2 phenotype, the latter being overrepresented within the tumor microenvironment (79). It is known that viral inflammation can polarize macrophages toward an M1 phenotype (80). This population promotes inflammatory conditions and supports the triggering of antigen-specific immune response. It has been shown that TAM depletion by chlodronate liposomes prevent intratumoral virus clearance resulting in increased replication and virus spread resulting in improved antitumoral effects (81). Like macrophages, tumor-associated neutrophils can be either assigned to an inflammatory N1 phenotype or an immune suppressive N2 phenotype, respectively (82). Though invading neutrophils belong to the first infiltrating immune populations at the site of inflammation (9), the role of neutrophil polarization in oncolytic virotherapies has not yet been addressed.

In recent years, myeloid-derived suppressor cells (MDSC) population has been described as one of the most important immunosuppressive within the tumor microenvironment. These cells have been observed in primary tumors as well as in metastases of patients (83, 84). Myeloid suppressor cells are attractive targets for therapeutic investigations (85). Related to oncolytic virotherapy, it was shown that the combination with gemcitabine, which is

a chemotherapeutic agent depleting MDSC populations, increases antitumoral immune responses (86, 87).

VIROTHERAPY IS A POTENT NK CELL ACTIVATOR

Among the cells of the innate immune system, NK cells play a crucial role in clearing viral infection and in fighting malignant cells. Trying to escape from adaptive immune responses by down-regulation of MHC, virus-infected cells, and tumor cells become a natural target of NK cells. In line with a role of NK cells in immunoediting of tumors, tumor-infiltrating NK cells correlate with a favorable prognosis in humans (88). NK cells belong to the first immune cell populations that are activated by a virus-mediated inflammation in order to identify and directly kill virus-infected cells (89). This suggests that NK cell inhibition will significantly support intratumoral spread of oncolytic viruses and effective tumor lysis. A study using oncolytic VSV showed that the replication of the virus could be enhanced by NK cell depletion resulting in more effective tumor killing (74). The supportive effect of NK cell inhibition was confirmed by the same group by application of a virus encoding for UL141, which blocks CD155 on infected cells thereby interfering with NK cell recruitment and activation (90). Furthermore, it was shown that the NK cell natural cytotoxicity receptors (NCR) NKp30 and NKp46 were highly activated during oHSV resulting in effective killing of oHSV infected cells thus impeding viral spread and oncolytic therapy (75).

On the other hand, several studies showed an antitumoral effect of NK cells after oncolytic viral treatment. Depletion studies with VSV in the B16 melanoma model revealed an NK cell and T cell dependent tumor regression (91). Furthermore, the remodeling of the immunosuppressive tumor microenvironment of prostate cancer by the infection with oncolytic reovirus demonstrated a strong NK cell involvement in antitumoral immune response (92). It was also observed that the antitumoral effect of an oncolytic parapoxvirus ovis (ORFV) was mainly NK cell-mediated (93). Using an adenovirus expressing IFNβ for systemic NK cell activation, Suzuki et al. could show that intratumoral virus treatment in a pancreatic cancer model resulted in strong NK cell-mediated antitumoral cytotoxicity, when MDSC were eliminated by gemcitabine (86). These data illustrate that other immunosuppressive populations within tumor microenvironment play an important role in the establishment of antitumoral immunity, which must be considered for the role of NK cells in oncolytic virotherapy. Promising reports come from observations on the application as adjuvant to surgical tumor removal. This is of particular clinical relevance since surgery is still the most frequent therapeutic option with curative intention. In a first therapeutic approach using virotherapy as perioperative agent in a surgical stress model, Tai et al. showed that virotherapy by vaccinia virus or ORFV can release NK cell suppression during surgical intervention (94, 95). Virus-mediated NK cell activation effectively inhibited the engraftment of metastatic cells. This finding suggests that NK cells seem to be in particular efficient to protect against tumorigenic cells when an established immunosuppressive tumor microenvironment is lacking. These observations are supported by the increased antitumoral NK cell efficacy, when it is used with chemotherapeutic approaches like gemcitabine or cyclophosphamide, which are well known immunomodulatory agents with selective depletion effects on immunosuppressive populations like MDSCs or regulatory T cells (Treg), respectively (86, 96, 97). It was also demonstrated that a novel oncolytic rhabdovirus (Maraba MG1) was able to boost NK cell activity for the reduction of postoperative metastases (98). Intriguingly, the authors revealed that the effect of NK cell activation was mediated via virus infection of conventional DC. This interaction refers to the important function of DC as functional interface to innate immune effector cells for triggering adaptive immune responses. It is known from patients treated with cetuximab that NK cells are involved in antibody-dependent cytotoxicity of tumor cells and assist DCs in priming of antitumoral T cell responses by an NK:DC crosstalk (99). This aspect could be relevant in oncolytic virotherapy since antibody-mediated cell killing of tumor cells has already been shown to play a yet underestimated role in human patients who have been treated with an oncolytic vaccinia virus (100).

TREGS AND TREG DEPLETION DURING ONCOLYSIS: GOOD OR BAD?

Regulatory CD4 T cells (Tregs) are an immunosuppressive cell population that has frequently been discussed as a critical contributor to the tumor microenvironment. It has been shown that the ratio of intratumoral cytotoxic T cells and Tregs is a prognostic factor for the patient's outcome and studies using antibodies blocking CTLA-4 (which is expressed on Tregs) for increased immune activation have shown that Tregs can be interesting targets for immunotherapeutic approaches (101, 102). The impact of viral infections on Tregs has been mostly studied in persistent or chronic virus infection, such as HCV or HBV whereas the role of Tregs during acute viral inflammations such as oncolytic virus infections is much less investigated. Studies showed that the number of Tregs significantly drops during acute viral inflammation to facilitate an effective antiviral immune response (103, 104).

To elicit enhanced immune stimulation, Treg depletion has therefore been considered a supportive measure during oncolytic virotherapy. Studies have shown that tumor preconditioning with IL-2 and Treg depletion using a depleting antibody or low-dose cyclophosphamide led to increased intratumoral uptake of systemically delivered reovirus or vesicular stomatitis virus. IL-2 in combination with Treg depletion generated "hyperactivated" NK cells with enhanced antitumoral activity and secreting factors that facilitated oncolytic virus spread throughout the tumor by disrupting the tumor architecture (105, 106). Survival benefit by this combination therapy was compromised when NK cells were depleted. Additionally, Cheema et al. could reduce regulatory T cell population in the tumor by arming an oHSV with the cytokine IL12 leading to increased survival in a murine glioblastome stem cell model. Survival benefit by additional expression of IL-12 was absent in athymic mouse indicating that antitumoral efficacy was T cell dependent (107). In contrast Treg depletion was demonstrated to have even a negative therapeutic effect on VSV therapy by relieving Treg-mediated suppression of antiviral immunity resulting in rapid clearance of the therapeutic vector (91).

However, the consequences of Treg depletion on long-term antitumoral T cell responses that can be induced by oncolytic virotherapy are not clear. Observations in classical infection models have shown that migratory activity of Tregs plays a central

role in eliciting a protective immunity to viral infection (108). Consistent with a positive function of Tregs in shaping antigen-specific immune responses, we have observed that Treg depletion abrogated the effective antitumoral T cell induction by an oncolysis-assisted, antitumoral DC-vaccine (109). We could also show that immunosuppressive MDSC expand in Treg-depleted tumors, which may explain the failure of antitumoral T cell priming. Supporting an important role of Tregs in the priming of antigen-specific T cells, it has been described that Tregs can undergo a conversion under acute inflammatory conditions to adopt a T helper phenotype (110). Converted Tregs express proinflammatory cytokines and activate additional functions to provide effective help for triggering T cell responses against new antigens. These findings described above indicate that Tregs can essentially modulate the course of tumor therapy with oncolytic viruses. A supportive role of Treg depletion on virus spread and therapeutic efficacy of oncolysis is still unclear and possible consequences on induction of sustainable tumor-directed T cell responses require further investigations.

HARNESSING ONCOLYTIC VIROTHERAPY AS IMMUNOTHERAPY

Observations in immunocompromised xenografts have tempted to overestimate the cytolytic effects that are achievable in human patients. The situation in the immunocompetent host is completely different with positive and negative consequences for the therapeutic efficacy of virotherapy. Since it is known that T cell responses against cross-presented cellular antigens upon viral infections trigger innate immune receptor pathways such as TLRs and MyD88 (11, 12), investigations on corresponding antitumoral immunity have been intensively pursued in oncolytic virus applications in immunocompetent models. The use of oncolytic VSV in the B16-Ova model strikingly demonstrated that antitumoral effects completely depended on Type I IFN responses, which mediate both antiviral protection and antitumor therapy, whereas VSV-mediated therapy was abolished in MyD88$^{-/-}$ mice (111). The relevance of both innate immune activation and subsequent triggering of adaptive responses was shown in experimental models with T cell depletion studies (10). Interesting observation have been reported using herpes simplex virus variants with different replicative properties. oHSV vectors that were more rapidly cleared from the tumor but induced higher levels of DAMPs resulted in best survival. This strongly indicates that replicative potency is not the dominating factor as believed before but emphasizes the impact of the initial immune induction (112), which needs to be considered in the rational designs of novel approaches aiming at increased antitumor immunity. DC are known to play a crucial role in the generation of tumor-directed T cell responses (113). First strategies on utilizing oncolytic virotherapy to engage intrinsic activity of DC were performed with an ICP34.5 deleted herpes simplex virus coding for GM-CSF (114). Tumor infection with this oncolytic virus led to regression and protected the mice against rechallenge with tumor cells. GM-CSF-expressing HSV then entered clinical development as OncoVexGM-CSF or T-Vec (14, 115). Furthermore, virus-encoded GM-CSF not only affected DCs, but also neutrophils which were shown to contribute to antitumor effects by a GM-CSF-expressing oncolytic measles virus in CD46 transgenic mice (70). The therapeutic benefit of engaging dendritic cell activity in virotherapeutic applications was confirmed using different cytokine setups. In a preclinical breast cancer model, systemic, and intratumoral delivery of a TRAIL-/E1A-expressing oncolytic adenovirus increased plasma levels of TNFα, IFNγ, and MCP-1, proinflammatory cytokines acting as maturation signals for DCs. Inclusion of FLT3L or GM-CSF-expressing adenovirus for expansion of DCs established systemic antitumor immunity and resulted in tumor elimination (116). We obtained consistent results in a mouse model of lung cancer using intratumoral delivery of an oncolytic Ad combined with vectors encoding FLT3L and MIP-1α. Tumor-directed T cells were significantly increased and improved tumor responses were obtained. However, adaptive immune responses against the viral vector were also strongly enhanced suggesting that the balance between tumor- and virus-directed immunity remains unaltered instead of generating a favorable tumor-directed response (117). Oncolytic viruses expressing cytokines for enhanced antigen cross presentation illustrate that virotherapy can be used as a tool for a generic *in situ* vaccination without the need for detailed information about specific tumor-specific antigens. However, the approach has limitations in shifting the predominant antiviral responses in favor of antitumoral responses.

ONCOLYTIC VIROTHERAPY IN DC-VACCINATIONS AND HETEROLOGOUS PRIME-BOOST SETTINGS

For focusing the immune system during virotherapy on the tumor requires the incorporation of tumor-specific antigen targeting approaches into the therapeutic scheme. We have investigated this aspect by combining viral oncolysis and a tumor-directed DC-vaccine (117). In another study, it has been shown that a CCL5 (RANTES) expressing oncolytic vaccinia virus significantly improved the therapeutic efficacy of a tumor-directed DC-vaccine (118). In a further study, it was demonstrated that the application of a replicating adenovirus allowed for highly effective DC-vaccination, when the vaccine is administered exactly at the time of apparent virus-induced tumor inflammation (109). This approach induced potent cytotoxic T cell responses leading to significant tumor regression and complete eradication of lung colonies in an aggressive tumor model that was otherwise resistant to the DC-vaccine. A further promising direction is the development of oncolytic virus-based prime-boost strategies that express the tumor-antigen. In a heterologous treatment sequence with an adenoviral TAA-endoding vaccine and an oncolytic VSV tumor expressing the same antigen significantly enhanced tumor-directed CD8 T cell immune responses compared to single treatments. Heterologous priming worked in both directions (119, 120). This approach shifted the immune responses from viral antigens to tumor-antigens and reduced viral replication in healthy tissues thereby improving efficacy and safety. Interestingly, the magnitude of tumor-specific responses after combination therapy was even higher in tumor-bearing hosts compared to tumor-free mice indicating the need of infected tumor tissue for priming antitumoral T cell reponses (120). The same group could also demonstrate that heterologous boosting not only resulted in higher numbers but also in functionally superior T cells (121). A further interesting variation of prime-boost vaccinations

comes from Brinkhoff and colleagues who elicited highest antitumoral responses when the boost step by an antigen-expressing infectious agent was preceded by a non-pathogenic prime using antigen-loaded PLGA-microspheres (122).

TARGETING THE TUMOR ANTIGENOME AND MUTANOME BY ONCOLYTIC VIROTHERAPY

The use of complete antigen libraries encoded by an oncolytic virus offers a promising approach to circumvent the limitations of antigen-specific vaccinations. In a preclinical study in prostate cancer, VSV-based cDNA libraries from xenogeneic healthy prostrate tissue were used for treatment of TC2 prostrate tumors. Application of VSVs coding for such a cDNA library [Altered Self-antigen and Epitope Library (ASEL)] cured established tumors after repetitive intravenous injections. The use of ASEL conferred significantly better protection against TC2 cells than a self-antigen library from normal mouse prostate tissue. Upon application of ASEL, a T_H17 response was detectable and TC2 rejection was dependent on CD4 cells, but not on CD8 T cells or NK cells (123). A subsequent study from this group demonstrated that an approach of virus-encoded melanoma cDNA libraries can be used to identify tumor-associated antigens that have the ability to cure melanoma (124). Virus-expressed cDNA libraries were effective against melanoma thereby inducing only mild signs of autoimmunity. The xenogenic, altered self-source is a precondition for successful tumor treatment due to additional adjuvant effects compared to a library from an autologous self-source. Again, the antitumoral effect was correlated with a tumor-specific IL-17 response, which was in turn utilized to screen for cDNA-viruses that induced IL-17 memory for identification of tumor rejection antigens. After validation of IL-17 inducing clones, three VSV-encoded tumor-antigens were tested to treat established B16 tumors. Intriguingly, injection of a single VSV-clone or a pool of two VSV-clones did not show a therapeutic response, only the combination of all three VSV-clones cured melanoma tumors to a similar extent as the whole melanoma-library did. Although, it remains unclear why only all three different TAA-coding VSVs contribute to therapeutic effects, this finding suggest that applications targeting multiple antigens at the same time should be preferred in immunotherapeutic strategies. These studies establish a rational approach to identify novel tumor-targets for immunotherapy and establish an effective generic virus-based ASEL-vaccine for defined tumor entities.

To date, identification of novel tumor-antigens that can be addressed by targeted therapeutics appears to be a crucial step toward the establishment of clinically effective immunotherapies and toward induction of sustained adaptive T cell responses. In the past, antitumoral vaccine research has focused on finding non-mutated, tumor-associated antigens such as telomerase or MAGE, which can be found either in a relevant numbers of patients and/or across several entities to promise broad applicability. Disappointingly, corresponding vaccination approaches have so far delivered insufficient effects in the clinic (125). A limiting factor is that non-mutated tumor-antigens may not reflect essential molecular functions required for tumor cell survival promoting the generation of escape variants (126). Furthermore, T cell precursors against this type of antigens are subject to thymus selection and self-tolerance mechanisms thus limiting the number of required high-affinity T cell precursors that are essential for effective antitumoral T cell responses. In this regard, triggering T cells that recognize immunogenic neoepitopes reflecting tumor-associated mutated proteins could be a more promising alternative. Data from melanoma patients indicate that autologous T cell responses to tumors are predominantly directed to neoantigens (127). In murine models as well, tumor rejection responses were also primarily induced by altered-self antigens (128, 129). However, this would require individualized (personalized) molecular diagnosis and therapy. Individual (solid) human cancers usually harbor about 30 to more than hundred of protein-encoded mutations referred to as mutanome (129–131), which can be nowadays rapidly and cost-effectively analyzed by Next Generation Sequencing (NGS) technology. Using this method, non-synonymous single nucleotide variants (SNV) can be identified, representing promising candidates for immunotherapies, since single amino acid variations in corresponding epitopes can be processed and presented by MHC to T cells.

In a pioneering study targeting the mutanome by vaccinations, NGS was used for immunoepitope identification in B16F10 melanoma cells. Selected from 563 non-synonymous SNV candidates, the immunogenicity of 50 validated mutations was determined using corresponding peptide immunizations in mice. The authors showed that immune responses could be raised against 60% of these epitopes and the vaccinations against these predicted and validated epitopes successfully raised antitumoral adaptive immune responses and significantly slowed tumor-growth (132). This illustrates the great potential of this method in identification of neoepitopes. However, the observation is also astonishing since those epitopes should be per definition of low immunogenic nature. In clinically manifest tumors, the remaining epitope spectrum is the result of a dynamic process termed cancer immunoediting, which acts on nascent tumors via different immune cell types to protect against cancer development and shapes the tumor at the same time toward decreased immunogenicity (129). In the study by Castle and colleagues, the key for successful induction of immune responses to immunoedited tumor-epitopes by DC-vaccination is most likely attributable to the use of adjuvants, i.e., poly(I:C) in the B16F10 model. Oncolytic virotherapy is likewise a potent trigger of innate immune receptors and inflammation and could be an interesting tool that enables identification of inflammation induced neoepitope-directed T cell responses and to cooperate with tailored neoepitope-directed DC-vaccines. However, it will be a challenging task to identify neoepitope-specific T cell reactivities that are involved in tumor responses induced by oncolytic virotherapy.

ONCOLYTIC VIROTHERAPY AND IMMUNE CHECKPOINT BLOCKADE

The recent clinical success of immune checkpoint blockade (133) has confirmed the curative potential of tumor immunotherapies. Checkpoint blockade using ipilimumab, a CTLA-4-blocking monoclonal antibody, has shown promising results in a phase III study (134). Remarkably, responses seemed to include even complete cures, but only a small proportion of patients benefited from therapy. In a case study which described a patient with

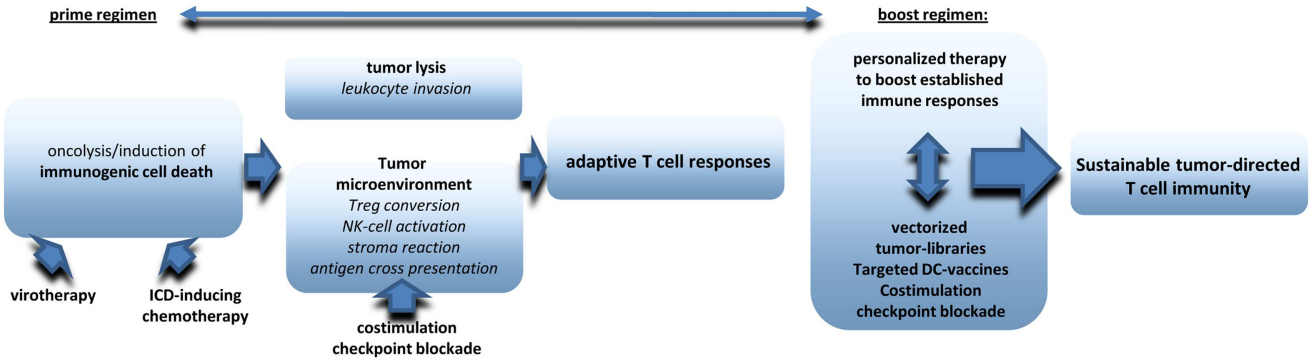

FIGURE 2 | The figure provides an overview on critical components to be included in multimodale virotherapy-based therapies that work like prime-boost strategies.

advanced melanoma experiencing tumor response under ipilimumab, neoepitope analysis by NGS and epitope prediction led to identification of a single ipilimumab-responsive neoepitope-specific CD8 T cell that increased fivefold under therapy and remained stable for a 10-month period (135). The fact, that only one epitope was triggered in a tumor displaying 448 potential T cell neoepitopes is remarkable but reflects that natural and thus immunoedited tumors are low immunogenic despite harboring a high number of mutations and may also explain why only small subgroups of patients respond to certain immunotherapies. Oncolytic viruses can serve as an ideal tool to augment tumor immunogenicity and could be ideally combined with immune checkpoint blockade. Gao and colleagues have investigated the application of a Her2/neu targeted oncolytic VSV in combination with a CTLA-4 antibody in mice bearing Her2/neu transgenic murine mammary tumors. This combination achieved cure in the majority of mice whereas the virotherapy alone only prolonged survival (136). Additionally, it has been tried to include an expression cassette for a CTLA-4-specific antibody into the backbone of an oncolytic Ad to enhance local concentrations and to avoid adverse events by systemic CTLA-4 inhibition (137). Recently, it has been reported that injection of oncolytic NDV in a preclinical model of B16 melanoma under CTLA-4 antibody treatment induces an inflammatory response in tumor tissue, leading to lymphocytic infiltration and antitumor effect in distant, non-virally injected tumors (138). Effective treatment induced activated CD4 and CD8 T cell infiltration in distant tumors and was dependent on $CD8^+$ cells, natural killer cells, and type I IFNs. Overcoming systemic resistance to immune checkpoint blockade by oncolytic virotherapy moreover led to protection from tumor rechallenge in poorly immunogenic tumors, even in a cell line refractory to NDV-mediated lysis. An alternative to checkpoint blockade is the direct activation of costimulation using oncolytic viruses expressing the costimulatory CD40L (53). Further approaches used oncolytic vaccinia viruses expressing the ligand for the costimulatory receptor 4-1BB (CD137) that achieved maximum antitumoral efficacy in lymphodepleted hosts (139). Strong antitumoral immune responses were also elicited by combining oncolytic vaccinia virus with systemic application of a 4-1BB agonistic antibody (140). An interesting immune checkpoint that has not yet been investigated with virotherapy is PD-1/PD-L1. PD-1/PD-L1-blocking antibodies are in a very promising clinical development (141). PD-1/PD-L1 inhibition primarily activates antigen-experienced T cell responses in the periphery, thus providing a mechanism that could be promising to combine with virotherapeutic treatments.

PERSPECTIVE: ONCOLYTIC VIROTHERAPY IN MULTIMODALE THERAPIES

There is increasing evidence that oncolytic virotherapy shows antitumoral efficacy in clinical application even as monotherapy. However, most preclinical data suggest that virotherapy can be ideally combined with other treatment options to raise significant therapeutic synergies on several levels (see also an overview in **Figure 2**). First of all, oncolytic virus treatment needs to be integrated in combined tumor-treatments leading to optimized induction of ICD. Excellent reviews already exist on this aspect (16, 142, 143). Next step should be additional measures that amplify, and prolong antitumoral immune responses. First data obtained in humans and in murine melanoma models suggest significant synergies when systemic immunotherapies, such as ipilimumab and virotherapy are combined in a well-coordinated manner (138, 144). A very promising but clinically challenging point will be the combination of viral oncolysis with surgical removal of the tumor. Finally, it still needs further investigations to establish follow-up therapies that work like classical boost strategies and may also pick up personalized approaches such as NGS of tumors, epitope prediction and and immunoanalysis in treated patients. Multimodal therapy schemes will be a clue to establish virotherapy in the clinic.

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Oncolytic virotherapy as emerging immunotherapeutic modality: potential of parvovirus H-1

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Human tumors develop multiple strategies to evade recognition and efficient suppression by the immune system. Therefore, a variety of immunotherapeutic strategies have been developed to reactivate and reorganize the human immune system. The recent development of new antibodies against immune check points may help to overcome the immune silencing induced by human tumors. Some of these antibodies have already been approved for treatment of various solid tumor entities. Interestingly, targeting antibodies may be combined with standard chemotherapy or radiation protocols. Furthermore, recent evidence indicates that intratumoral or intravenous injections of replicative oncolytic viruses such as herpes simplex-, pox-, parvo-, or adenoviruses may also reactivate the human immune system. By generating tumor cell lysates *in situ*, oncolytic viruses overcome cellular tumor resistance mechanisms and induce immunogenic tumor cell death resulting in the recognition of newly released tumor antigens. This is in particular the case of the oncolytic parvovirus H-1 (H-1PV), which is able to kill human tumor cells and stimulate an anti-tumor immune response through increased presentation of tumor-associated antigens, maturation of dendritic cells, and release of pro-inflammatory cytokines. Current research and clinical studies aim to assess the potential of oncolytic virotherapy and its combination with immunotherapeutic agents or conventional treatments to further induce effective antitumoral immune responses.

Keywords: immunotherapy, autonomous parvovirus, H-1PV, talimogene laherparepvec, T-VEC, JX-594, dendritic cells, CTLA-4

INTRODUCTION

Human tumors develop complex strategies to circumvent the human immune system and to become resistant to classical therapies like radiotherapy or chemotherapy (1). Besides the low immunogenicity of tumors, tumor-induced dysregulation of the immune response leads to loss of effective immune defense and uncontrolled tumor growth. Even though many classical chemotherapy or radiation strategies induce some extent of tumor surveillance (1), new approaches should be tested to overcome early tumor resistance and recurrence. Thus, the basic challenge of molecular immune targeting is to conquer local regulatory mechanisms in order to re-introduce tumor immune recognition and promote tumor cell apoptosis and immunogenic cell death (ICD) (2). Recently, loss of immune defense has been shown to be caused by expression of different immune suppressive receptors also called immune checkpoint pathways, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) (3). Its ligation is crucial to preventing immune overreaction by inhibiting T-cell activation (4). The inhibitory CTLA-4 antibody ipilimumab [Yervoy, Bristol Myers Squibb (BMS)], approved for the treatment of metastatic melanoma patients, blocks this negative immune stimulatory receptor, thereby preventing downregulation of T-cell activation (5).

Oncolytic virotherapy represents an emerging therapeutic modality that has achieved tumor regression in several pre-clinical models and in clinical trials (6). Preferential depletion of cancer cells by oncolytic viruses (OV) is based on the fact that more aggressive tumor cells show both impaired antiviral responses and higher permissiveness for virus replication. Therefore, these agents open up new horizons for the treatment of cancer types that commonly display poor prognosis (7, 8). Cancer virotherapy is an old concept that arose from observations of unexpected tumor regressions coinciding with virus infections. This can be exemplified by a report on Newcastle disease virus (NDV) in gastric cancer dating back to 1971 (9). It should be stated that viruses with natural or engineered effects on the immune system are highly potent candidates for cancer therapy (**Table 1**). Herein, oncolytic viruses can be engineered to deliver therapeutic transgenes to cancer cells, causing additional anti-tumor effects through cytokine secretion and induction of anti-tumor immune responses (10–14). For example, the oncolytic vaccinia virus pexastimogene devacirepvec (Jennerex, Inc., and Transgene SA; Pexa-Vec, JX-594) and herpes simplex virus (HSV) talimogene laherparepvec (T-VEC, Amgen) were "armed" with GM-CSF-expressing genes (15, 16) to initiate local and systemic immune responses. Recently a randomized, Phase III trial of talimogene laherparepvec or GM-CSF in patients (pts)

with unresectable melanoma with regional or distant metastases (OPTiM) met its primary endpoint by improving durable response rates versus GM-CSF alone, and showed a tolerable safety profile (17). A Phase II study of Pexa-Vec in primarily first-line liver cancer (HCC) patients demonstrated survival improvement in patients receiving intratumoral (it) injections of high-dose Pexa-Vec (18). The following randomized Phase IIb study in second-line HCC patients did not meet its primary endpoint of survival improvement for Pexa-Vec compared to best supportive care (BSC) (19). However, this trial was comprised primarily of patients with end-stage disease and significant comorbidities such as liver cirrhosis, therefore likely not the optimal population for successful OV therapy. Therefore, further studies of Pexa-Vec in a less advanced HCC population as well as other indications are warranted. Besides above-mentioned agents, various other viruses were shown to have oncolytic and/or immunostimulating properties, and are presently used in clinical trials. These include Parvovirus, Adenovirus, Vesicular Stomatitis Virus, Reovirus, NDV, Measles Virus, Seneca Valley Virus, Poliovirus, and Coxsackie Virus (**Table 1**).

The aim of this article is to provide an overview of upcoming oncolytic viruses and their potential immunogenic therapeutic effects. A first insight into this issue is provided through our pioneer studies showing that infection with the autonomous parvovirus H-1 (H-1PV) generated immunogenic tumor cell lysates (TCLs) (14). H-1PV-infected TCLS proved able to induce maturation of dendritic cells (DCs), release of pro-inflammatory cytokines, tumor-associated antigens (TAA) cross-presentation, and T-cell stimulation in an *ex vivo* human melanoma model (see **Figures 1** and **2**) (7, 14, 55, 56). On the basis of these observations, we present the prospects of H-1PV and other OVs activating the human immune system either alone or in combination with immunomodulators, such as antibodies blocking immune suppressive receptors.

METHODS

The human *ex vivo* melanoma model (**Figure 2**) represents a system that mimics the *in vivo* situation (14). Thus, it was used to investigate effects of H-1PV-infected or tremelimumab-treated tumor cells on immune activation. The human melanoma cells MZ7-Mel, SK29-Mel-1, and SK29-Mel-1.22 used were a gift from T. Woelfel (Mainz, Germany) (57). The SK29-Mel-1.22 cell line ($A2^-$) is an *in vitro* selected HLA-A2-loss variant of HLA-A2-positive SK29-Mel-1 ($A2^+$) line (58, 59). The cytotoxic T-cell clones CTL2/9 and CTL IVSB recognize different antigens of SK29-Mel-1 cells in association with HLA-A2 (57, 58), lyse SK29-Mel cells, and release interferon γ (IFNγ) upon specific recognition of SK29-Mel-specific TAA (58).

Peripheral blood mononuclear cells (PBMCs) were derived from buffy coats of healthy blood donors. Monocytes were isolated via adherence, and differentiation into immature DCs (iDCs) was achieved by stimulation with GM-CSF and interleukin-4. Matured DCs (mDCs) were generated by stimulation with a cytokine cocktail for 2 days (60). For coculture experiments, melanoma cells were kept in FCS-free medium. For induction of maturation and phagocytosis, tumor cells were co-cultured with iDCs at a ratio of 1:3 for 2 days. CTL-Coculture with DC was performed at 1:10 ratio (60).

RESULTS: ONCOLYTIC VIRUSES ARE ABLE NOT ONLY TO KILL HUMAN TUMOR CELLS BUT ALSO TO STIMULATE ANTI-TUMOR IMMUNE RESPONSES: THE CASE OF PARVOVIRUS H-1PV

Over the last years, OV therapy has shown promising results in both pre-clinical and clinical studies against various solid tumors (61). It is worth noting that besides their own anti-tumor efficiency, OVs can resensitize resistant tumors to chemotherapeutics, thereby highlighting the potential of OVs in multimodal treatments (12, 13). We were particularly interested in the oncolytic parvovirus H-1PV [for reviews, see Ref. (20, 62)]. The mode of action of H-1PV involves both direct oncolytic and immune-mediated components, making this virus an attractive candidate for inclusion in the cancer immunotherapy armamentarium (60). H-1PV is a small nuclear-replicating DNA virus, which preferentially multiplies in oncogene-transformed and tumor-derived cells (7). This oncotropism results at least in part from the dependence of H-1PV on proliferation and differentiation factors that are dysregulated in neoplastic cells (20). In consequence, H-1PV exerts oncolytic effects, which were documented in human cells from various tumor entities including melanoma, pancreatic (PDAC), hepatocellular (HCC), colorectal or gastric carcinomas, sarcoma, glioma, and other neuroectodermal tumors (7, 20, 21, 62–64). Most interestingly, the death mechanisms activated by parvoviruses allow them to overcome resistance of tumor cells to conventional cytotoxic agents (22, 65). Another intriguing aspect of H-1PV-mediated OV lies in the possibility of combining H-1PV with conventional cytotoxic drugs to achieve synergistic tumor cell killing effects, as demonstrated for instance in the PDAC system (13, 21, 22, 66).

Though not or poorly infectious for humans under natural conditions, H-1PV can be administered experimentally to patients, resulting in viremia and seroconversion (67). Infections with H-1PV appear to be clinically silent (68). It should also be stated that recombinant parvoviruses can be constructed, for example to transduce immunostimulatory cytokines (62). This arming strategy was found to increase the anti-tumor effects of parvoviruses in certain models (69–71).

BRINGING H-1PV FROM THE BENCH TO THE BEDSIDE

Recent work using an immunocompetent rat glioma model showed that H-1PV was able to efficiently cure gliomas, while raising an anti-tumor memory immune response. This oncosuppressive effect appears to rely on both the direct oncolytic activity of H-1PV and its handover to the host immune system (23). These pre-clinical data led to the current clinical evaluation of H-1PV it and intravenous (iv) administration to patients with recurrent resectable GBM progressing in spite of conventional therapies (27).

H-1PV-INDUCED TUMOR CELL LYSATES TRIGGER MATURATION OF iDCs AND EXERT IMMUNOSTIMULATING EFFECTS

H-1PV had little direct killing activity on human immune cells *in vitro*, in particular APCs and CTLs. Interestingly, the analysis of infected PBMCs revealed the induction of markers of both macrophage and Th1cell activation (**Table 2**). This Th1 bias is indicative of a possible direct immunostimulating capacity

Table 1 | Oncolytic viruses.

Oncolytic virus	Family	Pre-clinical data	Clinical trial	Selected reference
Parvovirus H-1	Parvoviridae ss DNA Icosahedral capsid	Oncotoxicity of the viral protein NS1 Virus replication-associated cytopathic/lytic effects Activation of immune responses Transgene expression (cyto/chemokines) Inhibition of neo-angiogenesis Ref. (12–14, 20–26)	Phase I/IIa glioblastoma multiforme (ParvOryx01)	Clinical: NCT01301430 (27)
Vaccinia/poxvirus	Poxviridae ds DNA Enveloped Pexastimogene devacirepvec (Pexa-Vec; JX-594): engineered from Wyeth vaccine strain GLV-1h68 (GL-ONC1): engineered from vaccinia virus Lister strain	Cell lysis caused by viral replication Thymidine kinase (TK) gene-inactivated, selective replication Transgene expression (GM-CSF) (28) Disruption of tumor-associated vasculature (29) Induction of antibody-mediated complement-dependent cancer cell lysis (30)	Phase IIB, hepatocellular carcinoma, Pexa-Vec Phase II, colorectal cancer, Pexa-Vec Phase II renal cell carcinoma, Pexa-Vec Phase I and II, malignant pleural effusion, peritoneal carcinomatosis (GL-ONC1)	Clinical: NCT01387555; NCT01394939; NCT01766739; NCT01443260
HSV-1	Herpesviridae ds DNA Icosahedral capsid Enveloped Talimogene laherparepvec: engineered from JS1 strain	Cell lysis caused by viral replication ICP34.5 functional deletion (neurovirulence factor) ICP47 deletion Activation of anti-tumor immunity Transgene expression (GM-CSF) (31)	Phase III complete, malignant melanoma (talimogene laherparepvec)	Clinical: NCT00769704 (32, 33)
Adenovirus	Adenoviridae ds DNA Oncorine based on H101-virus	Cell lysis caused by viral replication Activation of anti-tumor immunity Cytotoxicity by viral proteins (E4ORF4) (34) Transgene expression (GM-CSF by CG0070) (35, 36)	Phase II and III, bladder cancer (CG0070) Approved therapeutic (China), head and neck cancer (Oncorine)	Clinical: NCT01438112 (37, 38)
Vesicular stomatitis virus (VSIV, often VSV)	Rhabdoviridae ss RNA	Expression of IFN-β (39, 40)	Phase I, liver cancer (IFN-β expressing VSV)	Clinical: NCT01628640
Reovirus	Reoviridae ds RNA Icosahedral capsid	Cytopathic effect Activation of immune response (41)	Phase I-III, several entities, e.g., head and neck cancer, non-small cell lung cancer, prostate cancer, colorectal cancer (Reolysin)	Clinical: NCT01166542; NCT01708993; NCT01619813; NCT01622543
Newcastle disease virus	Paramyxoviridae ssRNA	Activation of anti-tumor immunity (42–47)	Phase I and II study in glioblastoma, sarcoma and neuroblastoma	Clinical: NCT01174537

(Continued)

Table 1 | Continued

Oncolytic virus	Family	Pre-clinical data	Clinical trial	Selected reference
Measles virus	Paramyxoviridae ss RNA	Cytopathic effect (48) Anti-tumor activity (49)	Phase I study in malignant solid tumor, breast cancer, malignant tumor of colon, GIST, ovarian cancer Phase I study in multiple myeloma and plasma cell neoplasm Phase I study in metastatic squamous cell carcinoma of the head and neck cancer Phase I in malignant pleural mesothelioma Phase I in brain and central nervous system tumors Phase I in ovarian cancer, peritoneal cavity cancer Phase I and II study in recurrent ovarian cancer	Clinical: NCT01376505; NCT00450814; NCT01846091; NCT01503177; NCT00390299; NCT02068794 (50–52)
Seneca valley virus	Picornaviridae ss RNA	Antineoplastic activity (53)	Phase I safety study, solid tumors with neuroendocrine features Phase II after chemotherapy in small cell lung cancer Phase II with cyclophosphamide in neuroblastoma, rhabdomyosarcoma	Clinical: NCT00314925; NCT01017601; NCT01048892 (54)
Cavatak virus (Coxsackie virus A21)	Picornaviridae ss RNA Capsid		Phase I study in non-small cell lung cancer, castrate resistant prostate cancer, and melanoma and bladder cancer Phase I study in melanoma, breast, and prostate cancer Phase I study in melanoma Phase I study in head and neck cancer Phase II study, malignant melanoma	Clinical: NCT02043665; NCT00636558; NCT00438009; NCT00832559; NCT01227551; NCT01636882

Oncolytic viruses in clinical trials (ds, double stranded; ss, single stranded).

of the parvovirus. Nevertheless, a major impact of H-1PV on the immune system appears to be indirect, i.e., mediated by infected tumor cells, as discussed in the following sections. H-1PV caused the death of human melanoma cells in culture, including the above-mentioned SK29-Mel-1 and SK29-Mel-1.22 lines. The extent of cell killing varied between tested lines, was dependent on the multiplicity of infection (MOI) and correlated with expression of the replicative viral non-structural protein NS1. In this system, H-1PV induced an apoptotic cell death, which was accompanied with the release of immunogenic HSP72 (63).

In further experiments it was shown that H-1PV-infected melanoma TCLs were phagocytosed by iDCs and induced their maturation, in particular the secretion of pro-inflammatory

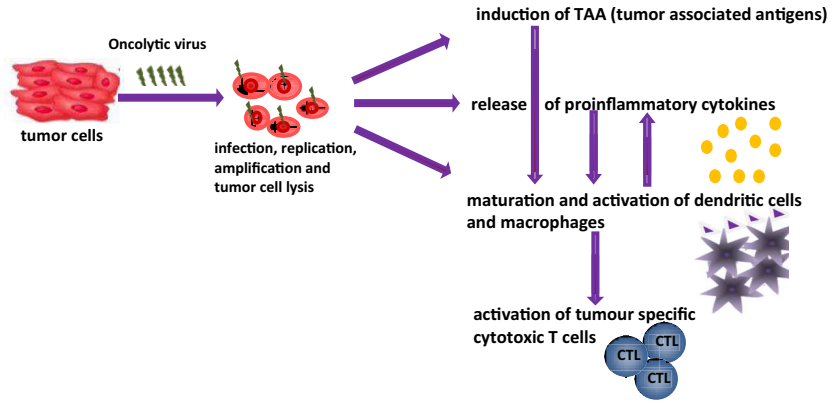

FIGURE 1 | Oncolytic viruses and their possible function in tumor therapy [changed after Ref. (14)].

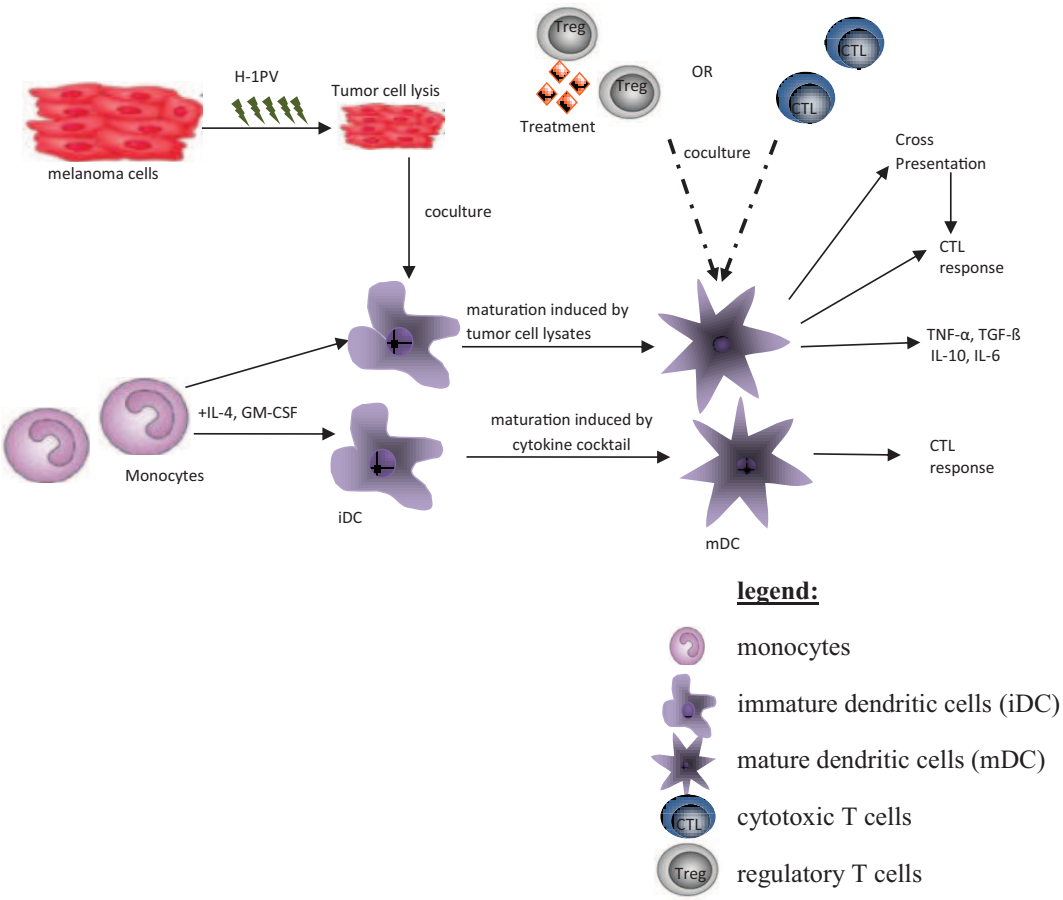

FIGURE 2 | The *ex vivo* human melanoma model.

cytokines such as TNFα and IL-6 (13, 63). Lysates of infected SK29-Mel-1.22 and MZ7-Mel cells were both competent for inducing DC maturation, although the former were more potent than the latter in this regard (13, 14). Primary immune cells were not permissive for H-1PV infection. Little direct killing effect, no apoptosis, and no progeny virus production could be detected in infected lymphocytes, monocytes, immature, and mature DCs (**Table 2**) (63).

We also demonstrated that human DCs coincubated with H-1PV-induced melanoma TCLs showed enhanced expression

Table 2 | Direct immunostimulating effects of parvovirus H-1PV.

Outcomes of infection of activated human peripheral blood mononuclear cells with parvovirus H-1PV

• **Abortive infection**	no progeny virus production viral RF DNA and transcripts detectable in T cells and macrophages
• **Cytotoxic effects**	indirect (TNF-α Ab protection)
	↑
• **Macrophage early activation**	induction of **TNF-α** release
	↓?
• **CD4⁺ Th cell late activation**	enhanced CD69, CD30 activation marker expression induction of Th1 and Th2 cytokine secretion (↑ **IFN-γ** → Th1 bias?)
• **CD4⁺ CD25⁺ Treg cell inhibition**	inhibition of suppressive activity (MLR test)
• **Stimulation of antiviral defense**	induction of type-I interferons through TLR9-mediated sensing

Moehler et al. Cancer Gene Ther, 2003
Grekova et al. Cancer Biol Ther, 2011
Moralès et al. PLOS ONE, 2012
Raykov et al. PLOS ONE, 2013

of TLR3, TLR9, and other maturation markers. This suggested that virus-induced TCLs contained molecular patterns triggering TLR signaling in DCs, as further evidenced by increased NF-κB levels and production of pro-inflammatory cytokines (12). Some of these immunostimulating patterns may consist of viral constituents, given the known ability of TLR3 and TLR9 for sensing viral determinants.

Combination of the oncolytic virus with cytostatic (cisplatin, vincristine) or targeted (sunitinib) drugs resulted in a further increase in melanoma cell apoptosis but failed to strengthen maturation of DCs. It was verified that the cytotoxic or targeted drug regimen used did not interfere with H-1PV infection (13). Interestingly, the interleukin profile of DCs was altered upon exposure to H-1PV plus sunitinib-cotreated TCLs. It therefore appears that H-1PV combination with this anti-angiogenic drug may reinforce its capacity not only for jeopardizing tumor cell survival but also for modulating the immune system.

H-1PV INDUCE ACTIVATION OF ANTIGEN-SPECIFIC CYTOTOXIC T-CELLS AND OTHER ANTI-TUMOR IMMUNE EFFECTORS

To further assess whether phagocytosis of H-1-infected TCLs by DCs induces cross-presentation of TAAs to antigen-specific CTLs in an HLA-class I-restricted manner, the above-mentioned human melanoma *in vitro* model was used (58, 72). Both melanoma-specific CTL clones tested were found to release increased levels of IFNγ after being co-cultured with DCs preincubated with H-1PV-infected SK29-Mel-1 or HLA-negative SK29-Mel-1.22 cells (14). Thus, H-1PV-induced TCLs stimulated cross-presentation of TAAs by DCs. This effect may contribute to reinforce the anti-tumor immune response by generating tumor-specific CTLs (14). In addition, several H-1PV-infected tumor cells were recently found to acquire an enhanced capacity for activating NK cells and getting killed by these cells (73, 74). The adjuvant effect of H-1PV was also evidenced *in vivo* by the virus-enhanced efficacy of an autologous tumor cell vaccine (24) and the adoptive transfer of anti-tumor immune cells from animals undergoing oncolytic H-1PV therapy (75).

ONCOLYTIC H-1PV VIROTHERAPY CAN BE COMBINED WITH IMMUNOTHERAPEUTIC AGENTS TO ENHANCE TREATMENT EFFICACY

Recent evidence for the expression of the immunosuppressing molecule CTLA-4 on regulatory T-cells (Tregs) and tumors generated widespread interest in the role of CTLA-4 in tumor escape and peripheral tolerance (3, 58). In particular, the human colon adenocarcinoma line SW480 was found to express CTLA-4 on the cell surface. This prompted us to extend the analysis of H-1PV anti-tumor effects to the SW480 system in combination with the anti-CTLA-4 antibody tremelimumab. When applied alone, this antibody had no detectable effect on SW480 cell viability and DC maturation. On the other hand, H-1PV alone was able to kill SW480 cells in a MOI-dependent manner. H-1PV-induced SW480 TCLs triggered iDC maturation in coculture experiments, as revealed in particular by increased release of the pro-inflammatory cytokines IFNγ, TNFα, and IL-6 (64). The secretion of IFNγ was stimulated to a low extent by treatment of the coculture with tremelimumab, recommend the use of the H-1PV/tremelimumab combination treatment to enhance tumor immunogenicity through both DC activation and CTLA-4 masking. It should also be stated that other (immuno)modulators, namely IFNγ (75) and HDAC inhibitors (76), were recently reported to cooperate with H-1PV for tumor suppression in human carcinoma animal models.

CLINICAL EVIDENCE OF OV-MEDIATED ACTIVATION OF IMMUNE RESPONSES IN HUMANS

Extensive analyses were performed to evaluate mechanisms-of-action of the oncolytic and immunotherapeutic vaccinia virus Pexa-Vec in patients. These include oncolysis (15, 77, 78), acute vascular disruption (29) as well as anti-tumor immune response induction. Pexa-Vec was engineered to express GM-CSF to stimulate white blood cell production and activate DCs. Detectable concentrations of GM-CSF in plasma were measured 4–15 days after treatment and associated with increased neutrophil, monocyte, and eosinophil production in patients receiving iv or it iPexa-Vec (77, 78). Inflammatory cell recruitment to tumors was confirmed on biopsy following Pexa-Vec administration in patients with melanoma (79, 80). Furthermore, functional anti-cancer immunity of Pexa-Vec treatment was demonstrated in patients by measuring induction of antibody-mediated complement-dependent cytotoxicity (CDC) utilizing a panel of tumor cell lines of different histologies (30). Low concentrations of serum *ex vivo* incubated with tumor cells resulted in a dramatic reduction in tumor cell viability; when normal cells did not exhibit decreased viability. This activity was shown to be dependent on both active complement as well as IgG antibody. Reproducible CDC activity was also observed in a Phase II study in HCC patient (18). Furthermore, T-cell responses to β-galactosidase peptides were detected in HCC patients treated with Pexa-Vec, as shown by ELISPOT analysis. In that way, the proof-of-concept provides that T-cell responses can be induced to transgenes encoded by oncolytic vaccinia viruses (18).

Talimogene laherparepvec is an oncolytic immunotherapy comprising a modified HSV type 1 engineered to selectively replicate in tumor cells and to express the immune-stimulating cytokine GM-CSF, while retaining sensitivity to antiherpetic agents (16). Local effects after intralesional injection include selective lysis of tumor cells and subsequent release of tumor antigen, as well as secretion of GM-CSF into the local environment, which results in the stimulation and maturation of DCs (32, 81). Antigen presentation by stimulated DCs to $CD4^+$ and $CD8^+$ cells may induce an adaptive systemic immune response (16, 82, 83). Recently a randomized, Phase III trial of talimogene laherparepvec in patients (pts) with unresected melanoma with regional or distant metastases (OPTiM) met its primary endpoint, demonstrating a significant improvement in durable response rate (defined as partial or complete responses that were maintained for ≥6 months starting within 12 months) versus GM-CSF alone (16 versus 2%, $p < 0.0001$) (17). Overall response rate was also higher in the talimogene laherparepvec arm (26.4 versus 5.7%, $p < 0.0001$). Subjects treated with talimogene laherparepvec showed a tolerable safety profile with the only grade 3/4 adverse event that occurred in >2% of patients being cellulitis (2.1%). A trend toward improved overall survival was seen based on a planned interim analysis (17). The primary overall survival results are pending. Evidence of durable responses together with the safety profile of talimogene laherparepvec supports evaluation of combinations with other immunotherapies, such as high-dose IL-2 or immune checkpoint blockade and with radiation therapy, chemotherapy, and/or targeted therapies that might amplify the anti-tumor response generated by talimogene laherparepvec (32).

DISCUSSION: POTENTIAL OF THE IMMUNOVIROTHERAPY CONCEPT

Despite recent improvements in surgical, locoregional, and systemic therapies, the prognosis of patients with gastrointestinal, hepatobiliary, and pancreatic cancers remains dismal, and treatment is limited to palliation in the majority of patients. These limitations indicate an urgent need for novel therapeutic strategies (13, 64, 66, 84). Combinations of oncolytic viruses with new targeted therapies draw much attention. It is however necessary to proceed with caution, as these therapies may interfere with pathways, which are needed for replication of genetically modified viruses. It was demonstrated that by interacting with the EGFR/RAS/RAF pathway, sorafenib inhibits replication of Pexa-Vec in liver cancer, when applied in combination. This is not surprising as Pexa-Vec replication is in part dependent on the EGFR/RAS/RAF pathway (85). Nevertheless, sequential therapy with Pexa-Vec followed by sorafenib resulted in decreased tumor perfusion and was associated with objective tumor responses for HCC (85). It is noteworthy that some oncolytic viruses such as parvovirus H-1PV also have potential to inhibit neo-angiogenesis. Therefore, OV-based combination treatments targeting both tumor cell proliferation and tumor angiogenesis represent a promising strategy for impeding the growth of various cancers (25).

Besides their low expression of TAA and low immunogenicity, tumors can induce an immune tolerance milieu by releasing anti-inflammatory cytokines such as IL-10 or TGF-β or recruiting Tregs to their microenvironment (86). T-cell activation relies on both, recognition of major histocompatibility complex (MHC) molecules by the T-cell receptor (TCR), and on costimulatory signals. Depending on the type of costimulatory receptor, T-cells can be activated or become anergic. For example, T-cell activation was prevented by engagement of CTLA-4 receptors with CD80 or CD86. In contrast, engagement of CD80 or CD86 with CD28 induced T-cell activation, often with a low affinity (87). Thus, a promising therapeutic option to achieve strong anti-tumor immune responses is the use of monoclonal antibodies against CTLA-4 and PD-1 alone or in combination. Herein, the constitutive expression of CTLA-4 and PD-1 on Tregs may play a crucial role in inhibiting anti-tumor T-cell responses. Tregs are often found in the peripheral blood of cancer patients and in the tumor microenvironment. These cells suppress an optimal anti-tumor immune response by preventing infiltrating $CD8^+$ T-cells from proliferating and producing cytolytic granules (88). BMS developed an anti-CTLA-4 monoclonal antibody named ipilimumab and an anti-PD-1 monoclonal antibody named nivolumab. Both antibodies were already tested in Phase III trials and found to achieve clinically significant benefits in median overall survival (89, 90). First pre-clinical studies of the combination of these antibodies to achieve blockade of both CTLA-4 and PD-1 showed increased tumor infiltration by $CD4^+$ and $CD8^+$ T-cells, enhanced IFNγ and TNFα production, and reduced amounts of Tregs (91). A Phase I study of nivolumab and ipilimumab combination in advanced melanoma patients showed an outstanding activity in 65% of patients with an objective response rate of 40% (92). As part of their further development and mechanistic understanding, these antibodies against immune check points would certainly deserve to be combined with OV in order to optimize anti-tumor

immune responses. Preliminary data from a Phase Ib trial combining talimogene laherparepvec with ipilimumab indicated that the combination was tolerable and devoid of unexpected toxicities (93). Exploiting these combinations represents a promising strategy to bring oncolytic viruses from bench to bedside and to establish oncolytic virotherapy as a new effective immunotherapeutic approach.

KEY CONCEPTS

- **Key concept$_1$**: There is a consistent need for immunotherapies in the treatment of human cancer.
- **Key concept$_2$**: Oncolytic viruses reduce tumor burden and show first clinical results in humans.
- **Key concept$_3$**: Oncolytic viruses, such as parvovirus H-1PV, induce effective anti-tumor immune responses.
- **Key concept$_4$**: Combinations of oncolytic viruses with immunotherapeutics are likely to achieve enhanced immune activation.

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GENERAL COMMENTARY
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How informative is the immune response against surrogate tumor antigens to assess antitumor immunity?

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A commentary on

The strength of the T cell response against a surrogate tumor antigen (TA) induced by oncolytic vesicular stomatitis virus (VSV) therapy does not correlate with tumor control
by Janelle V, Langlois M-P, Lapierre P, Charpentier T, Poliquin L, Lamarre A. Mol Ther (2014). doi: 10.1038/mt.2014.34

The last decade has seen the development of numerous antitumor therapeutic approaches. Concomitantly, the interest for using oncolytic viruses (OV) against cancer has grown tremendously and a number of promising candidates are now in preclinical and clinical studies. Tumor regression *in vivo* following viral infection has been shown to be a multifactorial process (1). The reductionist view of viruses simply causing direct lysis of infected cancer cells has now been replaced by a view including the complex interplay between viruses and the tumor environment. The important role of the immune response in either limiting or enhancing OV therapy is also now well recognized (2, 3). The prototypic *Rhabdoviridae* VSV has generated encouraging results in various experimental tumor models and is now used in a phase I clinical trial in patients with liver cancer (www.clinicaltrials.gov; #NCT01628640). VSV possesses intrinsic oncolytic properties as it replicates more efficiently in type-I interferon (IFN)-defective cells, a pathway frequently impaired during tumorigenesis (4). Cancer therapy using VSV has been shown to generate a variety of immune responses including tumor-specific $CD8^+$ T cells that are induced following the release of TA by infected cells (5). However, the tumor-specific immune response generated following VSV treatment is usually weak and often only leads to transient tumor control. Experimental tumor models expressing various surrogate non-self-TA have been developed over the years to more easily assess the magnitude and quality of immune responses generated against tumors. However, whether these responses are always representative of physiological antitumor immune responses is unclear.

Recently, our group characterized various VSV glycoprotein (G) mutants capable of interfering with host cell metabolism by inhibiting cellular transcription and translation in a kinetic similar to WT VSV as opposed to the prototypic matrix (M) mutant (M_{M51R}) that is slightly attenuated *in vitro* (6). Furthermore, VSV G mutants proved to be more cytolytic for B16 melanoma cells *in vitro* than the M mutant. To analyze their oncolytic potential *in vivo*, we used an immunocompetent mouse model implanted with B16 tumors transfected with a DNA minigene encoding the immunodominant $CD8^+$ T cell epitope of the lymphocytic choriomeningitis virus (glycoprotein aa 33–41) (7) as a surrogate non-self-TA (B16gp33) (8). Mice were injected subcutaneously into the flank with B16gp33 cells and when tumors reached a palpable size (day 7), animals were treated intratumorally every second day with three doses (days 7, 9, and 11) of WT VSV or of the G or M mutants. Tetramer and intracellular cytokine staining analysis revealed that $CD8^+$ T cells harvested from mice treated with WT VSV or the G mutants developed a polyfunctional gp33-specific immune response. Surprisingly however, the strength of the gp33-specific immune response generated did not correlate with the ability of a particular strain of VSV to slow down parental B16 growth and improve mice survival. Treatment with WT VSV was the poorest at controlling B16 tumor progression even though it induced a strong CTL response against gp33. On the other hand, M_{M51R} was more efficient than WT VSV at slowing down B16 growth despite the fact that this virus induced the lowest gp33-specific T cell response. We therefore determined whether $CD8^+$ T cell responses directed against endogenous self-TA were involved in limiting tumor progression. CTL responses against self-TA, such as TRP-1 and gp100, were barely detectable *ex vivo* when analyzed separately. However, adoptive transfer of purified $CD8^+$ T cells harvested from M_{M51R}-treated B16gp33 melanoma-bearing mice into naive mice provided better protection against parental B16 tumor implantation compared to CTLs taken from WT or G mutant-treated mice. These results suggest that the M mutant, despite being the weakest at inducing a T cell response against the surrogate non-self-TA gp33, induces the broadest antitumoral CTL response.

B16 melanoma is a highly aggressive tumor model in part because major histocompatibility complex class I (MHC-I) surface expression is very low on these

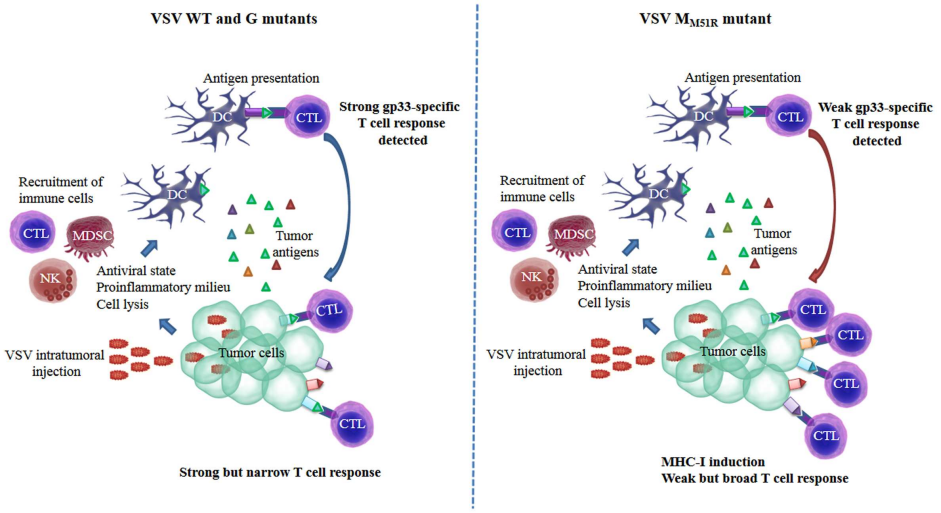

FIGURE 1 | Induction of a functional tumor-specific immune response is achieved through diverse mechanisms for different VSV strains: proposed model. VSV is quickly cleared from tumor tissue by the rapid induction of innate antiviral defense mechanisms and neutralizing antibodies. Nonetheless, the proinflammatory milieu generated in response to infection promotes leukocyte infiltration. Infection can result in tumor cell lysis either directly as a result of virus replication or indirectly through the action of innate immune cells generating a pool of tumor-associated antigens that may be taken-up by antigen-presenting cells such as dendritic cells and lead to T lymphocyte activation. Infection with WT VSV or glycoprotein (G) mutants induces a strong $CD8^+$ cytotoxic T lymphocyte (CTL) response against a surrogate non-self antigen (gp33) correlating with their ability to limit tumor growth (left panel). In contrast, the matrix mutant of VSV (M_{M51R}), although inefficient at inducing gp33-specific CTLs, is highly effective at slowing down tumor progression, likely through its capacity to induce the upregulation of MHC-I surface expression on cancer cells allowing for the induction of a broader CTL response (right panel). CTLs, cytotoxic T lymphocytes; DC, dendritic cells; MDSC, myeloid-derived suppressor cell; MHC-I, major histocompatibility complex class I; NK, natural killer; VSV, vesicular stomatitis virus.

cells. Strikingly, B16 infection with VSV M mutant induced the upregulation of surface MHC-I both *in vitro* and *in vivo*, a phenomenon that was not observed for WT VSV of the G mutants (8). The matrix protein of VSV was previously shown to alter trafficking of a molecule structurally similar to MHC-I, namely CD1d (9, 10). This leads to inhibition of antigen presentation to natural killer T (NKT) cells (11). Thus, VSV matrix protein could participate in the retention of MHC-I molecules within infected cells while the mutated protein in M_{M51R} may lack this ability. Thus, surface MHC-I upregulation following M_{M51R} treatment likely explains the significantly improved $CD8^+$ T cell-dependent survival despite the poor gp33-specific CTL response induced by this mutant. This may subsequently lead to presentation of a broader pool of B16 TA proportionally reducing the response against gp33 (see **Figure 1** for model).

In a recent study, Pedersen et al. compared vaccine-induced $CD8^+$ T cell responses directed against self and non-self-TA and showed that vaccination with adenoviral vectors encoding endogenous TA had little or no effect on the growth of B16 melanomas whereas vaccination with a similar vector construct expressing a surrogate non-self-TA induced efficient tumor control (12). Although vaccination against both self and non-self-TA induced comparable $CD8^+$ T cell responses in terms of cell numbers and effector functions, CTLs directed against self-TA were of lower functional avidity. These results are in agreement with our study and provide a potential mechanism explaining why T cell responses against self and non-self-TA are different and might not be induced at proportional levels during OV therapy.

Taken together, these results highlight a considerable limitation of many experimental systems used to assess antitumor immunity and warrant caution when extrapolating responses against surrogate TA to the overall antitumoral immune response. This may prove critical for the development of novel or improved OV, which may be biased by incorrectly estimating immune response correlates using such experimental systems. Therefore, great efforts will need to be made to develop improved methods for analyzing the antitumoral immune response induced by OVs against a broader array of TA in order to better appreciate their full therapeutic potential.

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Armed therapeutic viruses – a disruptive therapy on the horizon of cancer immunotherapy

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For the past 150 years cancer immunotherapy has been largely a theoretical hope that recently has begun to show potential as a highly impactful treatment for various cancers. In particular, the identification and targeting of immune checkpoints have given rise to exciting data suggesting that this strategy has the potential to activate sustained antitumor immunity. It is likely that this approach, like other anti-cancer strategies before it, will benefit from co-administration with an additional therapeutic and that it is this combination therapy that may generate the greatest clinical outcome for the patient. In this regard, oncolytic viruses are a therapeutic moiety that is well suited to deliver and augment these immune-modulating therapies in a highly targeted and economically advantageous way over current treatment. In this review, we discuss the blockade of immune checkpoints, how oncolytic viruses complement and extend these therapies, and speculate on how this combination will uniquely impact the future of cancer immunotherapy.

Keywords: oncolytic virus, cancer immunotherapy, immune-checkpoint inhibitors, CTLA-4, PD1, PDL1, PDL2, blockade of checkpoint inhibitors

INTRODUCTION

Tumors are difficult to treat and in many instances lethal. The treatment challenge is not surprising as they are genetically unstable and complex biological systems with an ability to adapt to and thrive in often harsh and changing environments. Furthermore, this plasticity increases the probability that subpopulations will acquire resistance to any one therapy. Thus one could argue that a disease with such a complex etiology must be met with an equally complex therapeutic approach. Appropriately, oncologists have for some time combined chemotherapy, radiation and surgery and complemented these strategies with more targeted approaches such as tumor selective antibodies and/or small molecule kinase inhibitors (1). More recently, two alternative therapeutic approaches, cancer immunotherapy and oncolytic viruses, have begun to show promise that should further complement the oncologist's repertoire of anti-cancer agents.

The area of cancer immunotherapy has had a long and complex history (2, 3). The idea that a patient's own immune system could remove a tumor in much the same way it so efficiently removes invading microbes has been around for more than a century. Through the years, however, this concept of immunosurveillance has fallen in and out of favor perhaps appropriately given the complex and dynamic role, it is now believed to play in cancer, acting anywhere from anti to pro-tumorigenic (4–6). Research is beginning to elucidate the mechanisms by which tumors evade the immune system and in some instances how tumors use it to their advantage. From this research several promising immune-checkpoint inhibitor targets that are now translating into exciting clinical trial results have emerged (7–9).

Like cancer immunotherapy, the concept of oncolytic viruses is not new dating back to at least the beginning of the twentieth century when it was observed that on occasion tumor regression would follow a viral infection (10, 11). Although over 100 years have passed since these initial observations, the idea of using a replicating virus to selectively infect and kill tumor cells remains understandably appealing. Theoretically, either naturally or through genetic engineering, such an agent would spare normal neighboring cells while killing cancer cells by viral lysis. Furthermore, the progeny released from the lysed cancer cells would result in a self-perpetuating and amplifying therapy. Adding to their appeal is the ability of such agents to deliver exogenous genetic material whose product or products could augment the oncolytic viral treatment (12–14). Despite their theoretical promise, the reality is that oncolytic viruses have had limited clinical success as monotherapies perhaps due to an imbalanced focus on safety over potency. Recently however, there are several late-stage clinical trials showing promise which may eventually lead to clinical acceptance (15, 16).

Here, we suggest merging immune-checkpoint blockers with oncolytic viruses. We will discuss not only how these approaches could complement one another biologically for increased therapeutic benefit, but also how they may represent a unique opportunity to employ alternative biological formats not normally utilized commercially (e.g., Fabs, scFv) to increase both the safety and therapeutic profile of these agents. Finally we will touch upon how, together, these attributes might translate into a more economically appealing and clinically active therapy resulting in a truly new and disruptive treatment for malignancies.

CANCER IMMUNOTHERAPY-BLOCKADE OF IMMUNE CHECKPOINTS

Immunotherapy works to direct the extensive repertoire of the host immune system to fight cancer. This approach strives to stimulate tumor suppression by (a) boosting the patient's immune

system, (b) decreasing the cancer-induced immunosuppression, and/or (c) increasing the immunogenicity of the tumor itself. If the immune system's ability to rapidly respond to and clear invading microorganisms could be extended to malignant cells then a powerful therapeutic may be realized. Such an approach may hold greater potential than current treatment approaches as it may prove to be more potent, benefit many more cancer types, offer long-lasting protection against the disease, and come with fewer off-target effects. Advances in cellular and molecular immunology have provided enormous insight into the inter-play between tumors and immune cells and from this research have come strategies by which the immune system might be harnessed to fight cancer (7).

The blockade of immune checkpoints is a more recent approach taken to decrease cancer-induced immunosuppression. Immune checkpoints refer to a number of inhibitory pathways that play crucial roles in maintaining self-tolerance and immune homeostasis. Their function is to down-regulate T-cell signaling in order to prevent uncontrolled T-cell proliferation thereby protecting tissues from auto-immune damage while maintaining tolerance to self-antigens. It is becoming increasingly clear that tumors commandeer certain immune-checkpoint pathways particularly against T cells that are specific for tumor antigens. Preclinical and clinical data have demonstrated that this is a major mechanism utilized by the tumor to evade the immune system. If this could be reversed, the resulting amplification of T cells and their activity would be highly beneficial to the patient given the central role T cells play in cell-mediated immunity. The immune checkpoints are controlled by ligand–receptor interactions, which can be readily blocked by antibodies or disrupted by recombinant forms of ligands or receptors making them appealing therapeutic targets. For a list of immune-checkpoint targeting antibodies that are currently in clinical trial see **Table 1**.

Table 1 | The most advanced clinically evaluated immune-checkpoint blocking antibodies.

Target	Antibody in development	Current clinical status	Reference
CTLA-4	Ipilimumab (MDX-010)	Approved for melanoma 2012. Multiple cancers (phase I, II, III)	(17–19)
	Tremelimumab (CP-675,206)	Multiple cancers (phase I, II)	(20–22)
PD1	Nivolumab (BMS-936558 or MDX1106)	Multiple cancers (phase I, II) Melanoma (recruiting phase III)	(23–25)
	CT-011	Multiple cancers (phase I, II)	(26, 27)
	MK-3475	Multiple cancers (phase I, II, III)	(28, 29)
PDL1	MDX-1105 (BMS-936559)	Multiple cancers (phase I)	(29)
	MPDL3280A	Multiple cancers (phase I, II)	(30)
	MSB0010718C	Multiple cancers (phase I)	
PDL2	rHIgM12B7	Melanoma (phase I)	
B7-H3	MGA271	Multiple cancers (phase I) Melanoma (phase I)	(31)
LAG3	BMS-986016	Multiple cancers (phase I)	

Above trial information from ClinicalTrials.gov.

The inhibitory receptor, Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), was the first checkpoint receptor to be extensively and successfully pursued as an anti-cancer target (32). The primary function of CTLA-4 is to regulate the magnitude of T-cell activation. It is expressed solely on T cells where it offsets the actions of CD28, a T-cell co-stimulatory receptor. Because CTLA-4 has a higher affinity for the CD28 ligands B7.1 and B7.2 it, in effect, out-competes CD28 for ligand binding resulting in an attenuated T-cell response (33–37). The lethal systemic immune hyperactivation phenotype of *Ctla4*-knockout mice clearly shows the importance of CTLA-4 and the need to keep T cells in check (38, 39). In 2011, an antibody against CTLA-4 (ipilimumab) was given FDA approval for the treatment of metastatic melanoma (20, 40–42). In a pivotal phase III randomized three-arm clinical trial, melanoma patients were treated with a glycoprotein 100 (gp100) peptide vaccine alone, ipilimumab alone, or the gp100 peptide and ipilimumab. Both ipilimumab groups demonstrated an increased survival of 3.5 months compared with the group receiving the gp100 peptide alone. Moreover, long-term survival was greatly increased with 18% of patients receiving ipilimumab surviving for greater than 2 years as compared with only 5% for the gp100 peptide alone cohort (17). Although ipilimumab treatment was relatively brief, spanning only 3 months, the finding of long-term progression-free survival supports the idea that immune-based therapies may actually result in a reprogramed immune system which can confer long-term antitumor immunity. Clinical trials are on-going evaluating the use of anti CTLA-4 antibodies in other cancer indications including lung, colorectal, renal, and ovarian (43).

The immune-checkpoint receptor, programed cell death 1 (PD1) and its ligands PDL1 and PLD2, are also emerging as promising targets. PD1 like CTLA-4 plays a role in regulating and maintaining the balance between T-cell activation and tolerance (44, 45). However, unlike CTLA-4, PD1 is more broadly expressed and can be found on other activated non-T-lymphocyte subsets including B cells and natural killer (NK) cells. Additionally while CTLA-4 primarily regulates T-cell activation, PD1 principally controls T-cell activity (46). The ligands PDL1 and PDL2 are commonly upregulated on the surface of many different human tumors with PDL1 being the predominant PD1 ligand on solid tumors. High expression levels of PDL1 have been shown on melanoma, lung, ovarian, and other human cancers (47, 48). PDL1 is also expressed on myeloid cells in the tumor microenvironment. *Pdl*, *Pdl1*, and *Pdl2*-knockout mice demonstrate a milder auto-immune phenotype than *Ctla4*-knockout mice (49–52). Preclinical studies have shown that blocking PD1 or its ligand PDL1 enhances immunity *in vitro* and mediates antitumor activity in preclinical models (53–55). Although the development of PD1 targeting antibodies is not as mature as that of CTLA-4 antibodies, preliminary clinical results look encouraging. In phase I trials of an anti-PD1 antibody (nivolumab), objective responses (complete or partial responses) were observed in those with non-small-cell lung cancer, melanoma, or renal-cell cancer with cumulative response

rates ranging from 18 to 28%. Responses were durable with 20 of 31 responses lasting 1 year or more (56). In a separate phase I trial of patients with various advanced cancers, an anti-PDL1 antibody (MDX-1105) also induced durable tumor regression (objective response rate, 6–17%) and prolonged stabilization of disease (12–41% at 24 week) (57).

Beyond CTLA-4 and PD1, molecular immunology has begun to reveal additional receptors and ligands that serve an inhibitory immune function. These include B and T-lymphocyte attenuator (BTLA), T-cell membrane protein 3 (TIM3), Lymphocyte activation gene 3 (LAG3), adenosine A2a receptor (A2aR), and the B7 family of inhibitory ligands (58–66). Each has been associated with the inhibition of lymphocyte activity in preclinical models and consequently antibodies against a number of these targets are being actively pursued (58–66). Additionally, because multiple inhibitory ligands and receptors contribute to the tumor's evasion of the immune system and appear to be non-redundant, there remains the possibility of further enhancing antitumor immunity by blocking multiple immune checkpoints. Currently several preclinical and clinical studies are on-going testing the effects of blocking a combination of immune checkpoints (**Table 2**) (67–73). In fact, a recently published phase I study in patients with melanoma that combined anti-CTLA-4 (ipilimumab) and anti-PD1(nivolumab) mAbs resulted in a rapid and deep tumor regression in a substantial proportion of patients (53% of patients had an objective response, all with tumor reduction of 80% or more) (74). These objective response rates exceeded the previously reported results with either mAb alone (17, 56).

Table 2 | The current clinical development of combined immune-checkpoint targeting agents.

Stage of clinical development	Targets	Antibodies in development	Target disease
Phase III	CTLA-4/PD-1	Ipilimumab + Nivolumab	Metastatic melanoma
Phase II	CTLA-4/PD-1	Ipilimumab + Nivolumab	Metastatic melanoma
Phase I	CTLA-4/PD-1	Ipilimumab + Nivolumab	Metastatic renal-cell carcinoma
	CTLA-4/PD-1	Ipilimumab + Nivolumab	Malignant melanoma
	CTLA-4/PD-1	Ipilimumab + Nivolumab	Non-small-cell lung cancer
	LAG3/PD-1	BMS-986016 + Nivolumab	Multiple cancers

Above trial information from ClinicalTrials.gov.

ONCOLYTIC VIRUSES AS (IMMUNO)THERAPIES

Oncolytic viruses can be RNA or DNA based and derived from human (e.g., herpes simplex virus, adenovirus, measles virus) or animal [e.g., vesicular stomatitis virus (VSV), Newcastle disease virus, myxoma virus] viruses. By definition they selectively replicate in, and kill cancer cells. This selectivity can be a natural property of the virus or an engineered trait (75–81). Oncolytic viruses can also be genetically armed to improve or generate more tumor selective cell killing. For example, cell death can be induced by delivering tumor-suppressors (e.g., p53, p16), pro-apoptotic proteins (e.g., TRAIL, IL-24), or small hairpin RNA targeting cell survival or proliferation factors (e.g., hTERT, survivin) (82–87). Arming can also sensitize the tumor to chemo or radiotherapy (Prodrug enzymes, NIS) (88–90).

Although direct oncolysis was envisioned as the primary desired outcome of this therapeutic approach, research and clinical data is supporting the assertion that these productive tumor-specific infections can elicit additional antitumor effects. For example there is evidence that oncolytic viral therapy can induce tumor vasculature shutdown resulting in tumor necrosis (91, 92). Data also suggests that because oncolytic viruses result in highly pro-inflammatory and immunogenic events (tumor cell death and the release of tumor-specific antigens) (93–95) they can elicit a tumor-specific immune response (96). Additionally, viruses encode products that can be recognized by immune and non-immune cells as Pathogen-associated molecular patterns (PAMPs) and can also cause the release of Damage-associated molecular pattern molecules (DAMPs) (97). PAMPs are structural motifs which serve as "danger" signals to the host indicating the presence of virus that trigger host defenses. These danger signals can be structural proteins and glycolipids but are mainly nucleic acids including double-stranded RNA (dsRNA), viral single-stranded RNA, and CpG DNA (98, 99). DAMPs are host nuclear or cytosolic proteins with defined intracellular function that activate effector cells from the innate immune system when they are released outside the cell (100). Virus-induced changes such as an increase in pro-inflammatory cytokines and chemokines, a decrease in immunosuppressive cytokines, and the release of PAMPs and DAMPs at the site of the tumor may diminish or reverse the established immunosuppressive microenvironment and initiate antitumor immunity.

Several oncolytic virus classes are currently in late-stage clinical trials (**Table 3**). The most advance of these, Talimogene laherparepvec (T-VEC, formerly OncoVex or JS1/ICP34.5-/ICP47-/GM-CSF; an HSV isolate selected for its potency over laboratory strains, it is deleted in both the ICP34.5 and ICP47 genes to further increase viral replication and tumor cell killing, it also expresses human GM-CSF for immune stimulation) has demonstrated some very promising clinical data. From recently announced results of a phase III trial in unresectable stage IIIB-IV melanoma receiving either T-VEC injected into the lesion or GM-CSF administered subcutaneously, the overall durable response rate (DRR) was 16.3% for T-VEC treated patients as compared to 2.1% for GM-CSF treated individuals (101). The objective overall response rate (ORR) was 26.4% for the T-VEC group (including 10.8% complete responders) compared to an ORR of 5.7% and a complete response rate of 0.7% in the GM-CSF alone group (101). Importantly, in a phase II trial, tumor shrinkage was noted in non-injected lesions, demonstrating that systemic immunity was induced (102). In addition, and across a number of viruses, studies have shown that both innate and adaptive immune responses are generated following viral tumor lysis (92, 103–111). This antitumor immunity is an important outcome of oncolytic viral therapy as it would lead to the destruction of tumor cells that escaped the initial viral lysis.

Table 3 | The most advanced clinically evaluated oncolytic viruses.

Virus	Name	Cancer type	Reference
Adenovirus	ONYX-015 H101	SCCHN Glioma Ovarian	(112–114)
	CGTG-102	Solid tumors	(115)
	CG0070	Bladder	(116, 117)
	ICOVIR-5	Solid tumors	(118–120)
	ColoAd1	Colorectal	(121)
Vaccinia virus	GL-ONC1	Solid tumors	(122–124)
	JX-594	Liver tumors Solid tumors IV	(125, 126)
Herpesvirus	G207	Glioma	(127–129)
	NV1020	Liver tumors IA	(130, 131)
	T-Vec	Breast SCCHN Melanoma IT Liver tumors	(132, 133)
Reovirus	Reolysin	SCCHN IT Solid tumors IV	(134–136)
Measles virus	MV-CEA	Ovarian IP	(137, 138)
	MV-NIS	Ovarian IP Glioma IT Myeloma IV Mesothelioma	(139–141)
NDV	PV701	Solid tumors	(142, 143)

Above trial information from ClinicalTrials.gov.

MERGING ONCOLYTIC VIRUSES AND IMMUNE-CHECKPOINT BLOCKING

The realization that oncolytic viral therapy can itself be an immunotherapy has in many ways reinvigorated the field and expanded the possible approaches that can be taken to treat cancer. Similarly, the discovery and targeting of immune checkpoints has opened a new immuotherapeutic avenue generating very promising clinical results. The potential to combine oncolytic viruses with a blockade of immune checkpoints is a very exciting strategy that may be beneficial on many levels and help overcome current shortcomings associated with either approach alone. To date, there have been only a few preclinical studies combining oncolytic viruses and immune-checkpoint blockers (anti-CTLA-4 mAb) (144, 145). However, results have been promising with one study showing that replication competent VSV in combination with anti-CTLA-4 mAb resulted in the elimination of macroscopic tumor implants in the majority of test animals, an outcome that could not be achieved by either treatment alone (145). The study went on to show that the response was CD4 and CD8 T-cell mediated (145). When combining these two approaches, the exact virus/checkpoint combination will likely need to be determined empirically with many factors including indication and immune status of patient playing a role. However, in general an argument can be made that the greatest synergies between these strategies would be realized by delivering the immune-checkpoint therapy directly from the oncolytic virus (**Table 4**).

Table 4 | The benefits of using an oncolytic virus to deliver immune-checkpoint blockers.

Viral attribute	Benefit		
	Safety	Potency	Economic
Immuno-stimulatory		x	
Targeted delivery	x	x	x
Delivery of alternative Ab formats	x	x	x
Multi-gene delivery	x	x	x

INCREASED PRIMING AND GREATER IMMUNE POTENCY

Preclinical studies have shown that in mice bearing partially immunogenic tumors, treatment with CTLA-4 antibodies could elicit significant antitumor responses whereas poorly immunogenic tumors were refractory to anti-CTLA-4 administration (32, 146). However, these refractory tumors could be made more responsive by administering granulocyte-macrophage colony-stimulating factor (GM-CSF) in combination with the anti-CTLA-4 (146). These findings suggested that a CTLA-4 blockade enhances an already existing endogenous antitumor response resulting in tumor regression. But when the tumor is poorly immunogenic and does not induce a robust enough immune response the anti-immune checkpoint is not as efficacious. Similar results have been found in the clinic where analysis of pre-treatment tumors indicated that patients with high baseline expression levels of immune-related genes were more likely to respond favorably to ipilimumab (147). Just as the GM-CSF is used to help boost the initial innate immune response, oncolytic viruses could have a similar effect as it is clear that the oncolytic viral infection has pro-inflammatory properties, eliciting both an innate and adaptive immune response.

ENHANCED SAFETY AND EFFICACY BY EXPRESSING IMMUNE-CHECKPOINT BLOCKERS FROM THE ONCOLYTIC VIRUS

The oncolytic virus and the immune-checkpoint blocker could be administered as two separate therapeutics but one of the most appealing aspects of the oncolytic viral approach is that it is localized to the tumor. This localization confers several advantages for both safety and potency. Clinical and preclinical data strongly suggest that a blockade of immune checkpoints is a very potent antitumor therapy. However, there are, in some cases, unwanted side effects. Given the importance of the immune checkpoints in maintaining immune homeostasis there is concern that a blockade of these receptors and/or ligands could lead to a break in immune self-tolerance resulting in autoimmune/autoinflammatory side effects (148). Blocking CTLA-4 as a therapy was initially questioned given its crucial role in the regulation of T-cell amplification. The phenotype of *Ctla4*-knockout mice also hinted at the possibility of a high number of unwanted immune-related effects. In the pivotal phase III trial of ipilimumab, Grade 3 or Grade 4 immune-related adverse events (including rash, colitis, hepatitis, and endocrinopathies) occurred in 10–15% of patients

treated with the anti-CTLA-4 antibody as compared to 3% of those treated with gp100 alone. During this trial, there were 14 deaths related to ipilimumab (2.1%), 7 of which were due to immune-related adverse events (17). Delivering the immune-checkpoint blocker (Ab, Ab derivative or modified ligand or receptor) from the oncolytic virus would localize the treatment and mitigate the risks inherent in systemic delivery. In preclinical studies of a replication competent adenovirus armed with the coding region of a full length CTLA-4 antibody a 43-fold higher antibody concentration in the tumor as compared to the plasma was noted (144). Moreover, plasma levels in treated mice remained below the reported human safety threshold (144).

It is also possible to make expression of these immune-checkpoint blockers contingent upon a productive viral infection (i.e., selective replication that is restricted to the tumor cell) further increasing the safety of the therapeutic. This can be done by utilizing endogenous late viral promoters that are dependent upon the uptake and replication within the target tumor cell to express exogenous genes and has been described for human adenovirus (12, 13, 149). In the normal cell, this expression would be blocked as replication would not be achieved consequently confining expression to target cancer cells. Potency, like safety also benefits from this localized delivery, concentrating the therapeutic to the tumor and its microenvironment. Accumulation of virally delivered transgenes (including reporter genes, prodrug converting enzyme, anti-angiogenic factors, immunostimulatory factors) at the site of the infected tumor has been shown in numerous studies (97, 115, 132, 150–153). For example, PET imaging experiments have dramatically demonstrated the tumor localized expression of thymide kinase following infection with an oncolytic virus armed with the enzyme (154, 155). This accumulation was translated into efficacy upon administration of the prodrug Ganciclovir (154). Additionally, the self-perpetuating nature of an oncolytic infection results in sustained transgene expression (156) that will continue until tumor regression is complete and the virus is eliminated from the tumor site by the immune system (157). Therefore the amount of material produced would be directly related, in theory, to the tumor load, personalizing the respective dose to the individual and their tumor burden. It is also appealing to consider that this may eliminate peaks and valleys associated with the intravenous administration of the therapeutic as the virus expressed molecule would be generated on a more constant basis that might also benefit the patient.

ENABLEMENT OF ALTERNATIVE THERAPEUTICS

Although viruses can be used to deliver an intact IgG, their focused delivery to the tumor site and their self-perpetuating nature allow for the use of alternative antibody formats such as diabodies, Fabs, and scFvs (144, 158). This could have a profound impact on any mAb-based antitumor therapeutic particulary immune-checkpoint blockers. From a safety standpoint, the use of these alternative Ab formats could be beneficial because IgGs, due to their size (150 kDa), have prolonged serum half-lives (>10 days) and are therefore more likely to have associated toxicities. If these alternative formats were to escape the tumor site their faster clearance reduces the risk for off-target events. For immune-checkpoint blockers, this could help to decrease the immune-related adverse events that have been associated with this therapeutic approach (148, 159). Additionally, smaller formats would potentially penetrate the tumor to a greater extent than a full length antibody. Studies have shown that an intact IgG molecule takes 54 h to move 1 mm into a solid tumor, whereas a Fab fragment travels the same distance in only 16 h (160). This enhanced penetration could increase overall efficacy. The diabodies in particular have been shown to provide rapid tissue penetration, high target retention, and rapid blood clearance presumably as a result of their multi-valent nature and intermediate size (55 kDa) (161). The use of alternative antibody formats also opens up the possibility of delivering multiple therapies from one oncolytic virus. This may have broad implications for the blockade of immune-checkpoint approach as studies are beginning to show that targeting multiple checkpoints may be more efficacious (67–71, 74). Without localized delivery, the use of these alternative formats would likely not be feasible as they would clear too rapidly (on the order of a few hours or minutes dependent upon the format) (162). This may necessitate the need for higher input doses or multiple injections of the Ab, which could potentially be cost prohibitive. Having localized delivery via the virus would avoid the need for full length Abs and make the smaller, faster-clearing formats viable therapies that are still capable of efficacious outcomes.

ECONOMICALLY ADVANTAGEOUS

Expression of immune-checkpoint blockers from an Oncolytic virus is economically appealing. If one assumes that the initial promising results seen with combination checkpoint blockers are maintained in larger phase II and III trials, the delivery of a combination of blockers from a virus would eliminate the need to commercially manufacture the molecules separately. This approach utilizes a single entity (the virus) to exploit the natural machinery of the virus and the tumor cell to continuously produce the therapeutic agents so long as the tumor cells continue to exist. Moreover, it has been demonstrated that multiple exogenous proteins can be delivered from a single virus (149). Due to their tumor selective localization, as mentioned previously, they would not need to express a full length antibody, making this approach potentially attractive and novel for delivering multiple-checkpoint inhibitors to the site. In addition, this therapy would have the potential added benefit of increased immunogenicity and/or direct tumor cell lysis offered by the oncolytic virus. Thus expressing a single biological agent with the ability to deliver multiple-checkpoint inhibitors that itself has anti-cancer activity is an interesting possibility. However, it should be kept in mind that the commercial manufacture of oncolytic viruses is behind that of antibodies and thus may be only a true economic advantage in the future with additional optimization.

CONCLUSION

In the fight against cancer, no single magic bullet has emerged. Despite several improvements in diagnostics and therapies nearly 7 million cancer-related deaths still occur every year worldwide (163). One reason is that cancer is complex and can evolve to thrive under harsh conditions and to evade the body's natural defenses. Two promising therapeutic strategies have emerged; the blockade of immune checkpoints and oncolytic viruses and we

Table 5 | The pros and cons of oncolytic viral, immune-checkpoint inhibition and combination therapy.

Therapeutic approach	Pros	Cons
Oncolytic virus	Selective for cancer cells Self-amplifying therapy Tumor burden dependent Pro-inflammatory/immunogenic Endogenous gene delivery	Selectivity is potentially cancer-type dependent Suboptimal potency as a monotherapy Pro-inflammatory/immunogenic Manufacturing challenges
Immune-checkpoint inhibitor	Potential to be non-cancer-type specific Potent/lasting tumor immunity Amendable to current biologics (antibodies, recombinant ligands, receptors)	Potential for adverse immunological events Dependent on immune status of patient
Oncolytic virus + immune-checkpoint inhibitor	Selective for cancer cells Self-amplifying therapy Tumor burden dependent Pro-inflammatory/immunogenic Endogenous gene delivery Potent/lasting tumor immunity	Selectivity is potentially cancer-type dependent Manufacturing challenges

believe that an argument can be made that the greatest potential for both of these therapies lies in the synergies that would be realized by delivering the immune-checkpoint therapy directly from the oncolytic virus (**Table 5**). We look forward to the continued evolution of these agents and to the exciting years ahead as we begin to see these agents come forward pre-clinically and clinically.

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Multimodal cancer therapy involving oncolytic Newcastle disease virus, autologous immune cells, and bi-specific antibodies

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This paper focuses on oncolytic Newcastle disease virus (NDV). This paper summarizes (i) the peculiarities of this virus as an anti-cancer and immune stimulatory agent and (ii) the approaches to further harness this virus as a vector to combat cancer. Special emphasis is given on combining virus therapy with cell therapy and on improving tumor targeting. The review will include some of the authors work on NDV, bi-specific antibodies, and cell therapy as building blocks for a new perspective of multimodal cancer therapy. The broad anti-tumor immune reactivation includes innate and adaptive, tumor antigen (TA) specific and TA independent activities

Keywords: Newcastle disease virus, T-cells, dendritic cells, bi-specific antibodies, tumor targeting, cellular therapy, hyperthermia

INTRODUCTION

Paramyxoviruses are a family of viruses that infect a diverse range of hosts. Animal pathogens, such as Newcastle disease virus (NDV), SV-5, and Sendai virus (SV), have been major subjects for basic research by virologists, immunologists, and molecular biologists. Previously, genetic manipulation of paramyxoviruses was not possible because the genome is not infectious alone and RNA recombination is essentially non-existent. During the last 15 years, methods of producing infectious paramyxoviruses from c-DNA clones (reverse genetics) have been developed. This review will focus on NDV, an avian paramyxovirus, because this has a number of very interesting anti-neoplastic and immune stimulating properties in mammalian cells, including human being, because it has a high safety profile for clinical application and because it can be harnessed by therapeutic transgenes.

NEWCASTLE DISEASE VIRUS, TRANSGENES, AND BI-SPECIFIC ANTIBODIES

ONCOLYTIC PROPERTIES OF NATURAL STRAINS OF NDV

Vaccine strains of paramyxoviruses such as mumps virus (MuV), measles virus (MV), and NDV efficiently infect and kill cancer cells and are consequently being investigated as novel cancer therapies (oncolytic virotherapy) (1). NDV wildtype (wt NDV) virus shows naturally tumor selective replication behavior (2). An abortive replication cycle by lentogenic strains leads eventually to tumor cell death. A lytic replication cycle by mesogenic or velogenic strains leads to fast tumor cell death (oncolysis) and further spread of the virus in the tumor tissue. The strong interferon (IFN) response of normal cells (2) prevents virus replication and cell death thus explaining the high safety record of NDV in cancer patients (3).

There are additional properties that make NDV a particularly interesting anti-neoplastic agent. It replicates and destroys in particular cancer cells that are resistant to certain types of chemotherapy (4–6) and apoptosis-resistant tumor cells from hypoxic tumor tissue (7). The oncogenic protein Rac1 was reported as a link between tumorigenesis and sensitivity of cells to oncolytic NDV (8). Furthermore, NDV triggers autophagy in glioma cells (9) and promotes Bax redistribution to mitochondria and cell death in HeLa cells (10). A time-course analysis revealed that NDV-induced apoptosis involved an early extrinsic pathway with TRAIL expression (peak at 24 h *p.i.*) and a later intrinsic mitochondrial pathway (peak at 48 h *p.i.*) (11).

NDV was reported to repress the activation of human hepatic stellate cells and reverse the development of hepatic fibrosis in mice (12). Liver fibrosis is a major health problem and the 12th most common cause of death in the United States (13).

HARNESSING NDV BY TRANSGENES AND BI-SPECIFIC ANTIBODIES

Recombinant NDV strains (rNDV) could be harnessed by transgenes to show enhanced oncolytic potential. This was achieved by F gene mutations (14, 15) or by addition of the NS1 (16) or Apoptin (17) gene. It could also be harnessed by genes coding for cytokines, such as IL-2 (18), GM-CSF (19), IL-15 (20), or IFN-γ (21) to express enhanced immune stimulatory properties. Other transgenes conferred resistance to complement (22). NDV was also capable of incorporating two transgenes, one coding for the light chain and the other for the heavy chain of a monoclonal antibody interacting with angiogenesis (23). The transfer of a gene coding for a tumor antigen (TA) created a vector with which the immune response could be targeted to a specific TA in order to compete with the usually stronger response to viral antigens (VA) (24). A recombinant oncolytic MV (MV-AC133) could be targeted to CD133+ cancer-initiating cells causing their specific elimination (25).

To augment the immune stimulatory properties of NDV infected tumor cells, another elegant approach was successful. It consists of the attachment of single-chain variable fragment (scFv) bi-specific antibodies (bsAbs). These attach with one arm to a VA and with the other arm to a target on immune cells. In case of

T-cells, such targets were CD3 (26), CD25 (27), and CD28 (28). The VAs of NDV were either HN or F. These served as universal anchor molecules through which T-cell co-stimulatory molecules could be attached to any type of tumor cell infectable by NDV (29).

In the following paragraph, we will present a perspective how such bsAbs can be further used in a multimodal approach for improvements of cancer therapy.

FUTURE PERSPECTIVE: COMBINING NDV WITH bsAbs AND WITH ADOPTIVE CELLULAR THERAPY

TUMOR TARGETING OF NDV

A major problem with the clinical application of oncolytic viruses is a proper targeting of tumor tissue. This can be achieved by intratumoral application (30) but metastases are often not accessible by this approach. Nevertheless, localized oncolytic virotherapy was reported to overcome systemic tumor resistance to immune checkpoint blockade immunotherapy (31).

Locoregional application (e.g., via the hepatic vain) was reported to be superior to systemic tail vain inoculation (32). Locoregional virotherapy was effective even against oncolysis-resistant tumor cells, thus suggesting that the anti-tumor effect was host mediated (32). Inhalation is another way of locoregional application. Inhalation of oncolytic NDV was applied to 33 advanced chemorefractory patients in a Phase II clinical study in Hungary as a means to affect their lung metastases (33). Virus inoculation into body cavities in case of tumor ascites is another way of locoregional application. For instance, intraperitoneal NDV virotherapy was effective against peritoneal carcinomatosis from human gastric cancer in a xenograft model (34) and intrapleural NDV virotherapy induced sustained remission of malignant pleural mesothelioma in an orthotopic model (14).

Upon systemic administration of NDV, its binding to normal cells could prevent it from reaching the tumor tissue and could cause undesired side effects. Since efficient distribution at the tumor site may be a very critical parameter for tumor selective gene delivery and for anti-tumor efficacy of oncolytic virotherapy (35), we have developed adaptor molecules that redirect the virus to tumor tissue (36). The targeting molecule used, anti-HN-IL-2, contains a scFv antibody cloned from a neutralizing HN specific hybridoma linked to the human gene for the cytokine IL-2. Selective virus entry was observed *in vitro* in a mixture of IL-2 receptor positive and negative human tumor cells (37). Retargeted virus infection of tumor cells required specific binding via the bi-specific fusion protein and membrane fusion via the viral F-protein. After systemic virus inoculation into tumor-bearing mice, the modification of NDV by the adaptor protein did not compromise the efficiency of gene delivery into target positive tumors but greatly reduced viral gene expression in target negative tumors and in normal tissues thereby reducing side effects (38).

UNIVERSAL ACTIVATION OF CANCER PATIENTS T-CELLS (NAÏVE AND MEMORY) VIA TUMOR CELL-BOUND BI-SPECIFIC ANTIBODIES

Infection of tumor cells by NDV leads to increase in tumor cell immunogenicity (39). A prospective, randomized, controlled clinical study of post-operative immunization with the autologous tumor vaccine ATV-NDV revealed evidence for clinical effectivity and long-term survival for colon cancer patients (40). Further augmentation of T-cell stimulatory capacity of the ATV-NDV vaccine was achieved by attachment of specifically designed bsAbs binding to viral HN or F on the infected tumor cells and to CD3 or CD28 on T-cells (41). The optimized vaccine ATV-NDV/bsHNxCD3/bsHNxCD28 appeared to be able to revert unresponsiveness of partially anergized TA-specific T-cells (42). It was also capable of *de novo* activation of anti-tumor activity from naïve T-cells, independent of TA recognition (**Figure 1A**) (42). The strongest potentiation of the T-cell stimulatory capacity of the ATV-NDV vaccine was observed upon attachment of a suboptimal amount of bsHNCD3 together

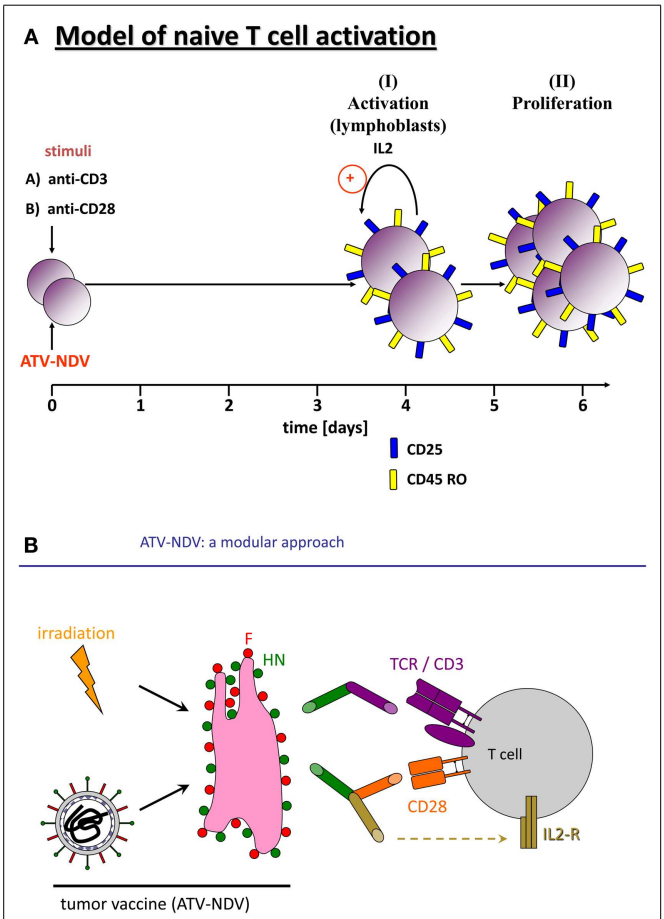

FIGURE 1 | Activation of naïve human T-cells by co-incubation with NDV infected irradiated tumor cells modified with bi-specific or tri-specific antibodies. **(A)** Time course of the induction of T-cell activation and proliferation by a stimulatory cell (NDV infected and γ-irradiated tumor cells) optimized for co-stimulation by attachment of the bi-specific fusion proteins anti-CD3 (anti-HNxanti-CD3) and anti-CD28 (anti-HNxantiCD28). Purified and CFSE-labeled naïve human T-cells were cocultivated for 5 or 7 days with the stimulatory cells. The CFSE signal intensities were compared with unstimulated cells by FACS analysis. We also followed by the FACS analysis the expression of the IL-2 receptor α chain (CD25) and of the memory marker CD45RO. **(B)** Diagram of the components of a tumor vaccine infected by NDV and modified by a bi-specific antibody (anti-HNxanti-CD3, suboptimal amount for signal 1) and a tri-specific immunocytokine (anti-HNxIL-2xanti-CD28, for delivery of two T-cell co-stimulatory signals via CD28 and CD25).

with the tri-specific (ts) fusion protein tsHNxIL-2xCD28. The latter delivers two co-stimulatory signals to T-cells, one via CD28 and the other via CD25 (26). **Figure 1B** illustrates the modular concept of the tumor vaccine infected by NDV and modified by bsAbs.

We suggest to use T-cell activation one universal GMP tumor cell line for patients. This will be modified by infection with NDV and by attachment of the above bsAbs and tsAbs. This universal T-cell stimulatory cell can be applied for non-specific activation of anti-tumor activity of T-cells from any type of cancer patient and is independent from a TA.

PROGRAMING OF CANCER PATIENTS DENDRITIC CELLS TOWARD DC1 VIA INFECTION BY NDV

We reported on polarization of human monocyte-derived DCs to DC1 by *in vitro* stimulation with NDV (43). Also, murine DCs upon infection by NDV differentiate into the immunogenic phenotype DC1 characterized by secretion of pro-inflammatory cytokines, in particular IL-12 and IFN-α and -β (44). Two receptor-initiated signaling cascades were involved: the first one is induced by triggering and upregulation of the intra-cellular cytoplasmic receptor RIG-1 upon recognition of viral non-capped RNA as ligand (45). The second signal cascade involves cell-surface expressed type I IFN receptor (IFNAR), which initiates a feedback loop cell activation upon interaction with extra-cellular type I IFN as ligand (31, 44). RIG-1/RNA ligand interaction not only activates type I IFN, but also induces inflammasome activation for IL-1β production (46). Type I IFN and IL-12 are critical mediators of cross-priming and Th1 polarization of CD8 T-cell responses (47) while IL-1β is critical for Th1 polarization of CD4 T-cells (48).

DCs can also be pulsed with NDV oncolysate. Such cells were superior in stimulating patients T-cells in ELISPOT assays compared to DCs pulsed with tumor lysate without NDV (49).

GRAFTING OF AUTOLOGOUS ACTIVATED T-CELLS AND DC1 BACK TO THE PATIENT

Our proposal for a multimodal cancer therapy involves the transfer of immune T-cells and of DC1 as professional antigen-presenting cells back to the patient. Activation of the tumor microenvironment by low dose irradiation (LDI) (50) or by local hyperthermia (LHT) (51) should improve tumor targeting of virus, T-cells, and DCs (52). Tumor destruction by the activated T-cells should release TAs, which would be taken up by co-injected DC1 to be then cross-presented to naïve or memory T-cells.

HITCHHIKING OF NDV ON ACTIVATED T-CELLS: COMBINING CELL THERAPY WITH VIRUS THERAPY

One way of further enhancement of the efficacy of this multimodal therapy concept consists in the loading of the activated T-cells with oncolytic NDV before grafting the cells back to the patient. In a tumor neutralization assay *in vitro*, monolayers of human tumor cells could be completely and effectively destroyed by the addition of polyclonally activated human T-cells loaded with oncolytic NDV (53). In this process, synergistic effects between cytotoxic T-cells and oncolytic virus in the tumor contact zone were apparent (53).

If activated T-cells are not available, a multimodal therapy could also consist of the combination of LHT, systemic application of oncolytic NDV and of DC1. Such approach resulted in long-term remission of metastatic prostate cancer (52).

TARGETING AN INTRODUCED VIRAL ANTIGEN IN TUMOR TISSUE BY GRAFTED T-CELLS AND DCs VIA CELL-BOUND TRI-SPECIFIC ANTIBODIES

Table 1 summarizes five steps that are essential for a new adoptive cellular cancer therapy strategy. Oncolytic NDV can be introduced into tumor tissue of the patient by various means as discussed before. The patients T-cells and DCs would be activated and polarized also as discussed before. The tsAbs have three different binding sites, each of which is only monovalent. To increase the avidity and stability of the cell surface attached ts fusion protein, we propose that two of the binding sites should bind to well-defined targets on T-cells or DCs. The addendum of the table lists some of the potential targets.

This approach is only meant as a perspective for the future and has not been tested experimentally or clinically. There should be a proper timing between virus-pretargeting of tumor tissue (including metastases) and the cell therapy. We envisage that 24–48 h after virus inoculation should be a good time period for grafting the cells for a VA targeted therapy. Excessive virus should

Table 1 | Adoptive cellular cancer therapy: targeting a viral antigen (e.g., HN) by grafted T-cells and DCs via cell-bound tri-specific antibodies.

Step 1	Pre-conditioning of the tumor microenvironment in the patient
Step 2	Local or systemic application of oncolytic NDV for introduction of the viral target antigen HN within the tumor tissue
Step 3	Universal activation ex vivo of the patients T-cells and loading with tri-specific antibodies thus exposing multiple anti-HN binding sites
Step 4	Generation of polarized DCs from the patient via infection by NDV or pulsing with NDV oncolysate; loading of the DC1 with tri-specific antibodies thus exposing multiple anti-HN binding sites
Step 5	Grafting the T-cells and/or DCs to the pre-conditioned patient

Addendum: The tri-specific single-chain antibodies should bind with two arms to targets on T cells or targets on DCs and expose the third arm anti-HN

Potential T-cell targets	Potential DC targets
CD3	CD11c
CD28	CD205
CD25	CD40
CD2	CD80
CD44	CD16a
CD45	CD83
CD69	CD116
CXCR4	IFNAR
CD107a	CD119

be cleared by then and the tumor tissue should be infected and expressing cell-bound VAs.

CONCLUDING REMARKS

We propose a multimodal approach for effective cancer therapy because previous monomodal approaches of chemo- or radiotherapy faced problems of tumor resistance mechanisms. Specific immunotherapies targeted to specific TA faced similar problems of tumor escape and resistance mechanism. There may be a long way to get a multimodal therapy such as the one proposed and established but we believe it is important to propose a viable perspective for future orientation. Oncolytic viruses, T-cells, dendritic cells, and bi-specific antibodies are all promising biologics whose intelligent combination holds a lot of promise for future cancer therapy.

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Attacking postoperative metastases using perioperative oncolytic viruses and viral vaccines

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Surgical resection of solid primary malignancies is a mainstay of therapy for cancer patients. Despite being the most effective treatment for these tumors, cancer surgery has been associated with impaired metastatic clearance due to immunosuppression. In preclinical surgery models and human cancer patients, we and others have demonstrated a profound suppression of both natural killer (NK) and T cell function in the postoperative period and this plays a major role in the enhanced development of metastases following surgery. Oncolytic viruses (OV) were originally designed to selectively infect and replicate in tumors, with the primary objective of directly lysing cancer cells. It is becoming increasingly clear, however, that OV infection results in a profound inflammatory reaction within the tumor, initiating innate and adaptive immune responses against it that is critical for its therapeutic benefit. This anti-tumor immunity appears to be mediated predominantly by NK and cytotoxic T cells. In preclinical models, we found that preoperative OV prevents postoperative NK cell dysfunction and attenuates tumor dissemination. Due to theoretical safety concerns of administering live virus prior to surgery in cancer patients, we characterized safe, attenuated versions of OV, and viral vaccines that could stimulate NK cells and reduce metastases when administered in the perioperative period. In cancer patients, we observed that *in vivo* infusion with oncolytic vaccinia virus and *ex vivo* stimulation with viral vaccines promote NK cell activation. These preclinical studies provide a novel and clinically relevant setting for OV therapy. Our challenge is to identify safe and promising OV therapies that will activate NK and T cells in the perioperative period preventing the establishment of micrometastatic disease in cancer patients.

Keywords: metastasis, postoperative period, oncolytic viruses, viral vaccines, cancer, perioperative immunostimulation, natural killer cells, surgical stress

SURGICAL STRESS PROMOTES THE FORMATION OF METASTASES

Surgical resection is the mainstay of therapy for most solid malignancies but, even with complete resection, many patients harbor microscopic residual disease and ultimately die of a recurrence (1). Our group (2, 3) and others have clearly demonstrated, using different animal and tumor models, that surgery promotes the formation of metastatic disease (4–11) and the number of metastatic deposits is directly proportional to the magnitude of surgical stress (6, 12). In clinical studies, a complicated postoperative course correlates with inferior cancer survival and increased incidence of metastases (13, 14). A number of perioperative changes have been proposed to explain the promotion of metastases formation following surgery including (1) dissemination of tumor cells during the surgical procedure (15–20), (2) local and systemic release of growth factors, such as vascular endothelial growth factor (VEGF) (21, 22), and (3) cellular immune suppression. The cellular immune suppression following major surgery appears to peak at 3 days (23) following surgery but may persist for weeks (7, 23–25). It is hypothesized to be mediated by secretion of stress hormones, such as glucocorticoids (26, 27), catecholamines (27–29), and prostaglandins (26). It is characterized by both plasma cytokine changes [a decrease in IL-2 (30), IL-12 (31) and an increase IL-6 (27, 30, 32, 33), IL-10 (34)] and a decrease in the number and function of circulating lymphocytes [cytotoxic T cells (35), dendritic cells (DC) (36) and natural killer (NK) cells (2, 3, 37)].

The postoperative stress response represents a diverse set of physiological changes that have evolved to ensure that the host can heal following major tissue trauma. These changes, however, involve pathways and mediators that can be exploited by cancer cells to facilitate metastatic spread. While a number of correlative studies have demonstrated an association between some of these changes and the enhanced formation of metastases following surgery, few mechanistic studies have been undertaken to understand it. This review will focus on the importance of both innate and adaptive postoperative cellular immune suppression, specifically NK and cytotoxic T cell postoperative dysfunction and make the case for the use of preoperative oncolytic viruses (OV) and viral vaccines to prevent the promotion of cancer metastases following surgery.

SURGICAL STRESS INHIBITS NK CELL FUNCTION AND ANTIGEN-SPECIFIC CD8$^+$ T CELL FUNCTION

Both the innate and adaptive immune system play a significant role in anti-tumor immunity. As integral members of the innate immune system, NK cells are involved in the direct killing of cells

displaying abnormalities linked to infection, malignancy, or transplantation (38, 39). Immunosurveillance of the host by NK cells for malignant cells results in direct cytotoxicity and the production of cytokines to enhance the immune response (39).

Natural killer cell dysfunction following surgery, as measured in a standard [51-Cr]-release assay, has been documented in both human patients (3, 25, 40–42) and animal models (3, 5, 7, 40, 41, 43). Postoperative NK cell suppression correlates with increased metastases in animal models of spontaneous (3, 9) and implanted (3, 10, 11) metastases, while in human studies low NK activity during the perioperative period is associated with a higher rate of cancer recurrence and mortality in a number of different cancer types (44–46). Despite the large number of studies that have documented postoperative NK cell dysfunction, very few studies have thoroughly characterized and directly explored the mechanism of this suppression (9, 11, 47). Our laboratory has clearly defined a role for NK cells in the development of postoperative metastases (2). Using several reproducible mouse models of surgical stress, including B16 melanoma, CT26 colon cancer and 4T1 breast cancer, our laboratory has demonstrated a consistent and significant (two- to fourfold) increase in the formation of experimental and spontaneous pulmonary metastases following surgery. In these experimental models, surgery markedly reduced NK cell total numbers in the spleen and affected NK cell migration. Further, *ex vivo* and *in vivo* tumor cell killing by NK cells were significantly reduced in surgically stressed mice. To establish that NK cells play the crucial mediating role in clearing tumor metastases following surgery, we transferred surgically stressed NK cells into NK-deficient mice (IL-2γR-knock out) and observed enhanced lung metastases in tumor-bearing mice compared to mice who received untreated NK cells (3). Transfer of NK cells labeled with the NK specific marker DX5 from surgically stressed and no surgery control donors into naive recipient mice represents the first *in vivo* evidence that links surgery to the spread of cancers via NK cells (3). In human studies, we have also confirmed that postoperative cancer surgery patients had markedly reduced NK cell cytotoxicity (3).

The adaptive immune system and more specifically $CD8^+$ T cells responses have received the majority of the attention from the cancer immunity field. Of recent interest in our lab is the impact of surgical stress on the development and maintenance of an acquired T cell-mediated anti-tumor immune response. A global reduction in T cell numbers and function post-surgery has been documented in preclinical studies and cancer patients (35). However, the effects of tumor-associated antigen (TAA)-specific T cells have not been evaluated and represent a current focus of research interest in our lab.

POSTOPERATIVE CELLULAR IMMUNE SUPPRESSION IS REVERSIBLE

Fortunately postoperative immune suppression is reversible, so while the postoperative period provides a window of opportunity for cancer cells to metastasize and grow, it also provides a window of opportunity to intervene, by supporting or further stimulating the immune system, and, in doing so, attenuate the development of cancer recurrences (48, 49). Based on promising preclinical results (8, 50, 51), clinical trials of preoperative non-specific immune stimulation with low-dose recombinant IFNα (52) or IL-2 (53–58) have demonstrated less NK and T cell suppression following surgery. In two randomized studies of patients undergoing resection of colorectal cancer (CRC) primary tumors (58) and hepatic metastases (57), preoperative low-dose subcutaneous (s.c.) IL-2 was associated with an improved prognosis. In the first study, 86 CRC patients with stage II or III disease were randomized to receive low-dose IL-2 twice a day for 3 consecutive days prior to surgery or no preoperative treatment. At a median follow-up of 54 months, there were significantly few recurrences in the IL-2 group (21.4 vs. 43.1%, $p = 0.03$) and a trend toward improved overall survival (OS). In the second study, 50 CRC patients with Stage IV disease, undergoing curative or palliative surgery, were randomized to the same two treatment arms. The median progression-free survival (PFS) and OS were significantly longer in the preoperative IL-2 group. While these studies were not designed to evaluate cancer outcomes, a Phase II trial in 120 patients undergoing resection for renal-cell carcinoma has demonstrated a significant improvement in 5-year PFS with preoperative IL-2 (74 vs. 62%, $p = 0.02$) (54). Moreover, in all of these studies, preoperative IL-2 was safe and well tolerated with adverse events limited to pyrexia (Grade I–III). A few other non-conventional immunomodulators have been evaluated for their ability to boost cellular immunity in the perioperative period including cimetidine (59, 60), mistletoe extract (61, 62), and granulocyte colony-stimulating factor (GMCSF) (63). Despite the paucity, the data are promising and perioperative treatment strategies, aimed at stimulating the cellular immune system warrants further study. As outlined in the remainder of this review, OV are an attractive agent to reverse perioperative immune suppression.

WHY USE PERIOPERATIVE ONCOLYTIC VIRUSES FOR IMMUNE STIMULATION? A MULTIPRONGED APPROACH FOR A MULTIFACTORIAL PROBLEM

Oncolytic viruses are not considered a "traditional" immunotherapy but their multiple mechanisms of action provide several advantages over traditional cytokine immune stimulants in the complex postoperative period. First, the immune stimulation provided by an OV is a more "physiological" immune stimulus, engaging and maturing DC, which in turn activates NK and T cells. The multitude of cytokines and chemokines, stimulate the appropriate picomolar concentration, by a systemic virus infection would be impossible to replicate even with the most carefully designed cytokine cocktail. Second, the OV will selectively replicate in and kill residual cancer cells, providing a direct cytolytic effect to remaining micrometastases, but also delivering the immune response to the tumor selectively. Finally, there is strong rationale to hypothesize that OV could infect and replicate better in the postoperative state because of the surge of growth factors such as VEGF, providing a therapeutic advantage for OV in postoperative cancer patients.

PRECLINICAL EVIDENCE FOR NK CELL ACTIVATION WITH PERIOPERATIVE ONCOLYTIC VIRUSES

Viruses, in general, are known to activate NK cells (64, 65) and OV are no exception. One of the first reports to support the anti-tumor activation of NK cells in response to OV therapy was

reported by Diaz et al. in which depletion experiments were performed to demonstrate that B16 melanoma tumor regression was achieved in a $CD8^+$ T and NK cell-dependent manner following vesicular stomatitis virus (VSV) intratumoral (i.t.) injection (66). Supporting these findings, oncolytic Reovirus treatment of prostate cancer produced an anti-tumor $CD8^+$ T cell response along with prominent NK cell infiltration (67, 68). Miller et al. also observed that i.t. therapy with oncolytic herpes simplex virus (HSV) for B16 melanoma was abrogated in syngeneic models lacking NK and T cell subsets (69). In mechanistic studies with oncolytic new castle disease virus (NDV), Jarahian et al. demonstrated enhanced NK cytotoxicity against human tumor cell lines infected with NDV. Further, soluble receptor binding and blocking assays suggest that NKp44 and NKp46 recognition of viral ligand hemagglutinin-neuraminidase on NDV infected tumor cells mediated NK anti-tumor activity (70). We have demonstrated that oncolytic ORF virus (ORFV) has a profound effect on NK cells following i.v. delivery and that this NK cell activation is the main mechanism by which ORFV exerts its anti-tumor effect (71). It is very likely that stimulation of NK cells play an important role in the therapeutic effect of many OV, not only by enhancing NK cell-mediated killing of tumor target cells but also by triggering a robust, T cell-mediated, anti-tumor immune response (72).

Given that surgery suppresses NK cell activity and OV activate NK cells, we explored the ability of preoperative OV to prevent postoperative NK cell suppression, and in turn prevent the development of postoperative metastases. Using our established murine model of surgical stress, we demonstrated that perioperative administration of novel oncolytic ORF and vaccinia viruses can reverse NK cell suppression following surgery and this correlates with a reduction in the postoperative formation of metastases (3). Similar effects were observed in 4T1-tumor bearing surgically stressed mice treated with perioperative OV. When NK cells were depleted, the effect was no longer present, suggesting that suppression of tumor metastases in a surgical stress model is mainly mediated through OV activation of NK cells and subsequent NK cell-mediated tumor lysis (3).

We demonstrated a similar effect with the novel oncolytic rhabdovirus, Maraba (MG1) and used this model to explore the mechanism of NK cell activation further. MG1 is a double mutant rhabdovirus with deletion in the G and M proteins (73). It is a clinical candidate OV that is scheduled to begin a Phase I clinical trial in 2014. MG1 infection in immune competent mice resulted in an immediate (24 h) and intense activation of NK cells, as evidenced by significantly increased NK cell cytotoxicity and cytokine secretion. Moreover, preoperative i.v. administration of MG1 overcame surgery-induced NK suppression and attenuated the development of postoperative metastases in the B16lacZ model of implanted lung metastases, as well as in the breast 4T1 model of spontaneous lung metastases (74).

Mechanistically, we demonstrated that MG1 activates NK cells through conventional DC (cDC) (**Figure 1**). Using an *ex vivo* NK:DC co-culture system, we showed lack of NK infection, activation, and cytotoxicity in the absence of cDC. Further, in cDC ablated mice (CD11c-Diphtheria Toxin Receptor Transgenic mice), NK cell cytotoxicity was significantly reduced following MG1 administration (74). While we demonstrated that MG1 does not directly infect or activate NK cells, this is not the case for other OV. For instance, vaccinia virus has been shown to interact directly with NK cells through Toll-like-receptor-(TLR)-2 (75).

As the interplay between OV and immune cells in the perioperative period is critically important for the eradication of tumors, we further explored these interactions in our preclinical models of tumor and surgical stress. In both B16 melanoma and 4T1 breast tumor models, we observed postoperative expansion of myeloid-derived suppressor cells (MDSC) (3), which are known regulatory cells that have been shown to expand following various pathologies to suppress innate and adaptive immunity (76–80). The role of MDSC on surgery-induced dysfunction of NK cells and antigen-specific T cells and its potential interaction with OV is part of ongoing research in our lab (**Figure 1**).

PRECLINICAL EVIDENCE OF TAA-SPECIFIC T CELL ACTIVATION WITH PERIOPERATIVE ONCOLYTIC VACCINE

Oncolytic vaccines (OVax) are OV that express TAA that can direct the host immune response toward the TAA while simultaneously performing viral oncolysis and creating an inflammatory tumor microenvironment (81, 82). Dr. Brian Lichty has pioneered this prime-boost OVax platform and demonstrated remarkable efficacy in the B16 model (82–86). B16 cells express the TAA, dopachrome tautomerase (DCT), which is a protein involved in melanogenesis and is present in normal melanocytes and melanoma. As previous studies have demonstrated, Ad-DCT is able to prime a DCT specific T cell immune response and protect mice from a B16 tumor challenge or tumor re-growth (87, 88), but has limited efficacy in a therapeutic model of lung metastases (89). Dr. Lichty's group engineered MG1, to express DCT upon productive infection and used these two viruses in a prime-boost strategy in tumor-bearing animals. They found that when Ad-DCT was allowed to prime an immune response, followed 9 days later by an MG1-hDCT boost, the results were remarkable, leading to a significant reduction in lung metastases with durable cures in >20% of mice, something not seen when MG1 expressing an irrelevant transgene (MG1-GFP, green-fluorescent protein) was used. Strikingly, ~27% of $CD8^+$ T cells were directed against DCT. Selective depletion of cytotoxic T lymphocytes (CTL) at the time of the boost abrogates the therapeutic efficacy, underscoring their central role. In the near and longer term, we will focus on using OVax, such as MG1-DCT in preclinical mouse tumor models of surgical stress to perioperative boost adaptive immune functions.

CLINICAL EXPERIENCE WITH PERIOPERATIVE OV IN CANCER SURGERY PATIENTS

The compelling preclinical and clinical data with oncolytic vaccinia virus, in particular the evidence that it can stimulate a potent anti-tumor immune response (90) led us to hypothesize that perioperative treatment with this OV could improve recurrence-free survival following surgical resection. We designed a single center Phase II clinical trial where patients with metastatic colorectal tumors within the liver were treated with a single i.v. dose of oncolytic vaccinia virus prior to surgical resection (91). This trial explored the mechanisms of action of oncolytic vaccinia virus through a series of correlative blood and tissue studies collected from patients pre- and post-OV treatment and surgery. In this

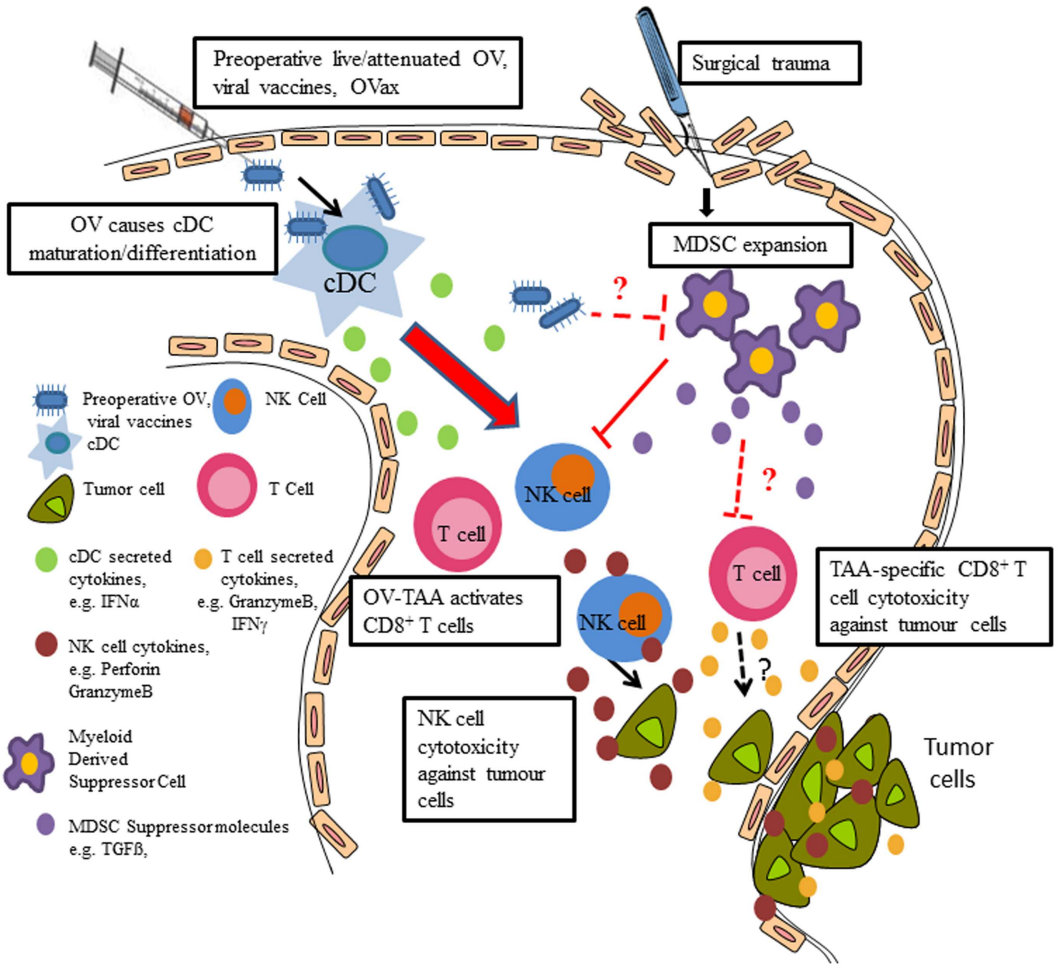

FIGURE 1 | Preoperative delivery of live/attenuated OV, viral vaccines, and oncolytic vaccines enhances innate and adaptive immune cell function to reduce postoperative metastatic disease. Preoperative administration of the live or attenuated OV results in NK cell activation via cDC; preoperative delivery of viral vaccines results in IFNα production (likely through cDC), which results in NK cell activation, thereby preventing surgery-induced dysfunction and removal of tumor cell emboli and micrometastases in the postoperative period. Postoperative MDSC expansion contributes to NK cell dysfunction and OV may reverse the suppressive effects of MDSC. Preoperative oncolytic vaccines may activate tumor antigen-specific $CD8^+$ T cells and reduce tumor burden and increase survival.

study, we confirmed that NK cell cytotoxicity improved in the setting of pre-operative oncolytic vaccinia virus compared to baseline control blood (3). Further, we detected genome copies of vaccinia virus in the tumors of patients following resection (unpublished data), which suggests that viral targeting of the tumor by i.v. injection may elicit an immune response in the tumor. These results demonstrated for the first time that oncolytic vaccinia virus markedly increases NK activity in cancer surgery patients.

In the same patient population of CRC, systemic delivery of oncolytic reovirus prior to planned surgical resection of liver metastases was undertaken by researchers in the UK (92) In this "window of opportunity" trial of 10 patients, Adair et al. was able to recover live reovirus from the blood cells, but not from plasma removed from these patients. In addition, reovirus protein was identified preferentially in resected tumor tissue, but not in normal liver tissue. Their results suggest that immune cells in the blood may protect virus from neutralizing antibodies, thus providing targeted delivery of OV to tumors. Importantly, preoperative treatment with oncolytic reovirus was well tolerated, with the most common side effects being flu-like symptoms and no reported grade 3 or 4 toxicities in any patients (92). In a study of perioperative oncolytic HSV delivery, virus was injected intratumorally pre- and post-surgical resection into patients with recurrent glioblastoma multiforme (93). Evidence of immune cell infiltration and viral replication in the resected tumors was reported by the authors. Notably, no patients developed HSV related encephalitis or required antiviral treatment (93). In a series of clinical trials using NDV-modified autologous tumor cell vaccine (NDV-ATV) for treatment of colorectal, renal cell, and glioblastoma cancer patients, researchers detected a significantly improved survival advantage compared to unvaccinated and historical controls. However, NDV-ATV was mostly administered postoperatively and

not preoperatively to prevent surgery-induced immunosuppression, which might further improve upon the survival advantage. Similar to the above studies, NDV-ATV was well tolerated, with the most common side effects being mild-fever/headache and no associated autoimmunity (70, 94–99). These reports demonstrate the feasibility of perioperative OV administration into cancer surgery patients.

THE IMPORTANCE OF TIMING FOR OV ADMINISTRATION IN THE PERIOPERATIVE PERIOD

While the postoperative period provides a window of opportunity for cancer cells to metastasize and grow, it also provides a window of opportunity to intervene, by strengthening the immune system and reducing recurrence of cancer following surgery in cancer patients. In our preclinical perioperative vaccine studies, we hypothesized that neoadjuvant delivery of vaccine immediately prior to surgery will allow for maximal NK cell stimulation to counteract surgery-induced NK cell suppression (100, 101). Indeed, we observed that influenza vaccine administered on the same day, immediately prior to surgery, reduced metastases most effectively. The results from NK cells isolated from cancer surgery patients also confirm that the timing of influenza administration is critical for its effect. In four out of four cancer surgery patients, NK cells isolated prior to surgical resection demonstrated enhanced cytotoxicity and IFNγ secretion following *ex vivo* pulsing with influenza vaccine, while in only one of these patients was similar activation demonstrated in NK cells isolated 1 day following surgery, suggesting that surgery-induced NK cell dysfunction can be prevented but not reversed by influenza. In humans receiving a flu shot as part of a vaccination campaign, NK cell activation peaked at 1–2 days following immunization (101). Based on this, it appears that a cancer vaccination strategy is probably best delivered the day before cancer surgery, in order to allow sufficient time to maximally activate NK cells prior to surgical stress.

Equally important for a replicating virus is the growth advantage that the postoperative state may provide, increasing oncolysis, viral replication, and spreading. Surgical stress results in a surge of VEGF with resulting angiogenesis to facilitate wound healing (21). Kottke et al. (102, 103) have previously demonstrated that a VEGF surge improved viral replication, viral cell lysis, and an innate immune mediated attack, in particular by NK cells, by allowing tumor-associated endothelial cells to transiently support viral replication during the VEGF surge. The sequential combination of oncolytic vaccinia virus and the small molecule B-raf and VEGF inhibitor, sorafenib, has also demonstrated efficacy in preclinical models and a few patients (104), further supporting the concept that OV and VEGF may act synergistically if the timing of viral administration is considered.

BARRIERS TO PERIOPERATIVE OV THERAPY AND STRATEGIES TO OVERCOME THEM

While these data are exciting, the perioperative use of OV is in preclinical and early stages of clinical investigation. In the design of our preoperative OV trial, we were confronted with multiple concerns associated with the use of a live virus immediately prior to surgery in cancer patients. In particular, concerns were raised about the potential for an overwhelming postoperative systemic inflammatory response, the risk of spread to members of the operating room team, and risk of meningitis with epidural analgesia. These safety concerns present real barriers to the development of perioperative OV. In their recent publication, Adair et al. demonstrated the feasibility and safety of perioperative live reovirus infusion prior to surgery in CRC patients. However, OV infusion was administered 6–28 days prior to surgery and not immediately before surgery. Further, three patients received fewer than their planned five doses of reovirus. In one patient, this was due to a decline in white blood cell count, while the remaining two patients opted to not receive their last doses of OV prior to surgery because of their own concerns that flu-like symptoms might interfere with the planned surgery, highlighting a strongly held belief that remains a theoretic barrier to immediate preoperative delivery of a replicating virus (92).

Given these very real concerns surrounding live perioperative delivery of OV, we subsequently focused on generating non-replicating MG1 viruses to characterize their ability to activate NK cells and attenuate metastases in a model of experimental (B16) and spontaneous (4T1) metastases following surgical stress. To accomplish this, we constructed a replication incompetent MG1 – MG1-Gless-eGFP, that is only capable of one infectious life cycle, thus offering a safe *in vivo* profile. Next, we compared these variations of MG1: (1) live MG1-productive infection and replication; (2) a G-less version (MG1-Gless) – capable of a single-replication cycle of virus; (3) MG1 exposed to ultraviolet (UV) for 2 min to 2 h – replication incompetent confirmed by plaque assay. MG1, MG1-Gless, and MG1-UV$^{2\,min}$ exhibited significantly higher NK cell function compared to PBS control, and they effectively attenuated *in vivo* B16lacZ lung metastases to near identical levels at high viral doses (1×10^8 PFU). However, at all lower doses studied ($1 \times 10^{5-7}$ PFU), live MG1 demonstrated better efficacy than attenuated MG1. Furthermore, we characterized this panel of MG1 viruses in terms of virus morphological structure and cell associated interaction via Electron Microscopy, qRT-PCR, and western blot and found that MG1-UV$^{2\,min}$ remains an intact virus particle (virus proteins, genetic materials) with cell-associated interactions, corresponding to the highest NK cell activation and least lung metastases, among MG1-UV viruses. Importantly, we demonstrated that preoperative i.v. administration of equivalent high doses (1×10^8 PFU) of live and attenuated MG1 (MG1-Gless or MG1-UV$^{2\,min}$) overcame surgery-induced NK cell suppression and reduced the development of postoperative metastases in the B16lacZ implanted lung metastases, as well as in the breast 4T1 model of spontaneous lung metastases. Taken together, these results suggest that the intact viral particle and cellular recognition, along with viral proteins and genomic RNA are essential for NK cell-mediated anti-tumor responses. Non-replicating forms of MG1, including MG1-UV$^{2\,min}$, are novel cancer therapies that can be safely used in the immediate preoperative period to prevent the formation of metastatic disease (74).

Parallel to our perioperative attenuated OV studies, we assessed a wide range of potential agents to provide perioperative non-specific immunostimulation including TLR ligands and inactivated vaccines against infectious disease. Firstly, we assessed a panel of routinely used immunizations, including vaccines against influenza, meningitis, measles/mumps/rubella,

diphtheria/tetanus/pertussis/polio, pneumonia, and influenza for their ability to activate (CD69 expression) and enhance NK cell function (cytotoxicity and IFNγ secretion). When directly compared, influenza was the most potent NK cell activator among the prophylactic vaccines, although, not unexpectedly, inoculating mice with live replicating viruses (such as vaccinia virus) induced higher levels of NK cell cytotoxicity. Using our mouse models of experimental (B16 melanoma) and spontaneous (4T1) metastases and surgical stress, we subsequently demonstrated that preoperative delivery of a single dose of influenza resulted in a dramatic reduction in lung metastases (101). In order to confirm that NK cells play a mediating role in preventing postoperative metastases following influenza treatment, we pharmacologically depleted NK cells and observed a complete abrogation of the therapeutic effect of influenza vaccination. Furthermore, we discovered that IFNα had the most dramatic increase following influenza vaccination after assessing a panel of serum cytokines following influenza administration. We also observed that low-dose preoperative IFNα was able to rescue surgery-induced NK cell dysfunction and metastases to the same degree as influenza vaccination. The central role for IFNα was underscored by demonstrating that influenza vaccination was not able to increase postoperative NK cell activity or attenuate postoperative metastases in IFNα receptor-deficient mice. In PBMC isolated from human donors, Type I IFN blocking antibody prevented influenza from activating NK cells (101). While our study did not explore the role of DC in the production of IFNα following influenza vaccination, it is very likely that they represent the primary source, resulting in secondary NK cell stimulation (see **Figure 1**).

CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

Surgical resection is the mainstay of therapy for patients with localized solid malignancies. Even with complete resection, many patients develop a metastatic recurrence and ultimately die of their disease. The immediate postoperative period provides an ideal environment for the formation of cancer metastases. Despite this, it remains a therapeutic window that is largely ignored. There are currently no standard perioperative anti-cancer therapies aimed at preventing postoperative metastases. We have demonstrated in preclinical models that perioperative OV therapy can activate both the innate and adaptive immune responses and attenuate metastatic disease. Early clinical trials confirm the feasibility of this strategy but these therapies must be rigorously characterized for safety and efficacy and then translated into thoughtfully designed clinical trials. This research supports the concept that neoadjuvant (preoperative) OV treatments can reverse postoperative immune dysfunction, while directly infecting and killing tumor cells and creating a favorable immune microenvironment. This treatment strategy has the potential to impact countless cancer patients who undergo surgical resection of their solid tumor every year.

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Sorting out Pandora's box: discerning the dynamic roles of liver microenvironment in oncolytic virus therapy for hepatocellular carcinoma

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Oncolytic viral therapies have recently found their way into clinical application for hepatocellular carcinoma (HCC), a disease with limited treatment options and poor prognosis. Adding to the many intrinsic challenges of *in vivo* oncolytic viral therapy, is the complex microenvironment of the liver, which imposes unique limitations to the successful delivery and propagation of the virus. The normal liver milieu is characterized by an intricate network of hepatocytes and non-parenchymal cells including Kupffer cells, stellate cells, and sinusoidal endothelial cells, which can secrete anti-viral cytokines, provide a platform for non-specific uptake, and form a barrier to efficient viral spread. In addition, natural killer cells are greatly enriched in the liver, contributing to the innate defense against viruses. The situation is further complicated when HCC arises in the setting of underlying hepatitis virus infection and/or hepatic cirrhosis, which occurs in more than 90% of clinical cases. These conditions pose further inhibitory effects on oncolytic virus (OV) therapy due to the presence of chronic inflammation, constitutive cytokine expression, altered hepatic blood flow, and extracellular matrix deposition. In addition, OVs can modulate the hepatic microenvironment, resulting in a complex interplay between virus and host. The immune system undoubtedly plays a substantial role in the outcome of OV therapy, both as an inhibitor of viral replication, and as a potent mechanism of virus-mediated tumor cell killing. This review will discuss the particular challenges of oncolytic viral therapy for HCC, as well as some potential strategies for modulating the immune system and synergizing with the hepatic microenvironment to improve therapeutic outcome.

Keywords: oncolytic virus, hepatocellular carcinoma, liver microenvironment, immunotherapy, viral engineering

INTRODUCTION

Hepatocellular carcinoma (HCC), representing over 90% of all cases of primary liver cancer, is the sixth most common form of cancer and the third leading cause of cancer-related mortality worldwide (1, 2). Due to the advanced stage at which most patients are diagnosed, only a small percentage are eligible for potentially curative resection, local ablation, or liver transplantation (3). HCC is highly refractory to chemotherapy and other systemic treatments, and local regional therapies such as transarterial chemoembolization (TACE) or selective internal radiation therapy (SIRT) are largely palliative. Recently, the multi-kinase inhibitor, sorafenib, was found to be effective in patients with advanced HCC and is currently the standard of care in these patients; however the prolongation of survival associated with sorafenib therapy is under 3 months (4), and the median survival for patients with advanced stage, unresectable HCC is less than 1 year (3). The lack of effective treatment options for HCC underlines the need for novel alternative therapies such as those employing oncolytic viruses (OVs).

We have previously demonstrated in a preclinical rat model that oncolytic vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) both replicate well and cause significant tumor-specific cell lysis in orthotopic HCC, leading to substantial survival prolongation (5–7). Based on preclinical data such as these, OVs have been applied in various clinical trials in cancer patients. However, as more and more data are accumulated from clinical trials, it is becoming evident that the significant efficacy reported for OVs in preclinical animal models is not readily translatable to the clinic, due to the vast complexities of spontaneous malignant transformation in the immune-competent setting in patients.

Although these challenges are universal to OVs regardless of the tumor target, the dynamic setting of the liver presents a unique set of hurdles, which viruses must surpass in order to exert their therapeutic effects against HCC. The liver microenvironment consists of a complex network of hepatocytes, stromal cells, inflammatory cells, and extracellular matrix (ECM). HCC is an inflammation-driven cancer (8), and the chronic inflammatory state, characterized by the recruitment of inflammatory cells and high levels of cytokine expression, not only promotes tumorigenesis (9), but it also serves to provide a basis for innate immunity against OVs. Although it may seem contradictory that hepatotropic viruses manage to escape immune surveillance and establish chronic infections in the liver, this paradox can be attributed to intrinsic

differences among viruses, whereby the hepatitis viruses are known to possess various mechanisms for evading or interfering with the immune system (10, 11). Whether or not the well-characterized feature of immune tolerance in the liver actually plays a role in promoting OV replication in liver tumors is not known; however, OVs are extremely sensitive to the anti-viral actions of type I interferon (IFN), and this is most likely the primary mechanism by which the replication of OVs is limited *in vivo*.

When HCC arises as a consequence of chronic hepatitis virus infection and in the setting of hepatic fibrosis, the liver milieu is distorted and provides a platform for dynamic interactions between OVs and the liver microenvironment. In Greek mythology, Pandora's box was actually a large and beautiful jar, which contained all the evils of the world. At first glance, the diseased liver can resemble a Pandora's box of sorts, filled with a variety of "evils" that present unique challenges to conventional therapies. As we learn more about the pathogenesis of liver disease, we can actually exploit the unique features of the local microenvironment to synergize with OV therapy and thereby transform Pandora's box from a vessel of evil into a platform of hope for new therapeutic targets. In this review, we will discuss the complex interactions between OVs and the liver milieu and present novel strategies for improving the therapeutic outcome.

THE COMPLEX LIVER MILIEU AND ITS IMPACT ON OV THERAPY OF HCC

The liver is arguably one of the most vital organs of the body, due to its diverse roles in metabolism, nutrient uptake, detoxification, and immune modulation. Because of the complexity of functions, the liver architecture is composed of an intricate network of cells and ECM to ensure that each task can be performed efficiently. Although this system is crucial for the proper functioning of the liver, it poses various barriers to the ability of OVs to infect and replicate well in hepatic tumors. In this section, the various aspects of the liver microenvironment, which challenge the fate of OVs in HCC therapy (summarized in **Figure 1**), as well as the unique interactions between OVs and the liver milieu, will be discussed.

THE HEALTHY LIVER SETTING

Although the majority of HCCs arise in the context of underlying chronic liver disease, a small percentage can develop in the absence of advanced hepatic fibrosis, or even in a healthy liver setting (12). Hepatocytes constitute the majority of the liver volume (approximately 80%), and they are protected from invading organisms in the bloodstream by non-parenchymal cells lining the liver sinusoids. The major sinusoidal components are Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), and natural killer (NK) cells. A unique microvasculature, including the fenestration of sinusoidal endothelial cells, acts as a filtration system to trap pathogens, waste products, and circulating tumor cells, making the liver a common site for tumor metastases. KCs are resident macrophages of the liver and are considered to be scavenger cells, playing a major role in removing foreign material from portal circulation (13). Together with NK cells and dendritic cells (DCs), KCs are important components of the innate immune system, providing a rapid first line of defense against invading pathogens and protecting the liver from bacterial and viral infections (14). Despite the crucial protective function of KCs in the liver, hepatic sequestration and destruction of viruses is a universal limitation to all systemically applied OVs, and they can pose a particular challenge to viral therapies targeting HCC cells, due to their close proximity. Following uptake, the KC, as well as engulfed viruses, are rapidly degraded, greatly reducing the bioavailability of the virus (15, 16). It is well established that therapeutic doses of adenovirus must first saturate the KC population before their effects can be seen in target cells (17, 18). To illustrate this point, it was demonstrated for adenovirus type 5 that up to 90% of injected viral particles are sequestered from the blood by KCs (19), and depletion of KCs via predosing with adenovirus or pretreatment with clodronate results in improved bioavailability and anti-tumor efficacy of adenovirus therapy (20, 21). In addition, activated KCs are potent producers of nitric oxide and cytokines such as IFN, TNF-α, IL-6, and IL-10 (13, 22, 23), all of which have potent anti-viral functions (24, 25) and likely contribute to the local control of OV replication in HCCs.

In addition to KCs, the LSECs, which are specialized endothelial cells lining the liver sinusoid, belong to the reticuloendothelial system and play a role in clearing materials from the bloodstream. They have been shown to be important in eliminating circulating adenovirus particles (15, 26) via scavenger receptors expressed on the cell surface (18). Although less information is available regarding the role of KCs and LSECs in the depletion of other OVs from the blood, it is speculated that the same mechanism identified for adenovirus applies to these viruses as well (27–29).

Natural killer or "pit cells," and NKT cells are enriched and constitutively activated in the sinusoid of normal, healthy livers, and are key players in innate immune surveillance (30, 31). These cells represent a distinct subset of the cytotoxic lymphocyte population and are crucial in the early defense against invading viruses (32), prior to the launch of adaptive immune responses (33–35). It is speculated that bone marrow-derived peripheral NK cells migrate to the liver (36), where they are stimulated by hepatic cells, such as KCs (37), causing them to differentiate and become activated and express DC markers (38). Liver-specific NK cells are immunologically, morphologically, and functionally different from peripheral NK cells, expressing higher levels of TRAIL, perforin, and granzyme B, and having a higher percentage of activated populations, presumably contributing to the increased cytotoxicity of liver NK cells (30, 39). Upon activation, NK cells mediate the direct lysis of target cells by releasing copious amounts of cytokines and cytotoxic granules, or by induction of apoptosis (40, 41). As crucial components of the cellular response to viral infections, it is not surprising that NK cells also have an inhibitory effect on OVs. To illustrate this point, it was demonstrated *in vitro* that NK cells rapidly and specifically lyse tumor cells at an early stage of infection with herpes simplex type 1 or vaccinia virus and prevent viral propagation and spread to neighboring cells (35). We have observed a significant intratumoral accumulation of NK and NKT cells in orthotopic, syngeneic HCC in immune-competent rats within 24 h of treatment with oncolytic VSV and have demonstrated that these cells play a major role in the rapid clearance of the virus (42). We believe that this rapid innate response is at least partially mediated by the large number of resident NK and NKT cells which are present in the liver and can immediately infiltrate areas

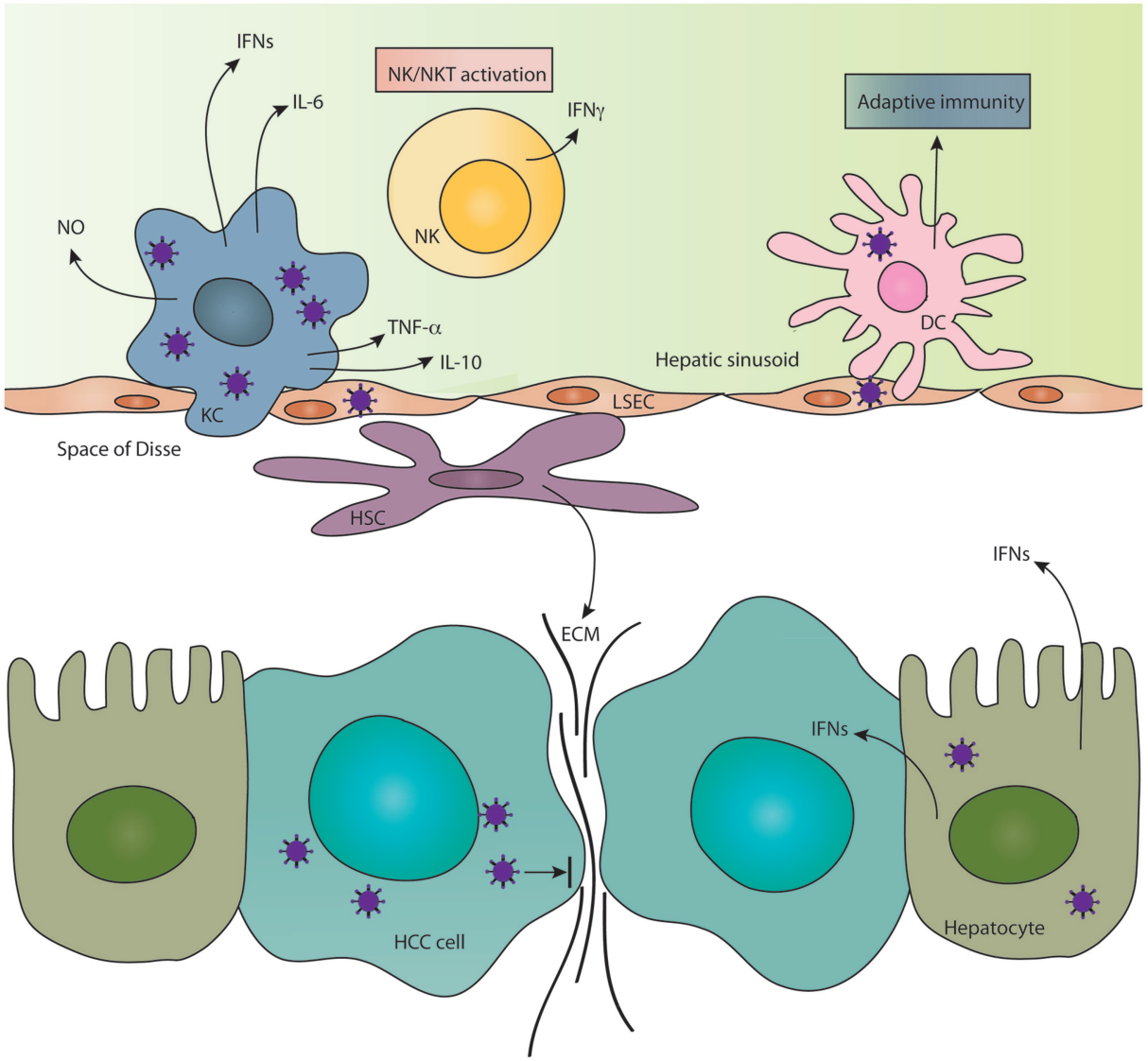

FIGURE 1 | Features of the hepatic microenvironment which challenge the fate of OVs. The innate immune response in the liver consists of scavenger Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) and resident NK and NKT cells, which are efficient at clearing invading oncolytic viruses from the liver. KCs and NK/NKT cells secrete a variety of antiviral cytokines in response to infection, which substantially limit the replication of OVs in hepatocellular carcinoma (HCC). Material from dying virus-infected cells mediates cross-priming of T-cell responses by dendritic cells (DCs) and thereby induces an adaptive immune response against the virus. Hepatic stellate cells, which reside in the space of Disse, become activated during tumorigenesis causing them to migrate and secrete copious amounts of extracellular matrix (ECM) components, which hinder intratumoral cell-to-cell spread of OVs. Infected hepatocytes enter an anti-viral state and secrete type I interferons (IFNs), which protect the neighboring liver cells from infection and could also infer protection to HCC cells that are partially sensitive to IFN.

of VSV infection to prevent productive replication and spread of the virus and thereby inhibit the therapeutic effect.

THE DISEASED LIVER

In nearly 90% of HCC patients, tumors arise as a consequence of chronic liver injury, which provides an ideal setting for carcinogenesis to occur (43, 44). Liver disease, caused by persistent viral, toxic, autoimmune, metabolic, or cholestatic impairments, results in a chronic inflammatory response marked by the secretion of a cocktail of cytokines and chemokines by infiltrating immune cells and the resident non-parenchymal cells. As a result, the hepatic architecture becomes disrupted, as evidenced by hepatocyte proliferation, the extensive deposition of ECM, nodule formation, and the increased risk of HCC.

When HCC occurs in the midst of a chronically injured liver, the already limited treatment options become even further restricted. Although the application of OVs is an attractive alternative to the palliative treatment options available to patients with advanced liver disease, the fate of therapeutic viruses administered in this complex setting is further challenged. Viral vectors targeting HCC

in a diseased liver face many unfavorable conditions, including accumulation of immune cells, constitutively activated cytokines, dense ECM, and altered blood flow.

During the fibrogenic wound-healing process, HSCs differentiate from the quiescent to the activated form with a myofibroblast phenotype, which is marked by the loss of intracellular vitamin A-rich fat droplets and expression of α-smooth muscle actin (α-SMA). These transdifferentiated HSCs promote ECM remodeling by deregulating the balance of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) and resulting in the degradation of the normal basement membrane and replacement with interstitial collagen (primarily type I and III) and scar matrix. In addition, HSCs migrate and proliferate in response to a variety of cytokines and growth factors elicited during hepatic injury to further promote the progression of fibrosis, resulting in the distortion of the normal liver architecture and leading to decompensated liver function.

The implication of the presence of hepatic fibrosis on the outcome of OV therapy for HCC is complex, due to the multifaceted nature of the interactions between OVs and the microenvironment of the chronically injured liver. The presence of fibrotic tissue throughout the liver likely provides a physical barrier to trap OVs and prevent efficient delivery of viruses to tumor beds, and altered patterns of blood flow limit the ability of systemically applied viruses to reach their tumor targets. The aberrant microenvironment within HCC, consisting of activated HSCs, inflammatory cells, and extensive ECM deposition, not only further promotes HCC growth, invasion, and metastasis, but also challenges viral infection and spread among HCC cells. Although HCC is not conventionally considered to be a fibrotic cancer, evidence has shown a correlation between poor differentiation of HCC and degree of ECM remodeling (45). Furthermore, the predominant components of the ECM of HCC are the fibril-forming collagens type I and III (46, 47), which are also dominant in hepatic fibrosis tissue. Although we have not yet specifically investigated this issue, it is likely that this intratumoral deposition of collagen plays a role in containing viral spread and leading to the well-defined foci of VSV replication that we observe in HCC lesions (6).

The inflammatory milieu associated with chronic liver injury, most often induced by hepatitis B or C virus infection, not only contributes to the pathogenesis of fibrosis and HCC, but it also threatens the ability of OVs to replicate and destroy tumor cells. The acute response to liver injury involves the activation of resident liver immune cells, followed by the recruitment of non-resident immune cells to launch a potent cytokine response in the liver in an attempt to lyse the infected or injured cells (48, 49). Hepatitis B virus (HBV) infection causes induction of NK cells and cytotoxic T-cells (CD8+), which then secrete anti-viral cytokines, such as IFN-γ and TNF-α (50, 51). Upon infection, the host recognizes the pathogen-associated molecular patterns (PAMPs) of viral products via pathogen recognition receptor (PRR) proteins, such as toll-like receptor 2 (TLR2). Activation of PRRs by HBV leads to induction of transcription factors, such as NF-κB, and the release of pro-inflammatory and anti-viral cytokines, such as TNF-α, IL-6, and IL-10 (51, 52), all of which can inhibit OV replication. Hepatitis C virus (HCV) infection causes an immune response characterized by cytokines and non-specific lymphocyte recruitment, which can also have inhibitory effects on OVs.

In addition to the potentially limited efficacy of oncolytic viral therapy for HCC in the context of liver injury, there are valid safety concerns associated with such a therapeutic approach. The local cytokine induction following OV application in an already inflamed liver could potentially cause a highly toxic "cytokine storm" and hepatotoxicity, strongly contraindicating this strategy. Furthermore, due to a lack of appropriate rodent models, the interaction of an OV with an underlying hepatic viral infection remains unclear. However, recent findings indicate that administration of OVs could potentially provide a therapeutic benefit in decreasing HBV load (53–55). In studies using inactivated *Parapoxvirus ovis* (Orf virus), it was shown that viral therapy inhibited human HBV and HCV, as well as herpes simplex virus infection, without any signs of toxicity, in preclinical mouse models (53, 54). In these studies, it was demonstrated that inactivated Orf virus-mediated induction of IFN-γ was a key mechanism in the anti-viral activity, and the absence of hepatotoxicity was associated with a down-regulation of antigen cross-presentation in LSECs. To further illustrate this phenomenon, it was recently demonstrated in a clinical trial in patients with HCC that, in addition to the anti-tumoral and anti-vascular activities of oncolytic poxvirus JX-594, virotherapy led to a suppression of underlying HBV replication and caused a transient decrease in viral load (55).

Along similar lines, an exciting new body of research has demonstrated antifibrotic effects mediated by OV therapy. It was first reported in 2009 that NDV replicates selectively in activated HSCs and causes reversal of hepatic fibrogenesis in mice (56). Our own work similarly demonstrated the antifibrotic properties of oncolytic VSV, via replication and subsequent apoptosis of activated HSCs, induction of NK cell infiltration, and gene modulation in favor of fibrotic regression (57). Furthermore, in addition to anti-viral activities, inactivated Orf virus has also been shown to elicit antifibrotic effects in two preclinical models of liver fibrosis (53, 58). These studies indicate that OV therapy in the context of underlying hepatic injury is not only safe, but also could provide additional therapeutic benefits to resolve liver disease.

Additionally, in light of these new findings, we may reevaluate our classical view of tumor stroma as being a barrier to OV therapy. Activated HSCs infiltrate the stroma of HCC and localize around tumor sinusoids, capsules, and fibrous septa (59), and increasing intratumoral density of activated HSCs is correlated with poor prognosis (60). Data demonstrating the ability of OVs to replicate specifically in activated HSCs imply that they may also replicate within HCC-infiltrated HSCs.

STRATEGIES TO IMPROVE OV THERAPY FOR HCC

Although the potential of OV therapy for HCC has been demonstrated, it is clear that novel strategies must be utilized in order to enhance viral replication and/or virus-mediated anti-tumor immune responses to improve therapeutic outcomes in the unique and complex setting of the liver. A prominent theme in OV development is the ongoing debate regarding the complex and contradictory roles of the immune system, which can be considered both inhibitory, in terms of the host's anti-viral immune response, or complementary, with respect to anti-tumoral immune responses

Table 1 | Barriers to intrahepatic OV therapy and potential strategies for overcoming them.

Barrier	Strategy	Reference
Non-specific uptake by Kupffer cells	Predosing with OV	(20)
	Kupffer cell depletion	(20, 21)
	Viral shielding	(64–67)
Innate anti-viral response	Kupffer cell depletion	(20, 21)
	OV-mediated expression of vCKBP	(42, 68, 69)
Poor viral replication and/or spread	Combination therapies	(62, 63, 70–72)
	OV as immunotherapeutic	(73–86)
Hepatic fibrosis	Utilization of antifibrotic OVs	(53, 56–58)
Underlying hepatic viral infection	Utilization of anti-viral OVs	(53–55)

which are crucial in clearing uninfected tumor cells and challenging tumor relapse. Although it remains to be seen whether inhibition or augmentation of the immune response is the more powerful therapeutic strategy, the ideal approach would undoubtedly involve selective inhibition of anti-viral immune components, while simultaneously inducing a strong anti-tumoral immune response. A comprehensive discussion of the competing roles of the immune response in the efficacy of OV therapy, as well as strategies to modulate the immune system to synergize with OV therapy has been thoroughly reviewed elsewhere (61–63), and therefore will not be recapitulated here. In this section, we will review the general approaches for improving viral-mediated oncolysis and/or modulating the immune system for optimization of oncolytic viral therapy, with an emphasis on strategies that have been employed specifically for treatment of HCC. These strategies are summarized in **Table 1**.

COMBINATION THERAPIES

The rational design of combination therapies involving OVs and existing clinical agents is a valuable strategy for improving therapeutic outcomes by employing synergistic mechanisms. Success with several combination therapies for HCC has already been reported. In an *in vitro* study, it was demonstrated that treatment with parvovirus could sensitize p53-negative HCC cells to the cytotoxic effects of cisplatin, and combination therapy resulted in increased HCC cell death in comparison to either individual therapy (70). Similarly, combination of adenovirus with the DNA-intercalating drug, doxorubicin, resulted in synergistic cytotoxic effects *in vitro* and significant inhibition of *in vivo* tumor growth in preclinical HCC models (71). These results were confirmed by an additional study in HCC, where it was shown that oncolytic adenovirus sensitizes tumors to chemotherapy, and combinations of adenovirus with 5-FU, doxorubicin, and paclitaxel all resulted in enhanced efficacy in killing of HCC cells (72). A telomerase-dependent replicating adenovirus (hTert-Ad) was also extremely effective at sensitizing resistant HCC tumors to chemotherapy via down-regulation of Mcl-1 expression, resulting in substantial tumor responses in mice treated with virochemotherapy (87). In another study using hTert-Ad, it was demonstrated that proteasome inhibition with bortezomib led to endoplasmic reticulum (ER) stress-induced apoptosis and improved anti-tumoral immunity, leading to improved oncolysis of HCC (88). It was further shown in this study that bortezomib inhibited anti-viral immune responses in immunocompetent mice, allowing enhanced viral kinetics of hTert-Ad, and indicating a dual benefit of the combination therapy (88).

As an alternative to combination therapies involving chemotherapy, we investigated the potential of applying a clinical embolization agent together with oncolytic VSV to treat HCC in an orthotopic rat model (89). In this study, we demonstrated significantly enhanced tumor necrosis and prolongation of survival in HCC-bearing rats treated by transarterial viroembolization, as compared to monotherapy, and we attributed this therapeutic effect to multiple mechanisms, including apoptosis, anti-angiogenesis, and induction of anti-tumor immunity (89).

INHIBITION OR EVASION OF INFLAMMATORY RESPONSES

The direct cytopathic effect elicited by OVs is dependent on their ability to evade immune surveillance long enough to allow viral propagation to high titers and efficient spread of the vector throughout the tumor mass. Because of the numerous physiological and immunological barriers to oncolytic viral therapy in the liver, several attempts were made to selectively block aspects of the anti-viral response to improve and prolong viral replication prior to the launch of an adaptive immune response. Although systemic suppression of immune responses has been successful in promoting enhanced OV replication and intratumoral spread in various tumor models, there have been concerns associated with the safety of such approaches (62). By incorporating genes encoding anti-inflammatory proteins directly into the virus, we speculated that the suppression of immune responses would be limited to the local area of virus replication within the tumor, thereby dampening safety concerns. In nature, many viruses have adapted themselves in various ways to counteract or evade anti-viral immune responses to promote their own survival (68). One such mechanism involves the viral production of chemokine-binding proteins (CKBPs), which are secreted proteins that competitively bind to and/or inhibit the interactions of immunomodulatory chemokines with their cognate receptors, to block the chemotaxis of inflammatory cells (69). Based on our observation that host inflammatory responses to VSV infection play a detrimental role in suppression of intratumoral viral replication in HCC, we exploited several heterologously expressed vCKBPs in order to enhance the oncolytic potency of VSV for the treatment of HCC. Specifically, we engineered recombinant VSV vectors encoding for the equine herpes virus-1 glycoprotein G and the M3 gene from murine gammaherpesvirus-68, both of which are broad range and high affinity vCKBPs (42, 90). Both recombinant vectors mediated the suppression of anti-viral NK cell and neutrophil infiltration, which resulted in prolonged kinetics of intratumoral VSV replication and significant survival prolongation in immune-competent, orthotopic liver tumor-bearing rats. In order to specifically target the NK cell population, we incorporated the UL141 gene from human cytomegalovirus into VSV, which specifically inhibits the NK cell-activating ligand CD155,

resulting in enhanced virus propagation and tumor responses corresponding to inhibition of NK and NKT cell migration to infected tumor sites (91). Importantly, none of these recombinant vectors resulted in any observable signs of toxicity to the host, indicating that this strategy has potential for clinical translation in HCC patients.

BOOSTING ANTI-TUMOR IMMUNITY

Due to the resistance of HCC to chemotherapy, and indications that immune responses have a direct effect on the clinical course of the disease (92), HCC has become an attractive target for immunotherapy. A variety of immunotherapeutics have been tested in clinical trials for HCC patients, including cytokines, adoptive immune cells, and antibody-based therapies, and the resulting data have indicated that these therapies are safe, even in the context of underlying hepatic cirrhosis and HBV infection (93, 94). Recent studies showing promising results involve adoptive DC therapy, targeting of glypican-3, which is a tumor-associated antigen (TAA) expressed by a high percentage of HCCs, or breaking immune tolerance via antibody-mediated inhibition of cytotoxic T-lymphocyte antigen 4 (CTLA4) (95, 96).

In addition to the direct cytopathic effect on tumor cells that is induced by OVs, the stimulation of the host's immune system to launch an attack against cancer cells is a potent mechanism of action that can be exploited by OV therapy. Particularly in tumors where conditions are unfavorable to virus replication, due to factors such as IFN sensitivity, inflammation, or a high degree of stroma and ECM, the effect of OV therapy can be rescued by utilizing the vector as a cancer vaccine rather than as a direct oncolytic agent. Although tumor cells express a variety of TAAs, a multitude of mechanisms allow tumors to evade rejection from the host immune system. The liver is a highly tolerogenic organ, due to features of the microenvironment which induce immune tolerance against foreign antigens (73), as evidenced by its susceptibility to infection by hepatic viruses and to carcinogenesis and metastases. OVs can serve to break the tolerance and enhance the immunogenicity of the tumor microenvironment as a potent therapeutic mechanism. Viral oncolysis is associated with the local release of TAAs, which can then be taken up by DCs. In addition, the release of intrinsic cell factors, such as uric acid, can be recognized as a danger signal to activate DCs (97). DCs are important components of the innate immune response, and are key players in the generation of adaptive immune responses via antigen presentation and priming of T-cells. Virus-infected cells are highly effective in delivering antigens for cross-presentation and cross-priming of adaptive immune responses (98). Therefore, harnessing the inherent ability of OVs to stimulate anti-tumoral immune responses is a logical approach, and several such strategies have been employed for HCC therapy.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine with strong immunostimulatory properties that is secreted by macrophages, T-cells, fibroblasts, mast cells, and endothelial cells. GM-CSF promotes progenitor cell differentiation into DCs and can generate tumor-reactive CTL (74). Gene transfer of GM-CSF to tumor cells augments tumor antigen presentation by recruited DCs and macrophages to mediate protective immunity against tumors (74, 75). To date, reports of recombinant vaccinia virus, adenovirus, HSV, measles virus, and NDV engineered to express GM-CSF have demonstrated improved therapeutic outcomes due to enhanced anti-tumor immune responses (76–80). In the context of HCC therapy, JX-594, a thymidine kinase-deleted oncolytic vaccinia virus armed with GM-CSF, resulted in partial responses with evidence of efficacy in non-injected tumors, indicating that viral-mediated immune stimulation played a role, in a phase I trial for therapy of primary and secondary liver tumors (81). JX-594 was then applied to a phase II clinical trial in patients with advanced HCC, where a median survival of 14.1 months with high dose therapy compared to 6.7 months for the low dose, was reported, implicating JX-594 as a highly promising vector for HCC therapy (82).

Along the same lines, other cytokines, such as IL-12, IL-24, IL-2, and IFN-β (83–85, 99, 100) have been incorporated into OVs. It has been hypothesized that virus-mediated expression of IFN-β would improve tumor specificity by inhibiting viral replication in normal tissues while permitting propagation in tumors, which possess various defects in type I IFN signaling. In addition, IFN-β can provide antiangiogenic effects (86) and therapeutic immune modulation via the induction of tumor-specific cytotoxic T-lymphocyte responses (101). A recombinant VSV expressing IFN-β was shown to enhance inflammatory cytokine production and NK cell activation, leading to enhanced bystander killing of tumor cells (100). Based on these results, rVSV-IFN-β entered a phase I clinical trial for sorafenib-refractory HCC in 2012 (NCT01628640). In a preclinical study, a conditionally replicative adenovirus (CRAd) was engineered to express IFN-γ, resulting in significant regression of HCC in mice through the combined effects of viral-mediated oncolysis, anti-angiogenesis, and anti-tumor immune responses (102).

Combination strategies involving the adoptive transfer of immune cells together with OVs are an exciting new approach which has shown striking efficacy in several models (103–105). Although this strategy has not been extensively explored for HCC, one study showed that a specific and strong immune response against HCC cells could be elicited *in vitro* via patient-derived DCs that were transduced with an adenoviral vector encoding α-fetoprotein, a TAA often expressed in HCC, and co-cultured with autologous cytokine-induced killer (CIK) cells (106). Strategies involving engineering OVs to express a TAA to prime T-cell responses have shown promise in other tumor models, such as an engineered VSV vector expressing a TAA that resulted in an antigen-specific CD8+ T-cell response in a murine melanoma model (107), and will likely be explored further for HCC therapy in the future.

OUTLOOK

Because of the rapid clearance of viruses in immune-competent hosts, the therapeutic window during which OVs have the opportunity to replicate and cause their cytopathic effect in tumor cells is relatively short. In the context of the liver microenvironment, where myriad other barriers to OV propagation exist, we believe that the immune system represents an essential tool, which must be harnessed in order to destroy the remaining tumor cells that have escaped viral infection. The combination of viral-mediated cytolysis with tumor-directed immune stimulation creates a potent

arsenal against hepatic tumors. Although two immunotherapeutic OVs are already in clinical trials for HCC, there are many other strategies for utilizing OVs to break immune tolerance and/or stimulate anti-tumor immune responses, which have shown efficacy in other tumor models but have not yet been tested in the context of HCC.

One such strategy involves the exciting new concept of incorporating new molecules called "T-cell engagers" into oncolytic viral vectors (108). In this approach, a secretory bispecific T-cell engager, consisting of antibodies directed against CD3 and a tumor cell-specific antigen, EphA2, was expressed by an oncolytic vaccinia virus, and resulted in improved anti-tumor efficacy via activation of T-cells within tumors and bystander cell killing (109). Another innovative approach involves the systemic application of oncolytic NDV, followed by intradermal vaccinations with DCs pulsed with viral oncolysate, to prime naïve T-cells against the patient's TAAs and establish a long-lasting memory T-cell repertoire (110). These novel strategies, which combine oncolytic virotherapy with immunotherapy have the potential to produce potent anti-tumor responses.

Alternatively, a prime-boost approach has been investigated, in which two different recombinant OVs are sequentially administered, the first one priming the immune response through expression of a TAA, followed by a boosted secondary response produced by a subsequent TAA-encoding virus, leading to a robust tumor-specific immunity (64, 111). A cDNA library has also been utilized to present a broad range of TAAs by a recombinant VSV vector, resulting in dramatic tumor regressions (112). These TAA-based approaches lead to significant tumor responses via complementary cell death mechanisms induced by the direct viral-mediated oncolysis in combination with TAA-specific CD8+ T-cell-mediated killing, causing additional TAAs to be released and presented by DCs to T-cells and resulting in further activation of tumor-specific immune responses, thereby conferring a potent arsenal against systemic metastases.

A ubiquitous problem in the field of OV therapy is the relative inefficiency of systemic application, due to virus inactivation by blood components, non-specific uptake by off-target cells, and sequestration by the liver and spleen. To address this issue, various approaches using synthetic polymers or cell carrier systems for viral shielding have been investigated (113). The innate ability of immune cells to home to tumors is a convenient feature, which affords them the opportunity to serve as OV cell carriers for the dual benefit of virus delivery and stimulation of anti-tumor immune responses. To this end, VSV has been loaded onto antigen-specific T-cells to simultaneously enhance adoptive T-cell therapy, while providing a vehicle for OV delivery to the tumor site (114). In similar studies, it was demonstrated that T-cells, mature DCs, and CIK cells could efficiently deliver OVs to their tumor targets to improve viral-mediated tumor oncolysis and prime anti-tumor immune responses (65, 66). The application of these approaches to HCC therapy will likely produce similar benefits, and are undoubtedly already under investigation by several groups.

As an alternative to the cell carrier approach for virus delivery, strategies involving the surface modification of OVs using synthetic polymers have been developed to shield oncolytic vectors from inactivation and non-specific uptake. VSV shielding via covalent modification with polyethylene glycol (PEG) has resulted in increased circulation times and a reduction of neutralizing antibody responses (115). Polymer shielding of adenovirus has been demonstrated to allow immune escape and a reduction of liver sequestration by increasing the diameter above the size of the hepatic sinusoidal fenestrae and by lowering KC uptake (67, 116). PEGylation of Ad5 with high molecular weight PEG (20 kD) resulted in improved efficacy of intravenously applied therapy for HCC, with reduced transduction of hepatocytes and KCs and a reduction of hepatotoxicity (117), making this an attractive approach for improving the specificity of OV therapies targeted to liver tumors.

CONCLUDING REMARKS

The complex liver milieu underlying HCC presents innumerable challenges to the development of effective therapeutic agents to produce significant tumor responses and prolongation of patient survival. However, by gaining a greater understanding of the dynamic roles of the hepatic microenvironment and the pathogenesis of liver disease and carcinogenesis, we can actually exploit the properties of the local liver setting to synergize with therapeutic agents. Because OVs exert their therapeutic effects via multiple mechanisms, including direct cytopathic effects, anti-angiogenesis, and anti-tumor immune stimulation, they represent ideal agents for contending with the liver microenvironment. This is evidenced by recent reports, which demonstrate that OVs not only provide potent anti-tumor effects, but they also possess antifibrotic and anti-viral properties, allowing them to provide therapeutic benefits against the underlying liver injury. Therefore, by discerning the complexities of the liver microenvironment and their roles in the pathogenesis of HCC, Pandora's box of evil is converted to a vessel of hope, for which OVs will surely play an important role in providing synergistic therapeutic outcomes.

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Pharmacological modulation of anti-tumor immunity induced by oncolytic viruses

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Oncolytic viruses (OVs) not only kill cancer cells by direct lysis but also generate a significant anti-tumor immune response that allows for prolonged cancer control and in some cases cures. How to best stimulate this effect is a subject of intense investigation in the OV field. While pharmacological manipulation of the cellular innate anti-viral immune response has been shown by several groups to improve viral oncolysis and spread, it is increasingly clear that pharmacological agents can also impact the anti-tumor immune response generated by OVs and related tumor vaccination strategies. This review covers recent progress in using pharmacological agents to improve the activity of OVs and their ability to generate robust anti-tumor immune responses.

Keywords: Oncolytic virotherapy, anti-tumor immunity, cancer, combination therapy, pharmacological therapy, chemotherapy, immuno-modulatory therapy

INTRODUCTION: ONCOLYTIC VIRUSES: MULTI-MECHANISTIC BIOTHERAPEUTICS AGAINST CANCER

Oncolytic viruses (OVs) are self-amplifying biotherapeutics that have been selected or engineered to preferentially infect and kill cancer cells. Generated from a multitude of viral species, OVs exploit cancer-associated cellular defects arising from genetic perturbations including mutations and epigenetic reprograming [reviewed in Ref. (1)]. Among others, these cellular defects lead to dysfunctional anti-viral responses and immune evasion, increased cell proliferation and metabolism, and leaky tumor vasculature (2). These characteristics in turn provide a fertile ground for viral replication and subsequent lysis of tumor cells and permit the growth of genetically attenuated OVs that are otherwise harmless to normal cells.

In addition to the direct killing of cancer cells, OVs can also trigger a potent anti-tumor immune response. Infected tumor cells induce the release of pro-inflammatory cytokines and expose both viral and tumor-associated antigens to patrolling immune cells, promoting the differentiation of antigen-presenting cells and T-cell activation (3–5). How much tumor infection and lysis are necessary to trigger these responses remains a topic of debate; however, it is clear that the combination of direct oncolysis and activation of anti-tumor immunity can lead to durable cures in pre-clinical mouse models of cancer.

A number of OVs are currently being evaluated in clinical trials to treat a range of cancer types. For a more comprehensive overview, the reader is invited to consult an excellent review by Russell et al. (6). Of particular note, herpes simplex virus-1 (HSV-1), vaccinia virus, reovirus, and adenovirus-based OV strains have made the most progress toward approval (7–10). Shanghai Sunway Biotech's oncolytic adenovirus (H101), deleted for the viral E1B gene and thought to target p53 deficient cancer cells, was the first approved OV in China as early as 2005, indicated for head and neck cancers. (11). Profound tumor regression is common following treatment with OVs; for example, durable objective responses were observed in 3/14 patients (hepatocarcinoma, lung cancer, and melanoma) following treatment with vaccinia virus JX-594 in a phase I trial (7). This virus has been deleted for viral thymidine kinase (TK), making it dependent on cellular TK that is overexpressed in cancer cells (7). In addition to the TK deletion that provides tumor selectivity, the virus also expresses granulocyte macrophage colony-stimulating factor (GM-CSF) to stimulate anti-tumor immunity. Most recently, Amgen's HSV-1-based talimogene laherparepvec (T-VEC) led to 16% durable response in a phase III clinical trial for late-stage melanoma, and it is expected that the company will file for FDA approval in North America in the coming year (12, 13). Like JX-594, T-VEC expresses GM-CSF but has deletions in viral genes ICP34.5 and ICP47 that confer tumor selectivity and promote antigen presentation, respectively (14).

While widespread approval and clinical implementation of oncolytic virotherapy are in the foreseeable future, heterogeneity in clinical response to OVs remains a significant challenge as evidenced from a number of early and late-stage human clinical trials (6, 15, 16). This heterogeneity in response can be attributed to factors that impact OV delivery and spread within tumors, such as pre-existing immunity and remnant tumor anti-viral responses, as well as to a variably immunosuppressive tumor microenvironment that can prevent the generation of an effective anti-tumor immune response. To overcome these challenges, it has been long recognized in the OV field that improvements to therapeutic efficacy either through viral engineering or through combination therapies will be critical (6, 17). In the current review, we will focus on advances in therapeutic strategies employing small-molecule pharmacological agents that ameliorate OV treatment *in vivo* by manipulating the innate and/or adaptive immune response to virus and tumor (summarized in **Table 1**).

Table 1 | Combinations of pharmacological and oncolytic therapies with demonstrated improvements in *in vivo* treatment efficacy.

Drug	Mechanism of action/molecular target	Reported immunomodulatory effect (systemic immunomodulation or specific modulation of anti-viral response	Oncolytic virus	Reference
CLASSIC CHEMOTHERAPY AGENTS				
Cyclophosphamide	DNA alkylation	Systemic immunomodulation	HSV	Ikeda et al. (20), Ikeda et al. (21), Wakimoto et al. (22), and Currier et al. (24)
			Adenovirus	Thomas et al. (25), Dhar et al. (26), Cerullo et al. (27), and Hasegawa et al. (28)
			Vaccinia	Lun et al. (29)
			Reovirus	Qiao et al. (30) and Kottke et al. (34)
			Measles	Ungerechts et al. (31) and Ungerechts et al. (32)
Gemcitabine	Nucleoside substitution and inhibition of DNA replication, ribonucleotide reductase inhibitor	Systemic immunomodulation	Adenovirus	Leitner et al. (38), Liu et al. (39), Onimaru et al. (40), Bhattacharyya et al. (41), Cherubini et al. (42), Wang et al. (43), and Kangasniemi et al. (44)
			Parvovirus	Angelova et al. (45)
			Reovirus	Gujar et al. (48)
			VSV	Hastie et al. (49)
			HSV	Watanabe et al. (50) and Esaki et al. (51)
			Vaccinia	Yu et al. (52)
			Myxoma	Wennier et al. (53)
Bortezomib	Proteasome inhibition	Systemic immunomodulation	VSV (VSV-mIFNβ)	Yarde et al. (61)
			Reovirus	Carew et al. (62)
			Adenovirus (hTERT-Ad)	Boozari et al. (63)
Mitoxantrone	Type II topoisomerase inhibition	Systemic immunomodulation	HSV	Workenhe et al. (69)
Irinotecan	Type I topoisomerase inhibition	systemic immunomodulation	HSV	Tyminski et al. (74)
			Sindbis	Granot and Meruelo (75)
Temozolomide	DNA alkylation	Systemic immunomodulation	Adenovirus	Alonso et al. (80), Holzmuller et al. (81), Liikanen et al. (82), and Tobias et al. (83)
			HSV	Aghi et al. (84) and Kanai et al. (85)
EPIGENETIC MODULATORS				
Valproic acid	Histone deacetylase inhibition	Specific modulation of anti-viral response	HSV	Otsuki et al. (106)
Trichostatin A	Histone deacetylase inhibition	Specific modulation of anti-viral response	HSV	Liu et al. (105)
			Vaccinia	MacTavish et al. (108)
Entinostat (MS-275)	Histone deacetylase inhibition	Both	VSV	Nguyen et al. (99) and Bridle et al. (109)
5-Azacitidine	DNA methyltransferase inhibition	Specific modulation of anti-viral response	HSV	Okemoto et al. (111)
PI3K/Akt/mTOR PATHWAY INHIBITORS				
LY294002	PI3K inhibition	Specific modulation of anti-viral response	HSV	Kanai et al. (116)

(Continued)

Table 1 | Continued

Drug	Mechanism of action/molecular target	Reported immunomodulatory effect (systemic immunomodulation or specific modulation of anti-viral response	Oncolytic virus	Reference
Rapamycin	mTORC1 and mTORC2 inhibition	Both	Adenovirus HSV VSV	Jiang et al. (120) Fu et al. (121) Alain et al. (122)
Everolimus (RAD001)	mTORC1 inhibition	Both	Adenovirus	Lukashev et al. (119)
OTHER				
Viral sensitizer 1 (VSe1)	Unknown	Specific modulation of anti-viral response	VSV	Diallo et al. (125)
Triptolide	Global transcription inhibition via RNA pol II inhibition	Specific modulation of anti-viral response	VSV	Ben Yebdri et al. (130)
Sunitinib	Receptor tyrosine kinase inhibition	Specific modulation of anti-viral response	VSV Reovirus Vaccinia	Kottke et al. (87) and Jha et al. (88) Kottke et al. (87) Hou et al. (89)
Ipilimumab	CTLA-4 inhibition	Systemic immunomodulation	NDV	Zamarin et al. (138)

Numerous studies have shown that combining oncolytic virotherapy and pharmacological therapy leads to improved outcomes in vivo. This table summarizes these reports, presenting the small molecule used in the study, its main mechanism of action or molecular target, its reported immuno-modulatory effect(s), and type of oncolytic virus used. Abbreviations: HSV, herpes simplex virus; VSV, vesicular stomatitis virus; mIFNβ, murine interferon beta; hTERT-Ad, human telomerase reverse transcriptase promoter-regulated adenovirus; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; CTLA-4, cytotoxic T-lymphocyte antigen 4.

STANDARD CHEMOTHERAPEUTIC DRUGS THAT BOOST OV ACTIVITY THROUGH SYSTEMIC EFFECTS ON IMMUNE CELLS AND THE IMMUNE RESPONSE

Most cancer patients with advanced disease will be subjected to some form of chemotherapy. This will largely depend on the type of cancer and other salient pathophysiological characteristics. Given that most patients enrolled in clinical trials to test the efficacy of OVs suffer from advanced disease (7), a natural trend in the OV field has been to test OVs in combination with chemotherapeutics that are currently the standard of care. Classic chemotherapy drugs typically capitalize on the fact that cancer cells are continuously replicating unlike most normal cells (18). However, some normal cell types have higher replication rates, leading to significant off-target effects. Hematopoietic cells among others can be affected and this can lead to systemic immunosuppression (discussed below). While the evaluation of chemotherapeutic drugs in the context of OV therapy has been fairly empirical for the most part, their immunosuppressive effects can inherently complement OV activity by increasing OV spread within tumor beds and/or increasing anti-tumor immune responses. The following sections provide an overview of classic chemotherapy drugs that have been evaluated in combination with OVs focusing on their anti-cancer mechanism of action, examples of OVs with which they have been tested, and the mechanism by which these agents suppress immunity and co-operate with OVs to improve therapeutic outcomes.

CYCLOPHOSPHAMIDE

Cyclophosphamide (CPA) is a nitrogen mustard alkylating agent that leads to cross-linking of nucleotides. Its active metabolite, phosphoramide mustard, interferes with DNA replication by forming guanine-to-guanine intra-strand and inter-strand crosslinks (19). Aldehyde dehydrogenase (ALDH) catalyzes the conversion of the immediate precursor of phosphoramide mustard, aldophosphamide, to an inactive metabolite. Normal cells, for example intestinal epithelial cells and bone marrow stem cells, have a high level of ALDH, protecting them from the effects of CPA's toxic metabolites. In contrast, some lymphocytes have a lower level of ALDH, which makes them more susceptible to the effects of CPA. CPA has been used in combination with several OVs including HSV-1 (20–24), adenovirus (25–28), vaccinia (29), reovirus (30), measles (31–33), and vesicular stomatitis virus (VSV) (33), leading to improved anti-tumor activity *in vivo*. Several studies suggest that CPA can be efficacious in combination with OVs by preventing immune-mediated viral neutralization through inhibiting or delaying the rise of neutralizing antibodies and depleting anti-viral immune cells including natural killer (NK) cells, monocytes, macrophages, and lymphocytes (20, 22, 23, 25, 26). For example, one study showed that CPA inhibits tumor infiltration of innate phagocytes (macrophages, microglia, and NK cells) following HSV treatment in a syngeneic rat glioma model, leading to increased viral persistence and improved overall efficacy (23). Other studies suggest CPA can also enhance the generation of

anti-tumor immunity by inhibiting regulatory T-cells (Tregs) (27, 34). Results from a first in-human clinical trial using Ad-GM-CSF (CGTG-102) to treat solid tumors suggest that metronomic dosing of CPA decreases Tregs without compromising the induction of anti-tumor T-cell responses. This was found to be associated with increased cytotoxic T-cell responses and the induction of Th1 type immunity in most patients. The best progression-free survival and overall patient survival rates were seen with the combination of metronomic CPA and intratumoral infection of adenovirus (27).

GEMCITABINE

Gemcitabine is a fluorinated deoxycytidine nucleoside analog. Incorporation of this analog into DNA prevents further addition of nucleosides during DNA polymerization and thereby halts DNA replication and cell division. Gemcitabine also binds irreversibly to the active site of ribonucleotide reductase. As a result, nucleotide production is halted and DNA replication ceases, leading to apoptosis in rapidly dividing cells [reviewed in Ref. (35)]. While gemcitabine can decrease neutralizing antibodies similar to CPA (36), it is thought to promote anti-tumor immune responses by off-target elimination of myeloid derived stem cells (MDSCs), which suppress T-cell responses. Gemcitabine treatment thereby increases the activity of CD4+ and CD8+ T-cells that recognize tumor antigens (37). This drug has been shown to increase the anti-tumor activity of a wide array of OVs including adenovirus (38–44), parvovirus (45, 46), reovirus (47, 48), VSV (49), HSV (50, 51), vaccinia (52), and myxoma virus (53). In the latter example, the anti-cancer activity of oncolytic myxoma virus was improved using gemcitabine in disseminated pancreatic cancer murine models (53). Interestingly, no sensitization occurred in immunocompromised mice, supporting the requirement for a virus-triggered anti-tumor immune response in mediating the combination effect. The combination of gemcitabine and reovirus was recently evaluated in a phase I clinical trial and while anti-tumor immune responses were not measured, neutralizing antibodies against reovirus were decreased by gemcitabine treatment. In this study, 80% of evaluable patients showed either partial response or stable disease (36).

BORTEZOMIB

Bortezomib is a proteasome inhibitor approved to treat multiple myeloma and mantle cell lymphoma. It reversibly binds the catalytic site of the 26S proteasome with high affinity and specificity (54). Bortezomib has been shown to inhibit NF-κB by preventing degradation of IκB-α in some cell types (55) although the opposite effect has also been observed (56). Other mechanisms of action by which bortezomib may kill cancer cells are through ER-stress and activation of the unfolded protein response (UPR) (57) and triggering apoptosis by preventing the degradation of pro-apoptotic proteins (56, 58). Some studies have shown that treatment of cancer cells using bortezomib increases surface expression of Hsp90 and Hsp60 in cancer cells leading to their more effective phagocytosis by dendritic cells (DCs), improving tumor vaccine effects (59). Bortezomib-treated mice also exhibit increased DC maturation and phagocytic potential (59). On the other hand, one study found that bortezomib treatment leads to apoptosis of allo-reactive CD4+ T-cells. Thus the net result on anti-cancer and anti-viral immune responses is likely context-dependent (60).

Bortezomib has been tested in combination with oncolytic VSV (61), reovirus (62), and adenovirus (63). Using VSV-mIFNβ, combined treatment with bortezomib was inhibitory to virus replication in myeloma cells *in vitro* but led to improved therapeutic efficacy compared to single treatments in syngeneic murine myeloma models (61). Given no observed effect on tumor viral load, this suggests bortezomib likely increases virus-induced cell death and/or potentiates the anti-tumor response mediated by the virus. Supporting the former, in combination with the oncolytic adenovirus hTERT-Ad, bortezomib enhanced infection-induced ER-stress and activated the UPR and UPR-associated apoptotic cell death *in vitro* (63). In subcutaneous hepatocellular carcinoma (HCC) mouse models, bortezomib refocused the immune response toward tumor-associated antigens by inhibiting immune recognition of the virus. This allowed for a reduction in viral dose in the combination therapy while maintaining similar efficacy. It was further demonstrated that bortezomib's efficacy is dependent upon a functional CD8+ T-cell response, as no response was seen *in vivo* upon depletion of CD8+ T-cells.

MITOXANTRONE

Mitoxantrone is a type II topoisomerase inhibitor and a DNA intercalating agent. Thus, it disrupts DNA synthesis and DNA repair in both healthy cells and cancer cells (64). Mitoxantrone was initially developed for treatment of cancer and has been notably approved to treat leukemia and prostate cancer. However, due to its immunosuppressive effects, mitoxantrone was also approved for the treatment of multiple sclerosis over a decade ago. Similar to other immunosuppressive chemotherapies, its activity can be attributed to its effects on proliferating immune cells, but it also has additional effects on antigen-presenting cells and enhances suppressor T-cell functions. Mitoxantrone treatment notably reduces the secretion of pro-inflammatory cytokines such as IL-2, interferon-γ (IFN-γ), and tumor necrosis factor alpha (65–68). This drug has been tested in combination with oncolytic HSV-1 in syngeneic murine breast tumor models (69) but only *in vitro* with adenovirus in prostate cancer cells (70–72). In the case of the HSV-1 ICP0 null OV KM100, mitoxantrone was found to induce immunogenic cell death and whereas no enhanced cell killing was observed *in vitro*, the combination treatment improved survival compared to single treatments in a Her2/neu TUBO-derived syngeneic murine tumor model. This effect was associated with increased intratumoral infiltration of neutrophils and tumor antigen-specific CD8+ T-cells. It was also observed that CD8+ and CD4+ T-cells as well as Ly6G+ neutrophils were important in mediating the improved anti-tumor efficacy.

IRINOTECAN

Irinotecan or more accurately its active metabolite SN-38 inhibits topoisomerase I leading to a blockade in DNA replication and transcription. It is mainly used in colon cancer as part of a regimen known as FOLFIRI, which also includes folinic acid and 5-fluorouracil. This course of therapy has been found to reduce the number of Tregs in colorectal cancer patients with minimal impact on total lymphocyte and CD4+ T-cells counts (73). Few studies

have used irinotecan in combination with OVs *in vivo*. One study showed that HSV-1 expressing CYP2B1, which converts irinotecan into SN-38, leads to improved survival in combination with irinotecan as compared to virus or drug alone in an immunodeficient mouse glioma model (74). While potential immunological effects were not assessed, a likely contributor to the effect of combination therapy is the increased conversion of irinotecan to active SN-38 due to the expression of CYP2B1 by the virus. Another study used oncolytic Sindbis to treat immunodeficient mice bearing human ovarian tumors (75). In this model irinotecan improved the oncolytic efficacy of Sindbis and this effect required NK cells.

TEMOZOLOMIDE

Temozolomide (TMZ) is an alkylating agent that leads to alkylation/methylation of DNA and has demonstrated clinical benefits in patients with glioblastoma (GBM) (76) and advanced metastatic melanoma (77). At higher doses, TMZ can be myeloablative and in these conditions, CD4+ and CD8+ T-cells, as well as Tregs are markedly reduced. Vaccination using an anti-tumor peptide vaccine following TMZ-induced myeloablation leads to improved CD8+ T-cell anti-tumor responses and prolongs survival in a murine model of established intracerebral tumors (78). However, Treg depletion has also been observed following low-dose TMZ in rats (79). Oncolytic adenovirus (80–83) and HSV (84, 85) have been tested *in vivo* in combination with TMZ, albeit immune effects have not been systematically explored. In one study with Ad5/3-D24-GM-CSF ± low-dose CPA (to reduce Tregs), treatment with TMZ increased tumor cell autophagy, anti-tumor immunity, and ultimately reduced tumor burden in murine models of xenogeneic prostate cancer (82). When used in chemotherapy-refractory patients, adenovirus infusion followed by TMZ treatment was found to increase tumor-specific T-cells and immunogenic cell death as well as overall survival compared to adenovirus treatment alone.

SUNITINIB

Sunitinib is an oral, small-molecule, and multi-targeted receptor tyrosine kinase (RTK) inhibitor that was approved by the FDA for the treatment of metastatic renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GIST) in 2006. Since then it has also been approved for use in neuroendocrine pancreatic cancer. Sunitinib inhibits cellular signaling by targeting multiple RTKs. These include platelet-derived growth factor receptors (PDGF-R) and vascular endothelial growth factor receptors (VEGF-R). Sunitinib also inhibits KIT (CD117), the RTK that drives the majority of GISTs. In addition, sunitinib inhibits other RTKs including RET, CSF-1R, and FLT3. Sunitinib has been recently shown to have additional off-target effects that block effector proteins of the IFN signaling pathway such as RNaseL and PKR (86).

Sunitinib has been evaluated in combination with VSV (87, 88), reovirus (87), and vaccinia virus (89). In the context of VSV oncovirotherapy, sunitinib decreased phosphorylation of the PKR substrate eIF2-α, leading to increased viral titers *in vitro*. Quite remarkably, combination therapy resulted in complete and sustained tumor regression in several immunodeficient and immunocompetent mouse tumor models (88). However, sunitinib may have additional effects on the infectivity of tumor vasculature. One study used sunitinib to transiently inhibit VEGF signaling, creating a "VEGF burst" upon treatment recovery. In combination with oncolytic VSV and reovirus, this led to increased viral infection and endothelial cell lysis as well as virus spread from blood vessels to cancerous tissues (87). A recent study looked at the combined effect of sunitinib and oncolytic vaccinia virus in syngeneic kidney and breast cancer mouse models, and found the combined treatment led to the most dramatic tumor reduction. Infection of tumors with oncolytic vaccinia as a monotherapy led to decreased VEGF expression (89), in line with the observation that vaccinia induces tumor vascular shutdown in both murine tumor models and in patients (90–92). Thereby, the combination effect in this study was attributed to enhanced tumor devascularization, although other potential effects of sunitinib on the cellular anti-viral response cannot be ruled out.

DRUGS THAT EPIGENETICALLY REPROGRAM IMMUNE RESPONSES TO ENHANCE OV THERAPY

Epigenetic changes in gene regulation and expression can lead to phenotypic heterogeneity in genetically identical cell populations. Through reversible modifications to DNA and chromatin structures by enzymes targeting DNA, histones, and the distribution pattern of nucleosomes, the ability of transcriptional factors to access their respective promoters can be deeply altered (93). Not surprisingly, many enzymes that are involved in epigenetic regulation are deregulated in cancer and manipulation of the cancer epigenome using small molecules has been explored successfully as a treatment modality for cancer. As will be discussed in the following sub-sections, modification of the cancer epigenome has also proven beneficial to improve oncolytic virotherapy through effects on the cellular anti-viral response, the anti-tumor immune response, and even viral gene expression [for a more extensive review, refer to Ref. (1)].

HDAC INHIBITORS

Transformed cells often have defective IFN signaling pathways due to the cytokine's ability to suppress cellular proliferation and stimulate immune responses, both of which cancer cells must bypass in order to evolve to full-blown malignancies (94–96). Indeed, it has been estimated that roughly three quarters of tumor cell lines within the NCI60 panel have defective IFN responses (97). Numerous reports have attributed dysfunctional IFN pathways in tumors to epigenetic silencing including DNA promoter hypermethylation and transcriptionally suppressive histone modifications [reviewed in Ref. (1)]. The extent to which interferon-stimulated genes (ISGs), the effector arsenal of the IFN-mediated anti-viral response, are epigenetically silenced can lead to differences in the sensitivity to virus infection (98–102). Importantly, transcriptional activation of ISGs has been shown to require histone deacetylase (HDAC) activity (103), which has spawned the evaluation of HDAC inhibitors (HDIs) in combination with several OVs.

HDAC inhibitors including valproic acid (VPA), trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and MS-275 have all been used in the context of OV therapy to effectively "reprogram" IFN-responsive tumors to become permissive to OV infection. HDIs such as VPA and TSA were found to enhance

HSV oncolysis in oral squamous carcinoma cells (SCC) (104) and glioma tumors (105–107). In one report, this was attributed to an inhibition of virally induced ISG expression, even in the presence of exogenously added IFNβ (106). The result of HDI/HSV combination therapy led to prolonged survival in several murine tumor models (105, 106). TSA also enhanced the oncolytic capacity of vaccinia virus, where the two agents synergistically increased cell killing *in vitro* in several cancer cell lines and the combination therapy led to improved survival responses in syngeneic lung metastasis and subcutaneous colorectal carcinoma mouse models (108).

Similarly, MS-275 (entinostat), SAHA (vorinostat), and other HDIs robustly sensitized resistant cells to VSV-mediated oncolysis by suppressing transcription of IFNβ and ISGs, increasing viral titers, and increasing cancer cell death. This potent synergy was cancer cell-specific and led to delayed tumor progression in xenograft models and improved viral spread within tumors in a syngeneic metastatic breast cancer model (99). While only evaluated *in vitro* in this study, HDI treatment of several cancer cell lines increased spreading of vaccinia and Semliki Forest viruses as well. This activity was ultimately linked to HDI-elicited dampening of the response to IFN (99).

In addition to the effects of HDIs on the response to IFN, evidence suggests HDIs can have additional immuno-modulatory properties. Particularly striking effects of HDIs have been observed in the context of a heterologous oncolytic prime-boost strategy, where mice with syngeneic B16 melanoma brain tumors were first primed with an oncolytic adenovirus expressing the tumor-associated antigen dopachrome tautomerase (hDCT, overexpressed in B16) then treated with oncolytic VSV expressing hDCT. MS-275 given along with VSV-hDCT potentiated the anti-tumor response to hDCT while suppressing the adaptive anti-viral response, ultimately redirecting the immune response toward the tumor. As a result, efficacy was dramatically improved, where the majority of mice given MS-275 in the prime-boost regime experienced long-lasting (>200 day) cures, compared to 100% mortality before day 50 in the mice given the same therapy minus MS-275 (109). In this study, it was also shown that MS-275 reduced virus neutralizing antibodies and memory CD8+ T-cells while maintaining prime-induced levels of humoral and cellular immunity against the tumor antigen (109).

5-AZA

DNA methylation and histone modifications are highly interdependent epigenetic processes (110). In addition to histone acetylation-mediated gene silencing, ISGs and other genes implicit in the IFN-mediated anti-viral response are often silenced in cancers by DNA hypermethylation at CpG islands in their promoter region [reviewed in Ref. (1)]. In addition to cellular genes, viral genomes can also be susceptible to direct epigenetic silencing. For example, oncolytic HSV rQNestin34.5 is transcriptionally silenced upon infection of glioma cells, due to increased DNA methylation levels at the virally encoded mammalian Nestin promoter (111). As such, some groups have investigated using OVs in combination with 5-AZA-2′-deoxycytidine (5-AZA): a DNA methyltransferase inhibitor that prevents DNA methylation and allows silenced DNA to regain accessibility to transcription factors. In the case of oncolytic HSV rQNestin34.5, treatment with 5-AZA was sufficient to de-repress transcription under control of the Nestin promoter, allowing viral gene expression, increased viral replication, and HSV-mediated glioma cell killing. This translated to increased survival in glioma bearing mice treated with both 5-AZA and the OV, compared to either treatment administered alone (111). However, it is interesting to mention that in the same study, VPA an HDAC inhibitor was sufficient to drive down DNA methylation at the Nestin promoter *in vitro* in infected glioma cells, highlighting the closely interrelated impact of DNA methylation and histone modification (111).

PI3K/Akt/mTOR PATHWAY INHIBITORS

The phosphoinositide 3-kinase (PI3K) pathway is critical to cell survival/apoptosis signaling in response to stress. Genetic mutations in the P13K pathway frequently occur in cancers resulting in dysfunctional apoptotic responses and pro-survival signaling (112). Various growth hormones and stress signals including IFN-α activate PI3K, which triggers a signaling cascade leading to Akt phosphorylation (112, 113). This activates the kinase, which then phosphorylates a number of cellular factors involved in cell survival and proliferation such as NF-κB, which is also involved in inducing the type I IFN cascade.

Several PI3K pathway inhibitors including GDC-0941 and NVP-BEZ235 are currently being clinically evaluated for the treatment of cancer (114). Both GDC-0941 and LY294002, a common PI3K inhibitor chemical probe, inhibit PI3K activity via competitive inhibition of an ATP binding site on the p85α subunit (115). The PI3K inhibitors LY294002, GDC-0941, BEZ235, as well as the Akt inhibitor tricibine, acted synergistically with oncolytic HSV MG18L to induce apoptosis in glioma cell lines *in vitro* in a cancer cell-specific manner. Remarkably, combination therapy resulted in durable cures in mice bearing glioblastoma multiforme (GBM) tumors, surpassing the efficacy of either therapy administered alone (116). Recent findings also indicated LY294002 increased killing of multiple myeloma cells *in vitro* triggered by the oncolytic adenovirus ZD55-TRAIL (117).

Mammalian target of rapamycin (mTOR), a master regulator of cellular translation, is downstream of PI3K and Akt signaling. Indeed, both GDC-0941 and NVP-BEZ235, a PI3K inhibitor developed by Novartis, have been reported to inhibit mTOR as well as PI3K (114). While mTOR controls translation of a host of cellular mRNAs and can also impact translation of viral proteins, evidence suggests it can control the anti-viral response by regulating translation of IFN and other key mediators of the anti-viral response such as IRF-7 (118). The mTOR inhibitor rapamycin, a well-known immunosuppressant, has been tested in combination with several OVs including oncolytic adenovirus (119, 120), HSV (121), VSV (122), and myxoma (123, 124). Treatment with rapamycin or closely related mTOR inhibitors such as everolimus (RAD001) has been reported to suppress the adaptive immune response to OVs by reducing levels of antibodies generated against the viruses (120), improving OV activity in several rodent models of cancer (119–121). In one study, enhancement of OV activity was also observed *in vitro* following treatment with rapamycin (121). This may be due to the impact of rapamycin on the IFN response as determined from another study where rapamycin was shown to reduce levels of VSV-induced IFN in rats, improving VSV efficacy

in an aggressive rat glioma model (122). Interestingly, oncolytic myxoma is enhanced by rapamycin in normally resistant human tumor cells *in vitro*; however, the mechanism by which this occurs is thought to be due to rapamycin-induced increases in Akt kinase levels optimal for sustaining myxoma replication (123).

OTHER PROMISING IMMUNO-MODULATORY OV-ENHANCING DRUGS

NOVEL VIRAL SENSITIZERS

The paragraphs above have shown countless examples of empirically or rationally selected combination therapeutic approaches aiming to improve the activity of OVs using well-characterized chemotherapeutics and signaling pathway inhibitors. A high-throughput screen was performed in an effort to expand this approach in an unbiased manner to identify previously uncharacterized small molecules that enhance OV activity. This screen was performed using oncolytic VSVΔM51 in the resistant murine breast cancer cell line 4T1 (125). Several molecules were identified as novel "viral sensitizers" (VSes) that were capable of boosting VSV replication and spread *in vitro*. One of these compounds, VSe1, boosted VSVΔM51 replication by up to 1000-fold, and was found to synergistically increase tumor cell killing. The mode of action of VSe1 is not fully understood but at a minimum it involves disruption of the IFN response. More specifically, ISGs typically triggered upon VSV infection remained silenced in cells pre-treated with VSe1 (125). When used as a combination therapy to treat an aggressive mouse colon carcinoma model refractory to VSVΔM51, VSe1 potentiated OV activity leading to delayed tumor progression in the context of the combination treatment, while either VSVΔM51 or VSe1 alone had no appreciable anti-cancer effects (125).

TRIPTOLIDE

Triptolide (TPL) is a naturally derived component of the Chinese herb *Tripterygium wilfordii* and has been used for centuries as an anti-inflammatory remedy that has also been found to have anti-cancer properties (126–128). TPL is known to be a global transcription inhibitor and has multiple effects including the inhibition of RNA polymerase II and the expression of genes involved in apoptosis and NFκB signaling (129). A recent report found that TPL also suppresses IFN signaling downstream of IRF3 (130). When combined with oncolytic VSV both *in vitro* in VSV-resistant tumor cells and *in vivo* in an aggressive mouse GBM tumor model, the two therapies synergistically improved tumor-specific virus replication leading prolonged survival and delayed tumor progression compared to either therapy given alone (130).

JAK KINASE INHIBITORS

Ruxolitinib (Jakafi) is a Jak1/2 kinase inhibitor (131) approved in 2011 for the treatment of myelofibrosis (132). Patients with myeloproliferative neoplasms often possess an activating mutation in the gene encoding *Jak2* (133), resulting in aberrant inflammatory cytokine release and splenomegaly. Treatment with ruxolitinib, while not targeting the genetic determinant of the neoplasm, led to profound resolution of severe symptoms in human trials to treat myelofibrosis (splenomegaly, weight loss, fatigue), and this clinical efficacy was associated with a potent reduction in inflammatory cytokine levels (134). Given that Jak1 is required for type I IFN signaling and induction of ISGs, Jak1 inhibitors have the potential to benefit OV therapy in IFN-responsive tumors. Both ruxolitinib and Jak inhibitor 1 were sufficient to sensitize VSV-resistant squamous cell carcinoma cells *in vitro* to VSV infection, and this sensitization was associated with marked decreases in ISG expression (135). Pre-treatment with the Jak inhibitor 1 also sensitized sarcoma and bladder carcinoma cells to VSV infection *in vitro* (136).

CHECKPOINT INHIBITORS

Targeting T-cell inhibitory check point molecules, including the T-cell inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programed cell death 1 (PD1), is a relatively new therapeutic approach to cancer therapy. During normal immune responses, T-cell checkpoint receptors such as PD1 and CTLA-4 prevent overactive T-cell responses, which can lead to harmful tissue damage. However in cancers, tumor infiltrating T-cells are often inhibited by both PD1 and CTLA-4 stimulation. As a result, T-cell anergy is a major barrier to immune-mediated tumor recognition and clearance. Given the ability of OVs to stimulate an anti-tumor immune response, combining OV with checkpoint inhibitors has emerged as a logical combination approach. While several groups are currently working on this approach, published studies to date have focused on ipilimumab, an anti-CTLA-4 antibody approved to treat melanoma in 2011. By targeting CTLA-4, ipilimumab blocks interaction with its ligands, CD80/CD86, leading to increases in T-cell mediated anti-tumor responses. Anti-CTLA-4 antibodies have been used in combination with oncolytic parvovirus *in vitro* (137) and Newcastle disease virus (NDV) *in vivo* to treat murine B16 melanoma (138). Remarkably, the combination therapy of NDV and anti-CTLA-4 led to nearly 70% cures in a B16 melanoma mouse model compared to 20% cures for anti-CTLA-4 antibody alone and no effect of the OV on its own (138). Notably, NDV complemented with anti-CTLA-4 led to an increase in the infiltration of activated CD8+ and CD4+ T-cells and a reduction in Tregs.

CONCLUSION

Successful therapy using OVs will ultimately depend on effectively navigating the delicate balance between the anti-viral response and the anti-tumor immune response such as to minimize the former in the short term and maximize the latter in the long term. As outlined above, several approved drugs and novel small molecules can be effective tools to dampen the innate and adaptive anti-viral responses, increase the anti-tumor immune response, or both. However, given the close interplay between the cellular antiviral response and the adaptive immune response that is required for prolonged tumor control, OV/drug scheduling is likely to be critical. To this end, it is probable that the combination of some of the agents described above may allow for additional flexibility and more effective therapy. For example, one can easily foresee first using a drug that specifically dampens the cellular antiviral to permit robust OV replication followed with another that promotes the generation of an anti-tumor response. However, given the efficacy of each approach is undoubtedly both context-dependent (e.g., tumor type and tumor site) and OV-dependent, more preclinical and clinical studies will be necessary to identify winning

combinations that can maximize the potential for curing cancers in a clinical context.

While many studies demonstrate therapeutic benefit of combination therapies at least in animal models, we can perceive a deficit in regards to systematic head-to-head comparisons of different combination therapies coupling OVs and the immune-modulatory drugs reviewed above. While such a feat may prove daunting experimentally, this exercise seems warranted and necessary to delineate a more educated choice of combination therapies to push forward into clinical trials. One clear trend overall is that evaluation of promising combination therapies with novel immuno-modulatory agents seems to stop at the pre-clinical level. There are likely several factors that contribute to this. For example, companies developing novel small molecules may be reluctant to explore combinations with OVs that are still relatively novel themselves. Similarly, novel small molecules need to be validated clinically, which complicates clinical trial design and adds additional risk from the perspective of those spearheading clinical translation of OVs. This is particularly challenging for novel small molecules such as VSe1, which have been selected for the sole purpose of enhancing OV activity (125). This type of small-molecule/OV co-development can only be reasonably achieved by pharmaceutical companies that have experience in developing both small-molecule and biological therapies separately. Hence, from a clinical perspective, it is likely that the combination of OV therapy with a chemotherapy drug that is part of current standard of care would be the easiest to implement as demonstrated with the combination of oncolytic adenovirus and CPA (27). With promising results emerging from the clinic showing benefits combining OVs with traditional chemotherapy drugs, and as pharmaceutical companies such as Amgen begin to take heed of the potential of OV therapy for the treatment of cancer, clinical evaluation of some of the more novel OV-synergizing compounds seems likely in the near future as a means to overcome heterogeneity in clinical response.

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Chemotherapy and oncolytic virotherapy: advanced tactics in the war against cancer

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Cancer is a traitorous archenemy that threatens our survival. Its ability to evade detection and adapt to various cancer therapies means that it is a moving target that becomes increasingly difficult to attack. Through technological advancements, we have developed sophisticated weapons to fight off tumor growth and invasion. However, if we are to stand a chance in this war against cancer, advanced tactics will be required to maximize the use of our available resources. Oncolytic viruses (OVs) are multi-functional cancer-fighters that can be engineered to suit many different strategies; in particular, their retooling can facilitate increased capacity for direct tumor killing (oncolytic virotherapy) and elicit adaptive antitumor immune responses (oncolytic immunotherapy). However, administration of these modified OVs alone, rarely induces successful regression of established tumors. This may be attributed to host antiviral immunity that acts to eliminate viral particles, as well as the capacity for tumors to adapt to therapeutic selective pressure. It has been shown that various chemotherapeutic drugs with distinct functional properties can potentiate the antitumor efficacy of OVs. In this review, we summarize the chemotherapeutic combinatorial strategies used to optimize virally induced destruction of tumors. With a particular focus on pharmaceutical immunomodulators, we discuss how specific therapeutic contexts may alter the effects of these synergistic combinations and their implications for future clinical use.

Keywords: oncolytic virotherapy, cancer immunotherapy, cancer vaccines, combination therapy, drug therapy, combination, oncolytic viruses

Do not repeat the tactics, which have gained you one victory, but let your methods be regulated by the infinite variety of circumstances.

–Sun Tzu, The Art of War

INTRODUCTION

Oncolytic viruses (OVs) can selectively infect, replicate in, and kill tumor cells with minimal impact on normal tissue. These tumor-specific properties, called oncotropism, is dependent on the expression of surface receptors that allow viral binding and entry, as well as, the permissiveness of the tumor cell toward viral replication. Genetic manipulation of the viral genome aims to improve the inherent therapeutic value of OVs by enhancing their capacity for targeted tumor killing (1, 2). Through transgene insertion, OVs can serve as directed gene-delivery vehicles, and thus accommodate a diverse array of therapeutic strategies. Arming OVs with additional weaponry, such as pro-apoptotic genes, tumor suppressors, or genes stimulating antitumor immunity, can enhance their killing capacity. With a broad arsenal, modified-OVs have the potential to target a wide spectrum of different cancer types. However, administration of OVs as a monotherapy has demonstrated varying degrees of success in clinical trials (3–5). This is likely due to host antiviral immune-mediated mechanisms that limit OV dissemination and promotes pre-mature viral clearance. Over an extended period, selective pressure on heterogeneous tumor populations can also lead to therapeutic resistance to OVs via receptor loss or mutation of essential signaling pathways required for viral replication (6). To overcome these barriers, many clinically established and novel chemotherapeutics have been used in combination with oncolytic virotherapy, showing synergistic effects that potentiate tumor killing (7–9). In this review, we summarize how immunomodulatory chemotherapeutic combinatorial strategies have been used to optimize virally induced destruction of tumors and discuss their implications for future directions and clinical use.

MECHANISMS OF ONCOLYTIC VIRUSES

TUMOR TROPISM AND ONCOLYSIS

The oncotropism of viruses is guided by cell surface receptors that enable viral binding and entry, and the permissiveness of the infected cell to viral replication. Surface receptors that are recognized by different types of viruses can be specific to neoplastic cells. These viruses target receptors characteristic of malignant phenotypes, such as Poliovirus that binds CD-155 that is almost exclusively present in high grade glioma cells (10, 11), and Sindbis virus that recognizes high-affinity laminin receptor overexpressed in many cancers (12). Other viruses, such as vesicular stomatitis virus (VSV) exhibit a remarkably robust and pantropic selectivity by binding to the ubiquitously expressed LDL receptor (13). Therefore, instead of relying on receptor specificity, tumor tropism of VSV is dependent on the permissiveness of malignant cells to viral infection. VSV belongs to a class of interferon (IFN)-sensitive

viruses, which preferentially infects tissues exhibiting reduced or absent IFN responsiveness (14–17). This is a typical feature of tumors, which often acquire defects in pathways involved in innate antiviral immunity, such as the IFN pathway, as a mechanism for immune escape. In fact, many of the biological pathways altered by viral infection are similar to cellular changes acquired during carcinogenesis. For instance, mutated oncogenes such as BRAF or Cyclin A, increases the infectivity of VSV and parvovirus, respectively (18, 19). As well, impaired apoptotic ability typically observed in neoplastic cells provides an opportunity for OVs to enhance their replicative capacity (20).

Selective retargeting of viruses to tumor cells can also be generated in viruses without innate oncolytic abilities. Adenovirus (Ad)-based vectors are a good demonstration of this approach, since they possess a wide tropism, but a lytic life cycle that can be exploited for oncolytic virotherapy (21). One method to restrict viral replication to tumor cells is the modification of E1A and E1B genes that results in conditionally replicative Ad. As a result, selective replication occurs in cells defective in p53 or Rb tumor suppressor pathways; a characteristic observed in 50% of human cancers (22). Alternatively, various transductional retargeting strategies exist that largely involve fusing tumor targeting ligands to the Ad fiber knob domain, summarized in Ref. (23).

Viral oncolysis directly destroys tumor cells through either their lytic replication cycle or the expression of endogenous cytotoxic gene products (24). To further enhance their oncolytic effects, transgenes encoding pro-apoptotic proteins are inserted into OVs to subvert cell death machinery. These proteins include various death-inducing ligands such as TNF-related apoptosis-inducing ligand (TRAIL) (25, 26), Fas ligand (FasL) (27), and tumor suppressor genes (e.g., p53, p16) (28, 29). Alternatively, small hairpin RNA targeting factors can be inserted to silence genes involved in cell survival or proliferation, including hTERT and ki67 (30) or MYCN oncogene (31). Oncolytic viral infection can also induce autophagy, a conserved catabolic process crucial in maintaining cellular homeostasis (32). Cellular autophagy machinery is disrupted by certain viruses to facilitate its own replication (33, 34) and enhance oncolysis (35, 36). By engineering viruses to express autophagy-inducing genes, such as Beclin-1 (37) and mTOR pathway regulators (38, 39), improved therapeutic outcomes can be achieved. This approach may be particularly useful for treating apoptosis-resistant types of cancer, thus warranting further development toward clinical application. Lastly, some OVs can exert indirect mechanisms of tumor killing, including tumor vascular shutdown (40, 41) and the induction of antitumor immune responses, the latter of which is described in further detail in the following section.

INDUCTION OF ANTITUMOR IMMUNE RESPONSES

The various mechanisms through which OVs are capable of lysing cancer cells result in the release of tumor associated antigens (TAAs), proinflammatory cytokines, chemokines, and other danger signals, which facilitates immune cell recruitment and activation within tumors. In particular, activation and maturation of dendritic cells (DCs) and other antigen presenting cells (APCs) allow for efficient cross-presentation to T cells, and subsequent initiation of antitumor and antiviral immune responses (42, 43). However, OVs induce only weak tumor-specific immune responses, due to premature viral clearance and immunosuppressive regulatory factors within the tumor.

To potentiate their immunogenic effects, genetic engineering strategies have been used to encode OVs with various cytokines, immunomodulators, and TAAs (44, 45). Evaluation of the antitumor efficacy of OVs expressing cytokines, such as IL-12, IL-2, IL-4, IL-18, IL-24, and TNFα, has shown improved therapeutic effects (46–49). One of the most promising cytokines tested within the OV platform to date, is the granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes DC maturation and induces tumor antigen-specific cytotoxic T cells. Three major viral vectors, Ad, VV, and HSV, armed with GM-CSF have been demonstrated to enhance antitumor immunity and cytotoxicity in several clinical trials (50–57). In particular, Talimogene laherparepvec (T-VEC), a GM-CSF-expressing oHSV-1 that has recently completed phase III trials in melanoma and head and neck cancer, are the first to demonstrate efficacy of OV immunotherapy, with an approximately 30% response rate against systemic disease, following local injection into accessible tumors (52, 53). Similar to GM-CSF, Fms-like tyrosine kinase-3 ligand (FLT3L) is a potent growth factor capable of recruiting and expanding DCs *in vivo* (58). OVs expressing FLT3L trigger DC and T cell infiltration into the tumor and enhance both antitumoral and antiviral immune responses (42, 59, 60), implicating potential benefits of using FLT3L as an adjuvant to cancer vaccination. Another strategy to boost the antitumor response involves genetically engineering OVs to express inflammatory chemokines, and thus increasing the number of tumor-infiltrating immune cells. Expression of CCL5, CCL3, and CCL19 by OVs enhances chemotaxis of immune cells within the tumor and improves overall therapeutic benefits *in vivo* (61–64). Interestingly, distinct effects on virus activity were also observed, in which VV expressing CCL5 or CCL19 resulted in increased persistence within the tumor and more rapid clearance from non-tumor tissues, respectively (61, 65, 66). Finally, cross-presentation of TAA to T cells through DC activation can also be achieved by arming OVs with co-stimulatory molecules such as CD40L (67, 68) and heat shock proteins (69).

A more direct approach to engage antigen-specific T cells is to engineer OVs to express TAAs, termed oncolytic vaccines (70). As such, TAAs are overexpressed in the tumor during viral replication, thus increasing the opportunity for immune responses to be generated toward tumor-specific antigens. However, successful antitumor activity has only been reported using model tumor antigens such as OVA or LacZ (71, 72) and the same approach was poorly effective against a self-TAA of low immunogenicity (70, 73). Altogether, these results suggest that overexpression of a TAA is insufficient to overcome immunosuppression in the tumor or immunodominant responses against viral antigens. Therefore, additional approaches are required to boost TAA-specific responses beyond these barriers. Indeed, significantly improved therapeutic efficacy can be achieved by adoptive transfer of TAA-specific transgenic T cells (74) or priming the host with a heterologous vector expressing the TAA (70), prior to oncolytic vaccination. Both approaches have been demonstrated to increase TAA-specific T cell frequency, by redirecting the focus of immune responses to the TAA, rather than the viral vector. Such

OV-based cancer immunotherapies show promise by harnessing both oncolytic and antitumor immune-mediated attacks. Clinical evaluation of adoptive T cell transfer and OVs are currently underway as monotherapies (4, 75), however their success as a combination therapy has yet to be determined in human cancers.

CHALLENGES OF ONCOLYTIC VIRUS MONOTHERAPY

Oncolytic viruses as a standalone therapeutic intervention have rarely been shown to induce complete, long-term regression of established tumors *in vivo* (76, 77). Tumors can develop multiple barriers to various anticancer therapies, including oncolytic virotherapy. Here, we detail several mechanisms that may hinder the therapeutic efficacy of OVs and the challenges they pose to the development of improved cancer virotherapies.

IMMUNOLOGICAL BARRIERS

The first line of defense against viral infection is the innate immune cells that patrol, detect, and rapidly eliminate foreign invaders. DCs express pattern recognition receptors that allow for the detection and subsequent uptake of viral particles. These activated DCs then migrate to draining lymph nodes to initiate the development of adaptive immune responses and to trigger NK cell activation. NK cells have a predominant role in impeding the early spread of viruses by directly lysing virally infected cells. Together, DCs and NK cells produce a range of cytokines that promotes T helper 1 (Th1) cell activity and potent cytotoxic T lymphocyte (CTL) responses that are necessary for clearing virus-infected cells (78). Additionally, humoral immune responses, namely the production of neutralizing antibodies by B cells and plasma cells, provide several lines of antiviral defense (79). Plasma cells derived from B1 cells imparts early defense against viral infection by producing polyspecific antibodies. $CD4^+$ T helper cells then stimulate naive B cells at later stages, in order to generate memory B cells and long-lived plasma cells that produce high amounts of specific neutralizing IgG antibodies. Finally, the complement system, composed of soluble factors and cell surface receptors, blocks viral infection by acting on both the innate and adaptive immune responses. These mechanisms include, enhancing humoral immunity, regulating antibody effector mechanisms, and modulating T cell function (80).

Altogether, these immunological barriers pose a particular problem for repeat administration of OVs, by further promoting the development of adaptive antiviral immunity and reducing of its oncolytic effects. Moreover, a large fraction of the population has previously been exposed to the naturally occurring viruses that are commonly employed for generating therapeutic strains. Therefore, the infectious potential of recognized OVs (e.g., Ad, HSV) becomes limited by high levels of neutralizing antibodies (81, 82). These circulating antibodies can limit viruses from ever reaching the tumor site, especially since some viral particles, including HSV-1- and murine leukemia virus-derived viruses, are particularly prone to inactivation by the complement system (83, 84).

TUMOR ENVIRONMENT

Tumors are a heterogeneous assortment of cells, composed of cancer cells, stromal cells, and infiltrating leukocytes, which promote tumor growth and maintain an immunosuppressive environment (85). Tumor-infiltrating leukocytes (TILs) can negatively regulate immune responses within the tumor, which include regulatory T cells (Tregs), myeloid derived suppressor cells (MDSC), and type 2 macrophages (M2). Their immunosuppressive functions can be exerted by secretion of cytokines (e.g., IL-10 and TGF-β), through inhibitory receptors (e.g., CTLA-4 and PD-L1) via cell contact, and secretion of amino-acid depleting enzymes (arginase and IDO) in the tumor microenvironment. Tumor cells themselves also have mechanisms to suppress antitumor immunity, such as the shedding of NKG2D ligands, MICA/B that blocks NK cell and T cell function (86) and facilitates the expansion of immunosuppressive $CD4^+$ T cells (87). Soluble mediators released by tumor cells can directly inhibit CTLs, which include TGFβ, IL-10, PGE_2, histamine, hydrogen peroxide, and adenosine (88), in addition to the hypoxic conditions and low extracellular pH that characterize the tumor environment (89, 90). Therefore, antitumor immune responses induced by modified-OVs may not be sufficient to combat a highly immunosuppressive tumor environment, unless additional therapeutic regimens are employed.

Preclinical and clinical evidence indicates that OVs often infect neoplastic lesions in a heterogeneous and incomplete fashion, irrespective of administration route and whether viruses are replication-competent or not (91–93). Physicochemical barriers to infection, including tumor size (94), the layers of dense intratumoral connective tissue (95), the elevated interstitial pressure (96), the poorly permissive vasculature (97), and the large areas of necrosis/calcification (98) play a prominent role in determining viral dissemination. As a result, oncolytic virotherapy may result in incomplete eradication of the primary tumor mass or possibly even promote metastasis of the tumor cells and eventually leading to recurrence of disease. Similar to what is observed in chemotherapy and radiotherapy regimens, malignant cells are also prone to become resistant to oncolytic virotherapy over time. This is presumably linked to the intrinsic nature of cancers to exhibit genomic instability and the propensity for accumulating mutations (99–101).

COMBINING IMMUNOMODULATORY CHEMOTHERAPY WITH ONCOLYTIC VIROTHERAPY

Chemotherapeutic drugs used in combination with OVs can potentiate their cytotoxic mechanisms (9), but may also act to remove barriers to successful oncolytic virotherapy. Counteracting immunological barriers can improve the persistence of viruses and/or weaken the immunosuppressive forces within the tumor microenvironment. In this section, we summarize how pharmaceutical immunomodulators may be used to promote adaptive antitumor immune responses induced by OVs.

EVADING ANTIVIRAL IMMUNE RESPONSES

Histone-deacetylase inhibitors (HDACi) are anti-inflammatory agents that can modulate immune responses to viral infection. By impeding the type I IFN response, a major component of the cellular innate antiviral response, HDACi's can enhance the spread and antitumor effects of OVs (102). In addition, HDACi's may also enhance OV efficacy through initial suppression of immune cell recruitment and inhibition of inflammatory cell pathways within NK cells (65). Similarly, a high throughput

screen of pharmaceutical agents identified a novel drug (Vse1) that could enhance oncolytic virotherapy by disrupting the IFN-induced antiviral response and repressing antiviral gene transcripts (103). Another drug that can be used for immune suppression is cyclosphorine A, which markedly increased and prolonged the therapeutic effect of reovirus therapy of metastatic cancer (104, 105). However, the most common immunosuppressant drug used in the context of oncolytic virotherapy is cyclophosphamide (CPA); a chemotherapeutic alkylating agent that also induces apoptotic cell death. CPA has complex immune-modulating effects, affecting humoral and cellular mediators of both the innate and acquired immune responses. These immunosuppressive functions have been shown to enhance viral oncolysis and improve antitumor efficacy of HSV (83, 106, 107), Ad (108), measles virus (109), reovirus (110, 111), and VV (112). More specifically, at high doses, CPA has been shown to limit neutralizing antibody titers below the limit of detection during herpes virus hrR3 infection (106). Furthermore, *in vivo* depletion of complement significantly improved survival of HSV and CPA treated tumor-bearing rats (83). Global immunosuppression has also been reported to occur as a result of CPA therapy, including significant decreases in total white blood cell, lymphocyte, neutrophil, and monocyte counts in tumor-bearing mice. This was accompanied by significantly improved survival and decreased tumor volume in mice treated with both Ad and CPA relative to treatment with either therapy alone (108). Host lymphodepletion can enhance the therapeutic efficacy of OVs, as demonstrated by the reduction of antiviral antibody titers and subsequent promotion of viral persistence (113).

COUNTERACTING THE IMMUNOSUPPRESSIVE TUMOR ENVIRONMENT

Regulatory T cells and MDSC are TIL populations that are a major component of the immunosuppressive tumor environment. Most pharmaceutical strategies that counteract immune resistance mechanisms within the tumor are aimed at depleting these inhibitory immune cell populations. Reduction of Tregs in cancer patients has been demonstrated to occur following treatment with fludarabine and paclitaxel (118, 119). Other chemotherapeutic drugs shown to decrease Tregs and inhibit their suppressive ability include CPA, paclitaxel, and temozolomide and cisplatin treatment, which enhances antigen-specific $CD8^+$ T cells in murine tumor models (114–117). In particular, CPA, paclitaxel, and temozolomide can successfully reduce Treg activity (120–122) when delivered as metronomic doses (i.e., repetitive, low doses). In the case of CPA, metronomic doses serve to minimize toxicity and avoid global immunosuppression resulting from administering a single, high dose. Comparison of metronomic and maximum tolerated doses of CPA revealed that deletion of proliferating tumor-specific CTLs occurred in both dosing schedules. However, at metronomic doses, slower kinetics of deletion and survival of cells with a $CD43^{lo}$ "memory" phenotype was observed, resulting in potent restimulatory capacity (122). This is supported by clinical evidence, in which metronomic CPA can deplete Tregs and restore T and NK cell effector function in advanced cancer patients (123). In the context of oncolytic virotherapy, preconditioning of mice with either CPA or anti-CD25 mAb to deplete Tregs enhances therapeutic benefits of oncolytic reovirus and VSV (111, 124). Furthermore, early clinical evaluation of metronomic CPA and oncolytic Ad combination treatment demonstrates improved antitumor efficacy, resulting from increased cytotoxic T cells and induced Th1 type immunity (125).

In healthy tissues, MDSCs play a protective role during inflammation to maintain homeostasis of pathogenic immune responses. However, accumulation of MDSCs in the tumor environment is also capable of promoting tumor growth by inhibiting antitumor effector T cell responses. They exert their effects through multiple immunomodulatory roles, such as upregulating the production of immune-suppressive factors (e.g., nitric oxide and reactive oxygen species), overexpressing anti-inflammatory cytokines (e.g., TGF-β and IL-10), suppressing proliferation and cytokine production by T cells and NK cells, and inducing apoptosis of $CD8^+$ T cells (126). Furthermore, MDSCs can mediate the expansion of other immunosuppressive Treg and M2 populations (127–129). Numerous chemotherapeutic drugs have been used to deplete MDSCs, including gemcitabine, sunitinib, 5-FU, docetaxel, and retinoic acid (130–134). Combinations of OVs with various MDSC depleting drugs have been investigated at length, overall demonstrating improved survival in preclinical studies. The therapeutic benefits of using these OV-drug combinations depend on several factors, including the type of OV-drug combination used, the timing, frequency, and dosage of drug administration, and the cancer type targeted. However, given that these immunomodulatory drugs have other antitumoral effects, few studies have directly assessed their ability to deplete MDSCs in each context (135, 136). Notably, use of these drugs to deplete MDSCs can also positively or negatively affect oncolytic virotherapy. For instance, metronomic treatment of either gemcitabine or 5-Fu with oncolytic Ad, increases viral uptake by upregulating the expression of internalization receptors (137). Moreover, sunitinib negatively regulates the antiviral OAS-RNase L pathway, thus enhancing viral replication of VSV in tumors (138). In contrast, concurrent therapy of 5-Fu with HSV-1 inhibits virus replication and oncolysis (139). Therefore, optimization of these OV-drug combination strategies to benefit both the oncolytic and antitumor immune effects of OVs requires further investigation.

Given that chemotherapies have non-specific effects, some drugs can also modulate tumor cell immunogenicity to benefit oncolytic virotherapy. For example, paclitaxel can upregulate MHC class I expression and antigen-processing machinery components (140). 5′-aza-2′-deoxycytidine and 5-Fu have been shown to enhance tumor antigen expression (141–143), while Ara-C (cytosine arabinoside) treatment results in the induction of co-stimulatory molecules that provide a greater chance of effective immune activation (144, 145). Furthermore, both doxorubicin and Ara-C decreases the expression of immune checkpoint molecules, such as PD-L1, blocking their inhibitory effects on infiltrating T cells (146, 147). Some drugs, namely CPA, 5-Fu, and Dacarbazine, can sensitize tumor cells to $CD8^+$ T cell-mediated apoptosis (148, 149), and thus may serve as ideal candidates for therapeutic combinations with various cancer immunotherapies.

EVALUATING THE LANDSCAPE OF OV-DRUG COMBINATIONS

Tumor cell heterogeneity as a result of DNA instability promotes the natural selection of tumor progeny with greater proliferative

capacity and invasive potential (150, 151). As a result, treatment methods that address a singular therapeutic strategy may be insufficient to completely eliminate tumor growth. OV-drug combinatorial strategies present countless different permutations, and consequently, numerous possibilities to mobilize multiple and simultaneous therapeutic approaches. However, previous studies that report synergistic outcomes from combining OVs with chemotherapy largely focus on a single therapeutic aspect, such as their effect on viral spread and persistence, cytotoxicity, or immunomodulation. As we become more familiar with how various chemotherapeutic drugs function, it is increasingly apparent that many drugs act in a multi-mechanistic fashion. In other words, chemotherapeutic agents can impact multiple biological processes, which in turn can further potentiate OV-drug interactions. For instance, rapamycin and its analogs have been shown to alter mTOR signaling to increase the tropism of OVs (152), inhibit angiogenesis (153), induce autophagy (32), and inhibit the function of M2 macrophages (154). HDACis such as Trichostatin A alter chromatin structure and regulate gene expression on an epigenetic level, leading to a wide range of biological effects like promoting tumor antigen presentation (155), improving tumor susceptibility to OVs (156–158), down-regulating the antiviral response (159), and targeting tumors and tumor vasculature (160). Lastly, receptor tyrosine kinase inhibitor sunitinib can down-regulate antiviral pathways (138), deplete MDSCs (131), inhibit M2 macrophages (161), and reduce tumor vascularization (162). Therefore, rather than evaluating individual therapeutic strategies that are complementary to oncolytic viral activity, combinatorial strategies using chemotherapeutic drugs should take into account of their entire functional repertoire, in order to determine the best overall approach. However, given the complex, interconnected biological pathways that regulate viral infection and tumor growth, assessing OV-drug combinations is not a simple task.

CHALLENGES OF COMBINATION THERAPY

As previously mentioned, the biological pathways that OVs manipulate to support their replication are similar to those utilized by cancer cells to become increasingly malignant (e.g., defects in the IFN pathway, apoptotic-resistance, immune suppression). In fact, targeting certain pathways with chemotherapy will also, by association, compromise the replicative capacity of OVs. As a result, discernable conflicts between virus-enabled therapeutic strategies and drug-enabled therapeutic strategies may limit the extent to which the two can be combined. For example, viruses require actively dividing cells to maximize their replicative efficiency, while many anticancer agents are either cytotoxic or cytostatic with death-inducing or anti-proliferative effects, respectively (9). Furthermore, studies suggest that the leaky vasculature of tumors is exploited by viruses to successfully extravasate into the tumor site (163, 164). Some OVs can actually stimulate angiogenesis to increase vascular permeability in tumors (165). Thus, anti-angiogenic therapy may thus adversely affect the localization of OVs to the tumor microenvironment. Finally, modulation of the host immune response through chemotherapy may conflict with the therapeutic function of the oncolytic virus. For instance, low dose CPA may remove immunosuppressive cells such as Tregs to improve vaccine-induced adaptive antitumor immune responses; however, it also promotes the antiviral immune response, leading to early viral clearance (166). Conversely, high dose CPA may enhance viral oncolysis through wide-spread immunosuppression of the innate and adaptive antiviral immune response, but also completely abrogate the antitumor immune response (167). These conflicting mechanisms (apoptosis vs. viral replication, anti-angiogenesis vs. viral trafficking, antiviral immune responses vs. antitumor immune responses) are further compounded when we consider that drugs often regulate multiple biological host processes. Nevertheless, OV-drug combinations that demonstrate therapeutic incompatibility are still efficacious in some models. In these cases, it is likely that the number of beneficial interactions between OVs and drugs outweigh the number of detrimental effects, resulting in an overall enhanced therapeutic outcome. While current combinatorial strategies have been able to identify unique synergistic OV-drug platforms, the challenge going forward is to obtain a greater understanding of OV-drug interactions. Based on these exploratory findings, we will be able to identify optimal treatment conditions that minimize therapeutic trade-offs.

SUCCESSFUL COMBINATION THERAPY IS CONTEXT-DEPENDENT

As previously mentioned, seemingly incompatible OV-drug combinations have shown therapeutic efficacy because their positive effects outweigh their negative effects. Based on these initial studies, it is also apparent that some factors can tip the OV-drug dynamic in favor of enhanced cancer therapy in one context, but also have the reverse effects in another. For instance, concurrent administration of 5-FU has been shown to inhibit the replication of wild-type HSV-1 strain KOS (139); however, the same drug has been shown to actually enhance viral replication of NV1066 (HSV-1 with a single copy of ICP0, ICP4, and $\gamma_1$34.5 deleted) in pancreatic cancer cell lines (168). Interestingly, growth arrest and DNA damage as a result of 5-FU administration upregulates the expression of DNA damage-inducible protein GADD34, which bears significant homology with the deleted $\gamma_1$34.5. As a consequence, GADD34 can functionally replace $\gamma_1$34.5, prevent premature shut-off of protein synthesis, and thus enhance viral replication (169). Another factor that is demonstrated to be context-dependent is the schedule and dosage of drug delivery given during OV-drug combination therapy. However, if their costs and benefits to oncolytic virotherapy are clear, we may adjust these variables for an optimized therapeutic outcome. For example, VEGF blockade through a variety of small-molecule chemotherapeutics decreases the tumor uptake of systemic oncolytic HSV, but can actually improve the treatment of sarcoma-bearing mice if anti-angiogenic therapy is given subsequent to virus administration (170).

Overall, specific strategies to optimize OV-drug combinations depend on the circumstances of the model system. To this point, we have previously shown that systemic vaccination with recombinant VSV encoding the xenogeneic TAA, human dopachrome tautomerase (hDCT), was unable to induce robust tumor-specific immunity because the host immune response was predominantly redirected toward viral antigens expressed on the vector. Therefore, by adopting a heterologous prime-boost system whereby mice were initially primed with recombinant Ad-hDCT and boosted with VSV-hDCT, substantive immunity was generated against the

tumor, while the antiviral response to VSV was dampened (70). The HDACi, MS-275, is an ideal candidate for combination therapy with this prime-boost system because it has previously been shown to decrease IFN responsiveness in tumors, thus augmenting viral oncolysis. However, MS-275 is also immunosuppressive and resulted in abrogation of the priming response if given concurrently with Ad-hDCT. Alternatively, if drug treatment was given concurrently with VSV-hDCT, the boosting response was unaffected and over 60% of mice challenged with intracranial melanoma were cured (171). Since MS-275 is an HDACi; an epigenetic modifier that can modify the expression of numerous genes, its range of effects have not yet been fully elucidated. As such, many unknown functional properties may still exist, especially in the context of oncolytic virotherapy.

CONCLUDING REMARKS

War strategy dictates methods in which to arrange and maneuver military forces during armed conflicts. Using the available resources and landscape to your advantage is a key aspect to defeating the enemy. The analogy of OVs as fighters, "targeting" cancer cells and being "armed" with various genes, is commonplace in the literature. Its ability to induce antitumor immune responses is akin to the call for air support, bringing in additional fighters that can help to identify and target enemy forces. The introduction of chemotherapeutic drugs to the battlefield is then, chemical warfare; a wide-spread, indiscriminate weapon. With our various forces at hand, how do we determine the best strategy to defeat our opponents? As with any war strategy game, finding the best approach begins with knowing the enemy (type of cancer), knowing our forces (viruses, drugs, and immune cells), their strengths and weaknesses (function), and finally how they interact with each other on the battlefield (combination therapy). Before you make a move, you postulate various scenarios in which your opponent may attack, but also how you can take the advantage. In a similar fashion, to identify the most suitable approach to OV-drug combination therapies, we should adopt a broader perspective to the treatment of cancer. Then and only then, will we not only win some battles, but we may also win the war.

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