Molecular Therapy
Oncolytics
Review

Current strategies in engaging oncolytic viruses with antitumor immunity

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Oncolytic virotherapy has produced promising yet limited results in preclinical and clinical studies. Besides direct oncolytic activity, a significant therapeutic mechanism of oncolytic virotherapy is the induction of tumor-specific immunity. Consequently, the efficacy of oncolytic viruses can be improved by the insertion of immune stimulator genes and rational combinatorial therapy with other immunotherapies. This article reviews recent efforts on arming oncolytic viruses with a variety of immune stimulator molecules, immune cell engagers, and other immune potentiating molecules. We outline what is known about the mechanisms of action and the corresponding results. The review also discusses recent preclinical and clinical studies of combining oncolytic virotherapy with immune-checkpoint inhibitors and the role of oncolytic virotherapy in changing the tumor microenvironment.

INTRODUCTION

Nearly all cells present fragments of their endogenously synthesized proteins on major histocompatibility complex (MHC) class I molecules on the cell surface, allowing immune surveillance of the contents of each cell.[1] Cytotoxic T lymphocytes (CTLs) can recognize viral or tumor antigens via association of the T cell receptor (TCR) with MHC class I antigen complex and subsequently kill the infected or cancerous cell. CTLs, therefore, can be potent anticancer agents. However, most tumors have means of evading recognition by CTLs and/or suppressing their activity. Numerous current research strategies are under investigation, which utilize the highly versatile yet specific nature of these cells to target cancer cells.

Oncolytic viruses (OVs), whether naturally occurring or genetically engineered, specifically infect and lyse cancer cells without damage to normal cells and are currently under investigation as a therapeutic strategy to engage the immune response against cancer. Their means of selectivity vary among different types of viruses. Some OVs only enter tumor cells by engaging with surface receptors exclusively expressed or upregulated by tumor cells.[2] Others have virulence genes deleted so that they depend upon defective signaling pathways found in cancer cells to be able to replicate. Upon viral infection, normal cells inhibit viral replication by initiating apoptosis and releasing type I interferons (i.e., IFN-α and IFN-β). Type I IFNs activate neighboring cells to halt translation, disabling the production of viral proteins. Whereas viral genes have evolved to inhibit apoptotic and IFN defense mechanisms, deletion of these genes renders viruses unable to replicate in healthy cells but still able to replicate in tumor cells, in which apoptotic or IFN signaling pathways are defective.[3–5] For example, the *ICP34.5* gene found in herpesviruses enables the virus to inhibit IFN signaling, and the gene products of the E1B region found in adenoviral genomes inhibit p53 and Rb, preventing apoptosis.[5] The *ICP34.5* and *E1B* genes are deleted in oncolytic herpesviruses and adenoviruses, respectively, restricting their ability to replicate in healthy cells. Other OVs utilize cell-specific promoters so that transcription of viral genes is dependent upon whether the host cell is healthy or cancerous.[3–5] OVs may also depend on the activation of specific cellular pathways that may be overactivated in tumor cells, such as the Ras pathway.[6] A wide variety of OVs are showing promise, both in efficacy and safety, in preclinical and clinical studies. Talimogene laherparepvec (T-VEC), a first-in-class oncolytic virus based on modified herpes simplex virus type 1 (HSV-1), has produced a measurable therapeutic response in a phase III clinical trial and has been approved by the US Food and Drug Administration (FDA) for treatment of melanoma.[7]

As illustrated in Figure 1, the therapeutic efficacy of OVs is not only due to the specific killing of tumor cells directly but more importantly, due to the immune response elicited toward uninfected cells, especially those of tumor-specific CTLs.[5,8] Tumor infection with viruses lacking immunogenic transgenes can produce tumor antigen-specific, CTL-mediated immune responses, likely through multiple contributing factors including support of dendritic cell (DC) maturation and release of pro-T cell cytokines.[8] Engineering of OVs to express immunomodulatory transgenes holds the potential for even further enhancement of CTL-mediated tumor immunity. In general, the more effectively an OV can transform the immunosuppressive tumor microenvironment (TME) into an immunostimulatory one, the more potent the tumor-specific immunity will be and the greater the therapeutic benefit. Of note, attempts to activate antitumor immunity must also consider potential side effects, and genetic engineering strategies have been used to abrogate the detrimental effects of immunomodulatory transgenes. For example, an oncolytic HSV that caused rashes through the expression of tumor necrosis factor alpha (TNF-α) was modified to contain a promoter that limited TNF-α

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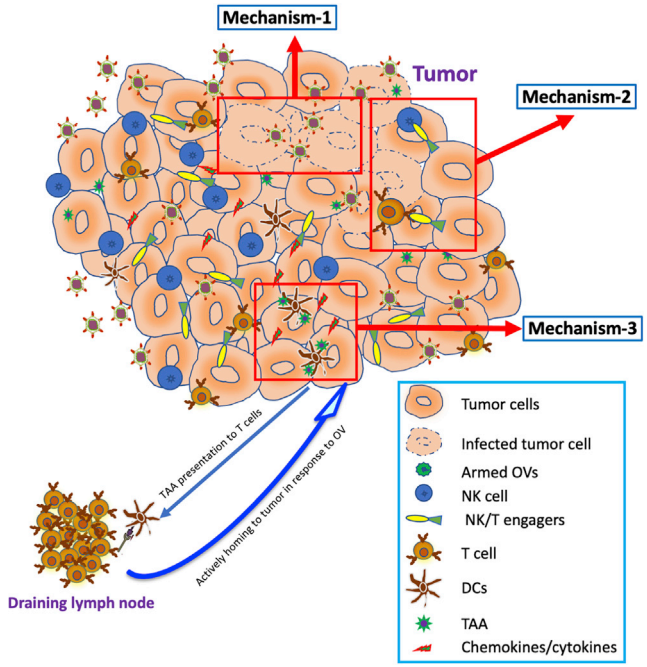

Figure 1. Armed OVs and their mechanism of action

Mechanism-1: Direct oncolysis by the virus, which also triggers NK and T cell infiltration. Mechanism-2: Specifically designed armed viruses can release NK/T cell engagers to activate these immune cells to kill more tumor cells. Together with Mechanism-1, abundant TAAs (including neoantigens) will be released from the lysed tumor cells. Mechanism-3: The activated NK cells can secrete additional cytokines/chemokines (e.g., XCL1-a C class chemokine also known as lymphotactin) to attract conventional DCs (cDCs) to capture TAAs. Armed viruses can also release other immune stimulators such as GM-CSF and interleukins that can further potentiate TAA presentation. Chemokines released from subsequent virotherapy can attract the migration of TAA-specific T cells back to the tumor site.

expression, reducing the side effects and improving anti-tumor efficacy.[9] Although this is an important aspect of oncolytic virotherapy, this review focuses on the molecular mechanisms behind the adaptive immune response to armed and unarmed OVs.

OVs AS A STRATEGY TO ENHANCE ANTIGEN PRESENTATION

In most instances, the process of antigen presentation begins as cytosolic proteins are degraded into peptide fragments by the proteasome and then transported into the endoplasmic reticulum by the transporter associated with antigen processing 1 and 2 (TAP-1 and TAP-2) proteins, where they are loaded onto MHC class I molecules.[10] The peptide-MHC class I complex then travels to the cell surface, where MHC class I becomes anchored in the plasma membrane, and the peptide fragment remains bound to the extracellular domain.[1] In the context of cancer, many tumor cells have lost MHC expression, which hinders recognition by immune cells.[11] Enhanced presentation of tumor-associated antigens (TAAs) on MHC class I would render tumor cells more likely to be recognized and lysed by CTLs, and numerous strategies have been employed to increase MHC class I presentation on tumor cells for this purpose. Results from the following studies indicate that various OVs can upregulate antigen processing and presentation in cancer cells.

Infection of mouse ovarian cancer cells with oncolytic reovirus was found to induce expression of MHC class I, TAP-1, and TAP-2, all of which are downregulated in the untreated cell line.[11] In another preclinical study, infection with an adenovirus triggered tumor cells to upregulate uric acid, stimulating DCs to release IFN-γ, which subsequently stimulated tumor cells to upregulate PA28,[12] a protein known to activate proteasomal cleavage of polypeptides to produce MHC class I antigens.[13] This process led to increased specific CTL lysis of infected tumor cells.[12] Similarly, Zamarin et al.[14] found that infection with an oncolytic Newcastle disease virus (NDV) stimulated uniform upregulation of MHC class I among infected and non-infected tumor cells. This was likely caused by increased type I IFNs, which are known to regulate MHC class I expression and were released by tumor cells infected by the same NDV.[14] A similar mechanism was observed in a murine lung cancer model treated with an adenovirus armed with an IFN-β transgene. IFN-β expression was shown to upregulate MHC class I expression in this tumor cell line. This alteration of the tumor cells was required for CTL-mediated tumor rejection.[15] These two studies suggest that the ability of IFN-β to upregulate MHC class I makes it a promising tool to increase the immunogenicity of tumor cells in the context of virotherapy.[14,15] In addition, inhibition of certain viral genes can promote antigen processing; for example, deletion of the HSV-1 gene *ICP47*, known to downregulate MHC class I expression by blocking TAP,[16] has successfully resulted in increased expression of MHC class I in infected tumor cells compared to mock treatment in mice.[5]

OVs CAN ENHANCE DC TRAFFICKING TO THE TME AND CROSS-PRESENTATION TO CTLs

Antigen-presenting cells (APCs), particularly DCs, have the unique ability to present antigens from exogenous proteins on MHC class I in a process known as cross-presentation.[1] As DCs reside in the tissues, they can take in these proteins through endocytosis and process them through various and poorly understood pathways.[17] Once tissue-resident DCs have matured, they travel to the draining lymph nodes to present MHC class I antigens by binding to the TCR of naive CTLs, a process called priming, inducing either tolerance or immunity against the presented antigen depending on the costimulatory molecules expressed.[18] For $CD8^+$ T cell-activating costimulatory molecules to be expressed, inflammatory cytokines and other "danger" signals must be provided during the maturation of APCs.[17] Therefore, enhancement of antigen uptake before DC maturation and provision of the appropriate maturation signals play essential roles in the induction of tumor-specific CTL immunity.

IFN-α/β signaling is required for the development of a systemic antitumor response.[19,20] It is essential for the accumulation of $CD8^+$ DCs in the TME and therefore, necessary for activation of tumor-specific naive T cells.[20] *In vivo* studies have also shown IFN-α/β signaling to be an important factor in generating DCs capable of efficient

cross-presentation to $CD8^+$ T cells.[2,19,20] *In vitro* studies have elucidated some of the mechanisms behind this connection between type I IFNs and DC activity that promotes antitumor immunity. The presence of IFN-α during DC maturation stimulates multiple immunogenic pathways, particularly those involved in phagocytosis and antigen processing. One upregulated protein in DCs matured in the presence of IFN-α is the scavenger receptor LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1), which mediates endocytosis of apoptotic cells, a necessary step for antigen cross-presentation to occur. The LOX-1 endocytic pathway induces immunity more efficiently than the non-specific mechanisms of endocytosis used by DCs.[21] IFN-α also triggers upregulation of MHC class I and TAP-1,[22] the latter of which is involved in at least one of the pathways for MHC class I cross-presentation. Non-OVs trigger the release of type I IFNs,[2] and OVs can do so likewise, although the magnitude of the release may differ among types of OVs, and the effects of the IFNs may differ among types of cancer cells. Release of type I IFNs may be a mechanism through which OV infection enhances cross-presentation by DCs. Multiple studies have found that oncolytic NDV infection enhances DC cross-presentation of tumor antigens and is accompanied by increased IFN-α secretion, but further research is needed to confirm a direct relationship.[14,23] In a murine lung cancer model, treatment with an adenovirus armed with an IFN-β transgene prolonged survival significantly compared to treatment with the adenovirus lacking the transgene. Tumor-bearing mice treated with the armed virus developed tumor-specific CTLs that contributed to the therapeutic effect and protected cured mice from subsequent tumor injections. Although a mechanism involving DCs was not confirmed by this study, further investigation into the effect of IFN release by this virus on DCs could be useful.[15] In another study by the same group, a vaccinia virus (VV) was armed with IFN-β and used to treat two different mouse lung cancer models. The transgene did not have the same treatment-enhancing effect relative to the unarmed virus as it did with the two adenoviruses, although even the unarmed virus caused IFN-β expression, which may have contributed to the CTL-mediated tumor regression observed.[24] Because IFN signaling also inhibits NDV replication,[25] future studies should consider the dual effects OV-induced type I IFN release on antitumor immunity and viral replication in order to optimize efficacy.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) controls both myeloid cell differentiation and the function of mature blood cells, including $CD8^+$ DCs. It has been found to be particularly important to nonlymphoid tissue migratory DCs, regulating homeostasis and promoting the survival of these cells. GM-CSF is also essential to T cell priming by $CD8^+$ DCs.[26] Its overexpression in transgenic mice has been shown to increase $CD8^+$ DC antigen presentation to naive $CD8^+$ T cells, leading to enhanced proliferation of $CD8^+$ T cells.[27] This effect has been replicated by treating tumors with OVs with GM-CSF gene insertions, producing specific immunogenic antitumor responses in multiple models. For example, the inclusion of the gene coding for GM-CSF in adenovirus Ad5-D24 caused tumor cells to secrete the gene product upon infection, inducing complete tumor eradication and protection from repeated challenge with the same tumor cell line in hamsters.[28] A tumor-specific CTL response in human patients was also shown.[28] Similar *in vivo* results have been found with the inclusion of GM-CSF in modified HSV-1,[5] NDV,[23] and VV[29] to treat various tumor models. GM-CSF levels have even been elevated by infection of tumors with viruses lacking the transgene, including ΔPK, an OV derived from herpes simplex 2 (HSV-2).[30] The arming strategy with this immune stimulator and other cytokines for OV is summarized in Table 1. Arming OVs to potentiate immunotherapy, therefore, has the potential to be a valuable contributor to long-term clinical benefits via specific antitumor immunity.

Tumor cells undergoing apoptosis or necrosis in response to effective, immune-activating cancer therapy are known to release damage-associated molecular patterns (DAMPs), which can trigger inflammatory responses from DCs and consequently CTLs.[31] In particular, adenosine triphosphate (ATP), high mobility group box 1 (HMGB1), and exogenous calreticulin (CALR) are considered to be important indicators of immunogenic cell death.[32] ATP released by dying cells can attract DCs to the TME.[33] It can also interact with purinergic P2X7 receptors on DCs, triggering the non-obese diabetic (NOD)-like receptor family, pyrin domain containing-3 protein (NLRP3)-dependent caspase-1 activation complex .[31] This complex is required for efficient priming of CTLs in response to dying tumor cells.[31,34] Upon interaction with Toll-like receptor 4 (TLR4) on DCs, HMGB1 is endocytosed and triggers signaling cascades that lead to activation of DCs.[35] By these respective mechanisms, the release of both ATP and HMGB1 by dying tumor cells is important for the maturation of DCs into cells capable of cross-presentation *in vivo*.[33,36] CALR marks cells to be targeted by scavenger receptor class A (SR-A) and scavenger receptor expressed by endothelial cell-I (SREC-1) on DCs for endocytosis and subsequent antigen processing and cross-presentation.[37,38] Through their interactions with antigen-presenting DCs, DAMPs are important drivers of tumor-specific immunogenicity induced by OVs. Tumor infection with oncolytic coxsackievirus (CV)B3 resulted in increased tumor production of ATP and HMGB1, and DCs with significantly high expression of the maturation marker CCR7 were recruited to the TME, indicating the potential for the generation of adaptive antitumor immunity.[39] Similarly, infection with adenovirus dl922-947 triggered mesothelioma cell release of ATP and HMGB1, as well as increased CALR cell-surface expression.[40] Treatment with both of these viruses induced significant tumor growth suppression and prolonged survival.[39,40] Increased ATP production has also been reported by an oncolytic HSV armed with phosphatase and tensin homolog (PTEN)α, an N-terminally extended isoform of PTEN that has the additional function of increasing electron transport chain activity by localizing to cytochrome C in the mitochondrial membrane. The PTENα-expressing virus improved the survival of brain tumor-bearing mice relative to the control virus in a manner dependent on $CD8^+$ T cells, and surviving mice were protected from tumor rechallenge. Consistent with the known effects of ATP on DCs, there was also increased DC infiltration in tumors treated with the PTENα-expressing virus.[41]

Notably, HMGB1 secretion triggered by OVs has also been shown to have other effects that may hinder therapeutic efficacy and must be

Table 1. OVs incorporated with immune modulators

Transgene	Type of OV	Effect on tumor microenvironment	Cancer type (preclinical model unless specified otherwise)	References
Cytokines				
IFN-β	AdV	upregulates MHC class I in tumor cells	mesothelioma; bronchogenic lung cancer	15
	MV	triggered CD68-positive immune cell infiltration; innate immune cell infiltration	mesothelioma	124
	VV		mesothelioma	24
	AdV		non-small cell lung cancer	125
	NDV		pancreatic adenocarcinoma	126
IFN-γ	NDV	increased cytokine expression; maturation of DCs	melanoma	127,55
GM-CSF	AdV, MV	promotes DC survival and T cell priming; $CD3^+$ T cell infiltration	solid tumors (clinical)	28,128
	HSV-1		breast adenocarcinoma	5
	NDV	improved peripheral blood mononuclear cell response	melanoma, breast cancer	23
	VV		melanoma, mammary carcinoma, and colorectal carcinoma	29
IL-12	HSV-1	prolongs expression of IL-2 receptor on $CD8^+$ T cells; infiltration of T helper (Th), CTL, NK cells, and macrophages	glioma	68
	reovirus (unarmed)		melanoma	8
	AdV	stronger antitumor activity; DC maturation	melanoma	129
	VSV		squamous cell carcinoma	130
IL-2	NDVHSV	promotes expansion and effector function of $CD8^+$ and $CD4^+$ T cells	colon carcinoma; hepatocellular carcinoma, melanoma	66,67
IL-15	VSV	increase in tumor-specific T cells	colon adenocarcinoma	70
	VV	infiltration of Th and CTLs	colon carcinoma; ovarian cancer	71
	NDV		melanoma	131
	IAV	anti-tumor immunity against rechallenge	melanoma	132
	HSV	increased survival, NK-mediated cytotoxicity	glioma	133
Chemokines				
CCL5	VV	improved DC maturation; improved infiltration of Th and CTLs	colon carcinoma	134
CCL2	HSV-1			135
CCL19	VV			136
CXCL9 (OV induced)	HSV-2	triggers migration of activated T cells to tumor site	pancreatic cancer	81
	HSV-1		ovarian carcinoma	82
CXCL10 (OV induced)	HSV-2	triggers migration of activated T cells to tumor site	pancreatic cancer	81
	HSV-1		ovarian carcinoma	82
CXCL11	VV	triggers migration of activated T cells to tumor site	mesothelioma	137
Checkpoint inhibitors				
CTLA-4	NDV	reverses inhibition of B7 costimulatory activation of $CD8^+$ T cells	melanoma	14,55
	AdV	decreased infiltration of Tregs		91
	MV	infiltration of Th and CTL		138
	VV	decreased infiltration of Tregs	renal adenocarcinoma; colon adenocarcinoma	92
PD-1/PD-L1	MV	infiltration of Th and CTL	melanoma	138
	MYXV	anti-tumor $CD8^+$ T cell responses		88
Co-stimulatory ligands				
B7	HSV-1	costimulatory activation of $CD8^+$ T cells	neuroblastoma	56,57
	VV	anti-tumor immunity	melanoma	139

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Table 1. *Continued*

Transgene	Type of OV	Effect on tumor microenvironment	Cancer type (preclinical model unless specified otherwise)	References
GITRL	AdV	increases proliferation and effector functions of $CD8^+$ and $CD4^+$ T cells	glioma	52
CD40L	AdV	inhibits Treg-immunosuppressive activity	melanoma	87,140
	VV	infiltration of Th, CTL, NK, DCs, and MDSCs	melanoma	141
	AdV	significant priming of T cells directed against TAAs	melanoma	142
OX40L	AdV	costimulatory activation of $CD4^+$ T cells	melanoma; colon adenocarcinoma	53
4-1BBL	AdV	increases proliferation and effector functions of $CD8^+$ and $CD4^+$ T cells	melanoma	58
	VV	infiltration of CTLs	melanoma	143
LIGHT	AdV	reduced Treg suppression	prostate	144
CD70	VV	tumor reduction	colon adenocarcinoma	145
Combinations				
GM-CSF + IL-12	AdV	shift from Th2 to Th1 response, infiltration of Th, CTL, NK, and DC	melanoma	146
4-1BBL + IL-12		infiltration of Th, CTL, DCs, and NK cells		147
B7.1 + IL-12				148
B7.1 + GM-CSF				149
IL-12 + IL-18				129
B7.1 + IL-18	HSV	reduced tumor growth	prostate; neuroblastoma	57
IL-12 + CCL2			neuroblastoma	135
Immune cell engagers				
CD3/EphA2	VV	induced T cell activation, increased cytotoxicity of target cells, and bystander killing of non-infected tumor cells	human lung cancer	106,116
			colorectal carcinoma	83
CD3/EGFR	Ad	T cell activation, proliferation and bystander cell-mediated cytotoxicity, and enhanced antitumor efficacy	human lung and colorectal cancer	109
CD3/EpCAM	EnAd	T cell activation	primary pleural effusions and peritoneal malignant ascites	107
CD3/CEA or CD20	MV	increased therapeutic efficacy	patient-derived primary colorectal carcinoma	108,110
CD3/FAP	VV AdV	T cell activation and killing of stromal fibroblasts	B16 models; human colon and lung cancers	116,118
CD3, CD206 folate receptor B	EnAd	T cell activation with preferential killing of M2-like macrophages	human cancer samples tested *in vitro*	119
Others				
ATP (OV induced)	coxsackievirus	promotes DC maturation and T cell priming	lung adenocarcinoma	39
	AdV		mesothelioma	40
	HSV-1		breast cancer, brain metastasis, and glioblastoma	41
HMGB1 (OV induced)	coxsackievirus	promotes DC maturation and T cell priming	lung adenocarcinoma	39
	AdV		mesothelioma	40
CALR (OV induced)	AdV	increases DC antigen processing and cross-presentation	mesothelioma	40
HSP70	AdV	increases DC antigen uptake; upregulates T cell costimulatory molecules on surface of DCs	prostate adenocarcinoma; melanoma	44
			various forms of gastric cancer	46
			pancreatic cancer	47

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Table 1. *Continued*

Transgene	Type of OV	Effect on tumor microenvironment	Cancer type (preclinical model unless specified otherwise)	References
HPGD	VV	expression of Th1 cytokines; secretion of IL-12	solid tumors; renal cell carcinoma	150
TRIF				
DAI		increased infiltration of $CD8^+$ T cells	melanoma	151

AdV, adenovirus; MV, measles virus; IAV, ; MYXV, Myxoma virus; MDSCs, myeloid-derived suppressor cells; LIGHT, lymphocyte activation gene 3 protein ; HPGD, hydroxyprostaglandin dehydrogenase 15-(NAD), 15-PGDH ; TRIF, TIR-domain-containing adapter-inducing interferon-β ; DAI, DNA-dependent activator of IFN-regulatory factor.

weighed against the beneficial effects. For example, inhibition of HMGB1 with both a small molecule inhibitor and genetic knockdown resulted in increased spread of an oncolytic HSV among fibroblast cells, implicating a role for HMGB1 in viral restriction.[42] In contrast, in a study on glioma, treatment with HMGB1-blocking antibodies had no effect on oncolytic HSV spread *in vitro* and *in vivo*. However, secreted HMGB1 triggered by oncolytic HSV infection was found to increase vascular leakiness and edema *in vivo*, whereas HMGB1 blockade rescued both conditions and prolonged survival.[43]

When secreted, heat shock proteins (HSPs) are also considered to be DAMPs, as they can support antitumor immunity by functioning as chaperones for receptor-mediated endocytosis of antigenic peptides by DCs.[44] For example, tumor-derived HSP70 can form a complex with tumor antigens and subsequently bind to various scavenger receptors, including LOX-1, on the surface of DCs. LOX-1 is particularly active in mediating cross-presentation of HSP70-complexed tumor antigens to elicit a specific CTL response.[45] HSPs can also upregulate costimulatory molecules necessary for T cell activation on the surface of DCs.[44] Arming an oncolytic adenovirus to express HSP70 dramatically improved the virus's immune-driven therapeutic efficacy. Treatment of mice with the armed virus completely eradicated weakly immunogenic tumors and induced a specific response against tumor rechallenge, whereas the virus lacking the transgene merely inhibited tumor growth and had no effect on tumor rechallenge.[44] Cell depletion revealed $CD8^+$ T cells to be the most important effector cells in the observed immunogenic effects of HSP70,[44] suggesting that viral expression of HSP70 enhanced cross-presentation by DCs, producing a tumor-specific CTL response against these tumor models. Other groups similarly found that adenoviral expression of HSP70 produced T cell-dependent antitumor effects in gastric cancer[46] and pancreatic cancer xenografts.[47] These findings do not contradict those that have found HSP70 to promote tumorigenesis, as it can function as an anti-apoptotic factor intracellularly and an immune stimulator extracellularly.[48] Nevertheless, as intracellular antiapoptotic factors, HSPs have effects on viral replication that should be considered in the context of OV therapy. For example, hyperthermia-induced HSP72 synergized with an oncolytic HSV-1, enhancing viral replication and increasing cytotoxicity against pancreatic cancer cells.[49] In another study, HSP90 was found to be required for efficient viral DNA replication and production of viral progeny during HSV-1 infection.[50] Although these results were obtained using non-tumor cells, HSP90 is known to be in an active state characterized by the formation of complexes with other HSPs and cochaperones in various types of tumor cells,[50] under which conditions it can inhibit apoptosis.[44] Taken together with the effects of HSP90 on HSV-1 replication, this suggests that HSP90 may play a role in promoting OV replication in tumors. Concurrently, HSP90 induction by the proteasome inhibitor bortezomib was found to synergize with oncolytic HSV-1 therapy in a variety of tumor xenografts by increasing viral replication and cell killing.[51] HSP transgenes in OVs merit further investigation for their dual function as immune-stimulating factors and their potential to promote OV replication by inhibiting apoptosis.

ARMED OVs CAN CAUSE THE RELEASE OF LIGANDS OF T CELL-COSTIMULATORY RECEPTORS

For $CD8^+$ and $CD4^+$ T cells to be primed against tumor antigens, they must receive not only an MHC-restricted TCR signal but a costimulatory signal as well, which is often provided by DCs.[52,53,54] These signals are often lacking in the TME, leaving infiltrating T cells in an anergic state.[52] Whereas oncolytic virotherapy has the potential to produce an inflammatory microenvironment that recruits T cells to the tumor site and potentiates their initial priming, arming OVs with costimulatory ligands, as summarized in Table 1, can increase activation of tumor-specific T cells.[55,56,57,52,53,58]

A critical costimulatory interaction is that of B7 expressed on the surface of DCs with CD28 expressed on T cells. B7-transfected melanoma cells have been shown to successfully provide this costimulatory signal, directly activating $CD8^+$ T cells against tumor antigens without the need for $CD4^+$ T cells.[54] As a single agent, B7.1 expressed by an oncolytic HSV-1 was found to be relatively ineffective in boosting antitumor immunity in a murine neuroblastoma model; however, it was able to significantly enhance antitumor effects in combination with HSV expression of interleukin (IL)-12 and/or IL-18 in a T cell-dependent manner.[56,57]

Upon TCR activation, $CD8^+$ and $CD4^+$ T cells upregulate glucocorticoid-induced TNF receptor (GITR), the activation of which increases their proliferation and effector functions.[59] One study found that treatment with an agonist of GITR produced these effects in mouse models; the agonist induced systemic $CD8^+$ T cell-mediated, tumor-specific immunity against secondary tumors, although significant effects on primary tumors were not observed.[60] Another group reported tumor growth inhibition or regression in nearly all treated mice, increased proportions of tumor antigen-specific $CD8^+$ T cells,

and protection from tumor rechallenge in response to GITR agonism.[61] Notably, these results were obtained using CT26 cells, which are known to naturally induce priming of naive T cells against their own antigens, albeit insufficiently for inducing tumor rejection. Upon repeating the experiment with a less immunogenic tumor cell line (E7), the same group observed that the GITR agonism alone did not produce significant effects on tumor-specific $CD8^+$ T cells or tumor regression.[61] To produce a significant antitumor response against E7 tumors, GITR agonism had to be combined with a peptide vaccination, indicating that GITR stimulation could activate antitumor immunity but was only effective when accompanied by a T cell-priming mechanism.[61] Thus, as OVs have been shown to enhance T cell priming, incorporation of GITR into the genome of an OV can be rationally hypothesized to enhance its antitumor effects. Concurrently, arming an oncolytic adenovirus with GITR ligand (GITRL) significantly prolonged survival of glioma-bearing mice compared to treatment with the parental virus, and the armed virus produced both cytotoxic and memory $CD8^+$ T cell responses against tumor antigens.[52]

Tumor-specific CTL activity can also be potentiated by stimulation of $CD4^+$ T helper (Th) cells.[53] MHC class II -restricted TCR engagement on the surface of $CD4^+$ T cells induces upregulation of the costimulatory receptor OX40 (CD134), the ligand (OX40L) for which is expressed by DCs. Tumor cell expression of OX40L mediated by an oncolytic adenovirus led to enhanced infiltration of $CD4^+$ and $CD8^+$ T cells to the tumor site. This resulted in improved tumor-specific lysis by CTLs, inhibition of tumor growth, and improved survival in mice with melanoma and colon adenocarcinoma. Tumor expression of OX40L significantly increased $CD4^+$ T cell IFN-γ production, and the antitumor effects of the OX40L-expressing adenovirus could not be replicated in either $CD4^+$ T cell- or $CD8^+$ T cell-deficient mice, indicating both cell types were required. These findings suggested that the OX40L-OX40 interaction on $CD4^+$ Th cells specific for the treated tumors stimulated IFN-γ release, which in turn activated tumor-specific CTLs, although more research is needed to confirm this mechanism.[53]

4-1BB is a TNFR expressed on activated $CD4^+$ and $CD8^+$ T cells. 4-1BB stimulation results in enhanced proliferation of activated $CD8^+$ T cells *in vitro* and subsequent generation of CTLs *in vivo*.[62] The expression of 4-1BBL in tumor cells has been shown to provide a costimulatory signal that drives both $CD4^+$ and $CD8^+$ T cell proliferation, leading to their expansion in the tumor site.[63] Although 4-1BB/4-1BBL interaction has this effect on isolated colonies of both $CD4^+$ and $CD8^+$ cells, optimal expansion of $CD8^+$ T cells in response to this costimulatory signal requires the presence of $CD4^+$ T cells. The ability of CTL effector cells to specifically lyse tumor cells can also be enhanced by 4-1BB stimulation.[63] As an adenoviral transgene, 4-1BBL has been found to significantly enhance the tumor-shrinking effect of virotherapy. Although 4-1BBL did not produce as strong an effect as an IL-12 transgene, the two transgenes were found to work synergistically to enhance tumor regression, likely by activating $CD4^+$ Th cells, which in turn activated CTLs.[58]

NDV infection has been shown to stimulate $CD8^+$ and $CD4^+$ T cell upregulation of various co-receptors, especially the inducible costimulator (ICOS). Compared to its wild-type counterpart, an NDV modified to express the ligand for this receptor enhanced $CD8^+$ and $CD4^+$ T cell infiltration as well as tumor regression in sites not directly treated with the virus. Viral ICOSL expression enhanced CTL expression of ICOS, suggesting increased activation. Increased CTL release of granzyme B was also observed in response to ICOSL expression, suggesting increased lytic function.[55]

OVs INDUCE THE RELEASE OF CYTOKINES THAT SUPPORT CTL EXPANSION AND FUNCTION

IL-12 and type I IFNs have similar functions as inflammatory stimulators required for optimal $CD8^+$ T cell activation.[64,65] Capable of functioning alone or synergistically, these signal 3 cytokines play important roles in T cell expansion, effector function, and ultimately in generating an antigen-specific response (Table 1). However, it has been noted that their requirement for optimal T cell expansion and effector function varies among different types of infections. For example, optimal expansion of T cells responding to vesicular stomatitis virus (VSV) infection depends on both signals, whereas only type I IFN is required in lymphocytic choriomeningitis virus (LCMV) infection. Neither IL-12 nor type I IFNs are necessary for the development of antiviral CTLs in VV infection.[65] Nonetheless, IL-12 and/or type I IFN signaling during $CD8^+$ T cell activation enhances the accumulation of effector cells by prolonging expression of the high-affinity IL-2R CD25, the presence of which increases cell sensitivity to IL-2. In the presence of IL-2, increased CD25 expression results in prolonged phosphatidylinositol 3-kinase (PI3K)-dependent cell division.[64] Thus, both IL-12 and IL-2 are potentially important factors for the accumulation of tumor-specific CTLs in the tumor site. Multiple studies with NDV have found that viral IL-2 expression increased infiltration of $CD4^+$ and $CD8^+$ T cells into TME, tumor-specific T cell function, and tumor regression in mice following infection compared to the virus lacking the transgene.[66,67] Oncolytic HSV-1 expression of IL-12 yielded similar results, enhancing the survival of tumor-bearing mice as well as tumor infiltration of natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells.[68] Tumor infection with a reovirus, without the need for a transgene, has been shown to increase levels of IL-12, suggesting IL-12 signaling to be one important factor in tumor-specific immunity induced by unarmed reoviruses.[8]

IL-15 binds to the same receptor as IL-2, which is commonly expressed on NK cells and T cells. Both cytokines activate, expand, and increase the cytolytic activity of these cells, but IL-15 lacks some of the immunosuppressive properties and clinical side effects of IL-2.[69] IL-15 treatment, especially with the IL-15 superagonist ALT-803, has shown promising preclinical results against cancer. Treatment of an experimental mouse glioblastoma with ALT-803 led to enhanced tumor growth inhibition and animal survival, which were found to be mediated by $CD4^+$ and $CD8^+$ T cells. Aligning with the known functions of IL-15, increased tumor-infiltrating $CD8^+$ T cells, as well as the increased function of these cells, were observed.[69] Editing a VSV and VV to express IL-15 and an IL-15

superagonist, respectively significantly increased infiltration of tumor antigen-specific $CD8^+$ T cells, tumor regression, and survival upon infection.[70,71] Cell-depletion assays in the second study revealed $CD8^+$ T cells to be more critical than NK or $CD4^+$ T cells in the observed therapeutic effects of the virus.[71]

Studies on the effects of IL-10 on the TME have yielded contrasting findings. In context with a persistent viral infection, IL-10 deficiency or IL-10R blockade was found to increase the number of virus-specific $CD8^+$ T cells, the function of $CD8^+$ and $CD4^+$ T cells, and viral clearance, suggesting that IL-10 suppressed antiviral $CD8^+$ T cell-mediated antiviral immunity.[72] Taking advantage of these immunosuppressive effects, arming an oncolytic VV with IL-10 enhanced the oncolytic effect of the virus by reducing anti-viral CTLs without reducing antitumor CTLs. Specific antitumor immunity was also observed and attributed to increased release of TAAs via enhanced viral oncolysis.[73] In another study, tumor-infiltrating DCs did not respond to the presence of molecules able to stimulate IL-12 and TNF-α secretion in typical immature DCs. Blockade of IL-10/IL-10R signaling, when combined with TLR9 activation, was able to restore the normal response, and the treated DCs were able to stimulate a tumor-specific CTL response, suggesting that IL-10 signaling was a contributor to inhibition of antitumor immunity.[74] In melanoma models, high levels of IL-10 can also inhibit antitumor CTLs by deregulating the CTL-activating ligand MHC class I polypeptide-related sequence A (MICA). ΔPK infection inhibited IL-10 secretion by melanoma cells, leading to restored expression of MICA.[30]

In contrast, tumor expression of IL-10 has also been shown to cause inhibition of tumor growth, with $CD8^+$ T cells playing a crucial role in the observed antitumor effects,[75,76] although these studies did not clearly prove that IL-10 acted directly on $CD8^+$ T cells. In a more recent study, however, IL-10 treatment was shown to enhance tumor rejection by increasing tumor-specific CTL proliferation and cytotoxic activity without the need for migration of new $CD8^+$ T cells to the tumor site. These immunogenic effects were shown to require IL-10 interaction with IL-10Ra on the surface of $CD8^+$ T cells only.[77] The effect of IL-10 likely depends on the environment in which it is expressed, and more research will be needed to elucidate its mechanisms of immune activation and/or suppression to inform the development of more effective OVs.

OVs CAN INDUCE THE RELEASE OF CHEMOKINES THAT ATTRACT CTLs TO THE TME

The IFN-γ-inducible chemokines CXCL9, CXCL10, and CXCL11 are known to directly attract effector CTLs to sites of infections or tumors via their interaction with CXCR3, which is highly expressed on activated T cells.[78,79] Downregulation of these chemokines is one way in which tumors evade immune responses,[80] and restoring their expression has consequently been considered as a possible way that OVs can engage tumor-specific CTLs. Listed in Table 1 are some reports of the effects of chemokines induced or expressed by OVs on the TME.

In one preclinical study, infection with an oncolytic HSV-2 triggered the release of CXCL9 and CXCL10, increasing migration of tumor-specific $CD4^+$ and $CD8^+$ T cells to the TME. This migratory effect enhanced tumor-specific immunity *in vivo*.[81] An oncolytic HSV-1 was found to similarly enhance $CD8^+$ T cell migration to murine ovarian carcinoma tumors via upregulation of CXCL9 and CXCL10 by both tumor cells and DCs, which also migrated to the tumor site in response to infection.[82] In yet another study, CXCL9 was inserted into the genome of an oncolytic VSV in an attempt to enhance migration of CTLs to the tumor site upon infection. Whereas this gene insertion increased tumor expression of CXCL9, it failed to increase $CXCR3^+$ T cell infiltration over that observed in response to treatment with the virus lacking the transgene. However, treatment with either virus increased both CXCL9 expression and $CXCR3^+$ T cell infiltration, and the authors hypothesized that oncolytic viral activity likely produced a sufficient chemokine gradient to optimally attract T cells to the TME, without the need for additional chemokine expression.[80] However, further research would be needed to definitively prove this, and chemokine gene insertion may have more immunostimulatory effects in the context of different OVs or different tumor models.

Expression of CXCL11 by an oncolytic VV enhanced the therapeutic efficacy of the virus against mesothelioma via increased migration of tumor-specific T cells to the TME as well as increased activation of systemic tumor-specific $CD8^+$ T cells.[81] In a murine colorectal cancer model that is weakly immunogenic, the induced CXCL11 enhanced tumor infiltration of $CD8^+$ T cells, but therapeutic efficacy was not significantly increased compared to treatment with a virus lacking the transgene.[83] However, the CXCL11-expressing virus was shown to enhance therapeutic efficacy when combined with a cytokine-modulating (CKM) drug cocktail capable of increasing intratumoral CCL5 and CXCL9. Combination therapy induced greater CXCL11 levels, more $CD8^+$ T cell infiltration, and longer survival than either therapy alone. Although further study would be required to fully elucidate the mechanism of the synergy between these two therapies, the viral therapy likely induced activation of CTLs, and both therapies likely played a role in enhancing CTL migration to the tumor site, whereas the CKM drug cocktail functioned by promoting prolonged T cell activity in the tumor site.[83]

OVs CAN SUPPORT CTL ACTIVITY BY INHIBITING T REGULATORY CELLS (TREGS)

Tregs, often characterized as $CD4^+CD25^+Foxp3^+$ T cells are particularly active toward self-antigens. By expressing CTL-associated antigen 4 (CTLA-4), they can decrease APC expression of the costimulatory ligands CD80 (B7-1) and CD86 (B7-2), resulting in apoptosis, anergy, or dormancy in $CD4^+$ and $CD8^+$ T cells.[84] They also induce immune tolerance via their high-affinity IL-2Rs, which deprive responder T cells of IL-2 signaling.[84] Whereas these functions are essential to prevent autoimmunity, they can be detrimental to antitumor immunity and have been associated with poor prognosis.[84] For example, one study found that infection with a particular virus increased $CD4^+$ T cell populations expressing T regulatory markers

and that these cells inhibited the ability of mice to reject tumors that could otherwise be rejected through $CD8^+$ T cell activity.[85] Injection of a splenic cell suspension depleted of $CD25^+$ cells into athymic mice followed by injection of leukemia cells resulted in tumor-specific rejection requiring the presence of $CD8^+$ CTLs.[86] Similar results were obtained by systemic administration of antibodies against CD25 in immunocompetent mice, using a variety of tumor cell lines.[86] It is therefore reasonable to expect that Treg-targeting strategies could prove effective in enhancing the adaptive immune effects of virotherapy.

Tregs highly express GITR. A study found that tumor infection with an adenovirus containing the gene for the GITRL could augment tumor infiltration of $CD4^+$ and $CD8^+$ T cells.[87] Another study similarly found that tumor-bearing mice treated with a GITRL fusion protein had decreased proportions of Tregs with respect to total lymphocytes, as well as increased antigen-specific $CD8^+$ T cells in the tumor site, leading to tumor rejection and protection from rechallenge.[61] Taken together with the findings that Treg-immunosuppressive activity can be inhibited by GITR-specific agonists,[87,61] the expansion of antigen-specific T cells was likely due, at least in part, to Treg inhibition. *In vitro*, the GITRL could enhance proliferation of $CD4^+$ and $CD8^+$ T cells in the presence and absence of Tregs, allowing the possibility that the proliferative effect was mediated by a direct action of GITRL with GITR expressed by T cells,[87] and further research is required to clarify which mechanisms contribute to the therapeutic effects of GITR therapy.

A myxoma virus armed with a programmed cell death protein 1/programmed death ligand 1 (PD-1/PD-L1) blocking molecule, while able to regress the injected tumor via $CD8^+$ T cell activity, was ineffective against metastatic lesions. However, when $CD4^+$ T cells were depleted, the virus had enhanced efficacy against the injected tumor and could significantly reduce the number and size of metastatic lesions. This was likely due to inhibition of Treg-mediated suppression of CTLs, although more data are needed to confirm this mechanism.[88] In a study that found anti-PD-1 treatment to synergize with oncolytic VV therapy to elicit tumor-specific immunity, anti-PD-1 decreased the proportion of total $CD4^+$ T cells characterized as Tregs.[89]

ONCOLYTIC VIROTHERAPY IS ENHANCED BY TARGETING CTL CHECKPOINTS

Throughout the past decade, various antibodies that target immune-checkpoint molecules CTLA-4 and PD-1 have been approved by the FDA for the treatment of certain types of cancer.[90] Because immune-checkpoint molecules are often highly expressed by tumor-infiltrating T cells, and their ligands are often expressed by tumor cells, oncolytic virotherapy may be more efficacious in combination with these immune-checkpoint inhibitors (ICIs) or as gene-delivery vehicles of immune-checkpoint antagonizing molecules (Table 1).[55,89,90]

CTLA-4 is expressed by T cells upon TCR activation. It binds B7, competitively inhibiting it from interacting with the costimulatory receptor CD28.[90] CTLA-4 antagonism prevents suppression of $CD8^+$ and $CD4^+$ T cell activity and deactivates Tregs.[91] Tumor treatment with NDV has resulted in an inflammatory response in both directly treated and distal tumors characterized by increased infiltration of various immune cells, including $CD8^+$ T cells. This was accompanied by increased $CD8^+$ T cell expression of activation marker ICOS, proliferation marker Ki-67, and lytic function marker granzyme B. The immunosuppressive marker CTLA-4 was also upregulated. Although the immune activity triggered by NDV mediated some tumor regression and protective antitumor memory, these therapeutic benefits are limited in NDV treatment alone but were profoundly enhanced by combination treatment with anti-CTLA-4 monoclonal antibodies (mAbs). The combination treatment also yielded more favorable results than the antibody alone.[14] Similarly, in a study of ICOSL-expressing NDV, the highest $CD8^+$ T cell tumor infiltration was achieved with combination therapy with anti-CTLA-4.[55] Systemic CTLA-4 blockade has also produced synergistic therapeutic benefits with an oncolytic VV, at least in part by increasing the presence and activity of tumor-specific CTLs.[92] Combinations of OVs with CTLA-4 blockade are therefore an attractive prospect for cancer treatments that optimally activate the adaptive immune system. However, systemic delivery of anti-CTLA-4 produces adverse side effects; a better approach to combination therapy may be to modify an OV to express anti-CTLA-4. This has been shown to increase tumor concentrations of the antibody without affecting systemic levels, and the blockade was shown to successfully activate tumor-resident T cells.[91]

PD-L1 interaction with PD-1 expressed on the surface of T cells has been shown to promote apoptosis and suppress activation.[89] Despite the ability of virotherapy to engage the immune system therapeutically, an optimal CTL response can be suppressed by tumor expression of PD-L1.[88,89,93,94] Various OVs have been shown to upregulate tumor expression of PD-L1.[89,93] Combined with knowledge of the ability of OVs to attract T cells to the tumor site, these data have led researchers to hypothesize that PD-1 blockade would synergize with oncolytic virotherapy by enhancing the antitumor immune response.[88,89,93–130] In one study, PD-L1 knockout in a melanoma model was shown to increase the efficacy of an oncolytic myxoma virus, resulting in complete tumor eradication in almost all treated mice, whereas the OV treatment in wild-type mice merely stabilized tumor growth.[88] Blockade of PD-1/PD-L1 interaction has been shown to restore $CD8^+$ T cell function during viral infection[89] and to enhance immune responses to cancer by preventing exhaustion of antitumor T cells.[88] Antibodies against PD-1 have been approved for the treatment of some malignancies, although they are only therapeutically effective in a relatively small proportion of patients, particularly those whose tumors highly express PD-L1 and have high T cell infiltration.[88,89] Virotherapy has the potential to modify the TME to resemble that of patients who respond to checkpoint blockade treatment, as has been suggested by multiple studies reporting synergistic effects between virotherapy and PD-1/PD-L1 blockade.[88,89,93] For example, a study in which tumor-bearing mice were treated with a combination of anti-PD-L1 and an oncolytic VV reported that the VV was capable of attracting T cells to the tumor site and causing various types of tumor cells to upregulate PD-L1 on the cell surface.

Combination therapy with the virus and immune-checkpoint blockade reduced tumor burden, improved survival, increased the ratio of $CD8^+$/Tregs, and significantly increased cytolytic activity compared to either monotherapy. The increased immune response was shown to be specific to tumor antigens.[82,89] Another VV armed with IL-2 and TNF-α was proven to work synergistically with PD-1 blockade to increase the presence of $CD8^+$ T cells in the tumor site, shrink tumors, and increase long-term survival to 100% of mice treated with combination virotherapy and anti-PD-1.[94] Delivered before surgical tumor resection, an oncolytic Maraba virus was shown to improve survival, and the response of tumor-specific T cells rendered the mice significantly more responsive to post-surgery PD-1 blockade therapy.[93]

Although combination virotherapy and immune-checkpoint blockade have shown promising therapeutic results in preclinical studies, systemic administration of PD-1 has been associated with toxicity and autoimmunity. As such, PD-1-antagonizing molecules have been incorporated into the genome of OVs to attempt to mitigate these side effects while maintaining or improving therapeutic efficacy. In one such study, treatment of melanoma with a myxoma virus armed with a soluble splice variant of PD-1 with similar blocking effects to an antibody led to significantly better tumor regression than the combination of the parental virus with the antibody or with either monotherapy. $CD8^+$ T cells were the most responsible for the observed therapeutic effects of the recombinant virus, and although all viral therapies produced similar infiltration of these cells, their activation was highest in mice treated with the armed virus.[88]

The ability of various oncolytic adenoviruses, HSV-1s, VVs, CVs, reoviruses, VSVs, and Maraba viruses to activate antitumor immunity in preclinical and clinical studies has recently led to a plethora of clinical trials that combine OVs with ICIs.[96] The two treatments are expected to work synergistically, with the general hypothesis that virotherapy will trigger an inflammatory response in the TME that includes increased infiltration of tumor-specific CTLs, and ICI therapy will enhance the immune response by preventing those CTLs from becoming anergic.[96] Many of these trials are still underway and have not yet published results, although some have released limited interim results. A few completed clinical trials of this nature have also been published. Although melanoma is the most frequent cancer type among these trials, combination treatment is also being tested on others including pancreatic cancer, liver cancer, glioma, and advanced solid tumors.[96]

A 2016 clinical trial in which patients with advanced melanoma were treated with a combination of the approved OV T-VEC and the CTLA-4 inhibitor ipilimumab reported an objective response rate (ORR) of 50%, whereas the ORR for previous phase III trials for monotherapy with T-VEC and ipilimumab were 26.4% and 10.9%, respectively.[97] Although a direct comparison of these values suggests that the combined therapy may be more efficacious against melanoma than either monotherapy, such a conclusion cannot be definitively made without further evidence, because there were differing patient characteristics between the studies, and the dual therapy trial had a very small sample size (n = 19).[97] A 2018 phase II trial in which 198 melanoma patients were placed into randomized groups to receive either T-VEC plus ipilimumab or ipilimumab alone reported a significant increase in ORR in the combination arm versus the monotherapy arm (39% versus 18%), providing stronger evidence for the higher efficacy of the combination therapy relative to monotherapy.[98] A 2019 trial on a mutant HSV-1 known as canerpaturev (C-REV) combined with ipilimumab reported that the virus alone resulted in significantly increased infiltration of $CD4^+$ and $CD8^+$ in over one-half of melanoma patients and that the combination therapy provided an ORR of 41% and disease control rate of 68%, which were higher than the corresponding values of 4% and 16% observed in a trial of ipilimumab monotherapy.[84,96,99] The trial also found that responders had higher levels of ICOS on $CD4^+$ T cells,[86] which has been indicated as a pharmacodynamic biomarker for anti-CTLA therapy.[31] Another trial for the treatment of advanced melanoma with CVA21 combined with ipilimumab released interim results stating that tumors had increased immune cell infiltration and expression of genes associated with IFN-γ and immune checkpoints, supporting the rationale behind the combination therapy.[100]

In a small (n = 21) but promising 2017 phase 1b clinical trial, patients with metastatic melanoma were treated with T-VEC, followed by a combination of T-VEC and the anti-PD-1 antibody pembrolizumab.[95] The ORR was 62%, and the complete response rate was 33%. Biopsies from patients who responded to this dual therapy revealed an increased density of infiltrating $CD8^+$ T cells, increased tumor cell expression of PD-L1, and increased T cell expression of PD-1. Increased IFN-γ mRNA and granzyme B post-treatment specifically indicated an increase in cytotoxic T cell activity. These trends were observed from biopsies taken between virotherapy and combination therapy, as well as those taken after both therapies had been administered. Thus, this study supports the hypothesis that oncolytic virotherapy leads to recruitment of cytotoxic T cells that could be further activated by PD-1 blockade and that combination therapy modulated the TME to favor antitumor immunity.[95] Interim results of a phase II study of treatment of recurrent glioblastoma with the oncolytic adenovirus DNX-2401 combined with pembrolizumab have also reported that the therapy is generally well tolerated with promising effects on disease control and survival.[101]

OVs CAN ALSO BE ENGINEERED TO MEDIATE DIRECT ENGAGEMENT OF TUMOR CELLS WITH IMMUNE CELLS IN THE TME

Studies in recent years from us and others have shown that virotherapy can impact the immune cell landscape by attracting the migration of immune cells to TME,[81] the so-called converting "cold tumors" to hot ones.[95] Other studies have shown that there is an early influx of innate immune cells, including macrophages and NK cells, in response to tumor virotherapy. Several strategies have been developed to exploit the changes in the immune landscape during virotherapy by engaging the infiltrating immune cells to attack tumor cells (Figure 1). First among them, are the bispecific T cell engagers (BiTEs). These are

bispecific antibodies, consisting of two single-chain variable fragments (scFvs) with one binding to the CD3 receptor on the surface of T cells and the other engaging a target antigen on the surface of cancer cells. This results in activation of T cells and consequential killing of tumor cells independent of the MHC-peptide-TCR complex. BiTEs have shown impressive results in the treatment of hematological malignancies;[102–139] however, their application in the treatment of solid tumors is limited. This may be due to penetration issues into the TME or toxicities associated with off-target activity. These problems can potentially be overcome by encoding BiTEs in OVs.[105]

The possibility of tumor-restricted expression, combined with the infiltration of immune cells into the TME, renders OVs as promising gene-delivery tools for intratumoral expression of BiTEs. The first BiTE-armed OV to undergo preclinical evaluation was a double thymidine kinase (TK)-deleted VV with a secretory BiTE, specific for CD3 and the tumor cell-surface antigen ephrin type A receptor 2 (EphA2), and has been demonstrated to significantly enhance antitumor immunity.[106] BiTE has since been incorporated into other OVs such as adenovirus[107] and measles virus[108] and others that have been described in Table 1. The dynamic design of BiTEs offers flexibility in replacing scFvs to target various receptors on immune cells and various antigens on the tumor cells. Freedman and colleagues[107] modified the oncolytic group B adenovirus enadenotucirev (EnAd) with BiTEs targeting epithelial cell adhesion molecule (EpCAM) and crosslinking them to CD3 on T cells and demonstrated clustering and activation of CD4 and CD8 T cells. Taking a similar approach, another group[109] has armed an oncolytic adenovirus (ICOVIR-15K) with an epidermal growth factor receptor (EGFR)-targeting BiTE. The authors reported improved efficacy in two xenograft mouse tumor models. Other BiTE-armed OVs, which are currently under investigation, are the oncolytic measles virus, encoding carcinoembryonic antigen (CEA)-[110] and CD20-targeting BiTEs.[111]

In 2017, the FDA approved the use of BiTE targeting both CD19 and CD3, blinatumomab, for the treatment of a rare type of acute lymphoblastic leukemia (ALL).[104] Moreover, trispecific antibodies binding to NK or T cells have also been explored to treat cancer. Vallera and group[112] designed a unique IL-15-trispecific killer engager (TriKE) that contains a scFv against CD16 and CD33. This engager links NK cell with $CD33^+$ myeloid targets, creating an immunological synapse that also combined with an IL-15 crosslinker that promotes NK cell expansion and survival. Another similar strategy has also been explored, where OVs are used to express T cell-activating molecules. These activating molecules are called membrane-integrated T cell engagers (MiTEs), which were expressed in an oncolytic adenovirus, selective to CD46-expressing tumor cells.[113] This approach can potentially overcome immune suppression in the TME by antigen-independent activation of T cells. MiTEs thus can mediate the engagement of infected cells with the tumor cells to spark the host immunological responses, leading to a broader anti-tumor immune response. We have shown in our recent studies that arming an oncolytic HSV with a novel chimeric molecule that can engage NK cells with tumor cells via protein L and a TAA ligand can also enhance the antitumor efficacy of the virotherapy.[114]

Although arming OVs with cancer cell-targeting BiTEs/trispecific T cell engagers (TriTEs) or bispecific killer engagers (BiKEs)/TriKEs is promising, there is a risk of premature clearance of infected tumor cells hampering OV replication and spread. An alternate approach is to target non-transformed cells (cancer-associated fibroblasts [CAFs], adipocytes, endothelial cells, as well as a range of immune cells such as macrophages, myeloid-derived suppressor cells, Tregs, and neutrophils), all in co-existence in the TME that plays a critical role in enhancing tumor growth, immune suppression, and metastasis. Therefore, targeting the TME using armed OVs encoding BiTEs is a viable option. But challenge remains; e.g., lack of tumor-restricted surface antigen of TME cells limits the therapeutic application. Fibroblast activation protein (FAP)-α is overexpressed in CAFs. It thus represents an attractive target for the TME-focused generation of OVs. Consequently, researchers have investigated the use of FAP as a target for BiTE, to which, Chen and Song[115] and Song and team[116] have constructed an oncolytic VV encoding BiTE specific for murine CD3 and FAP- fibroblast activation protein (mFAP-TEA-VV). It has proven to exhibit potent antitumor activity in an immunocompetent mouse melanoma model, which is a result of BiTE-armed OV spread and destruction of tumor stroma. Based on the same principle, another group also constructed an OV encoding a BiTE that targeted FAP on CAFs and CD3e on T cells, leading to the death of the fibroblasts and simultaneously activating T cells within the TME.[117] Another BiTE-targeting FAP on CAFs and CD3e on T cells was constructed by inserting it into the oncolytic adenovirus (ICO15K-BiTE).[118] The engagement of the CD3 T cell with the CAFs led to T cell activation, proliferation, and the cytotoxic death of FAP^+ CAFs in the TME. Overall, BiTE-armed OVs enhanced intratumoral infiltration/accumulation of T cells and decreased the FAP expression in the treated tumors.[118] Engineering OVs to express TME-targeted BiTEs offers a unique advantage, as this synergizes the immune-stimulating activities of the OV with direct oncolysis from viral spread and infection. Scott et al.[119] developed BiTE- and TriTE-armed adenoviruses, in which the molecules were designed to recognize CD3e on T cells and CD206/folate receptor b on M2-like macrophages/tumor-associated macrophages (TAMs). This strategy guides T cells to kill tumor-associated macrophages in the TME instead, thus likely improving the therapeutic benefit.

There are also reports on exploring the possibility of combining armed OVs with chimeric antigen receptor (CAR)-T cell therapy. Suzuki and team[120] have constructed a BiTE molecule designed to target CD44v6 on cancer cells that can engage CAR and crosslink TCR. This molecule was incorporated into an oncolytic adenovirus, together with IL-2 and an anti-PD-L1 antibody, making it a CAdTrio. CD44v6 BiTE expressed from CAdTrio engaged human epidermal growth factor receptor 2 (HER2)-specific CAR T cells with $CD44v6^+$ cancer cell lines to induce cytotoxicity, leading to rapid and sustained disease control of orthotopic $HER2^+$ and $HER2^-$ $CD44v6^+$ tumors. This approach ensured dual targeting for two

tumor antigens by simultaneously engaging native TCR and CAR, resulting in improved therapeutic efficacy.[120]

Efforts are made in our lab in designing novel chimeric molecules that engage either NK cells CD16 receptor[114] or both NK and T cells via NKG2D- Natural killer group 2 member D receptor [121]. Instead of use of the traditional scFv antibodies, we chose to use ligands as the targeting moieties. The reason for such a design is the concern on the high binding affinity of scFvs used in either BiTE or CAR-T cells and its potential link to the enhanced release of cytokines and the consequential cytokine storms.[99,122] The affinity of ligand binding is usually significantly lower than that of a scFv. As such, this design mimics "affinity tuning" that has been applied for increasing the safety of BiTE.[123] Our data showed that such engagers, once incorporated into a HSV-2-based OV, can significantly potentiate the overall antitumor activity of the OV.[114] Most importantly, our data revealed that the combined effect from the direct oncolytic effect of the virus and the engaged NK cells could lead to the induction of neoantigen-specific antitumor immunity.[114]

All of these studies suggest that arming OVs with immune cell engagers is a versatile approach that comes with multiple forms of engagers (BiTEs and TriTEs), immune cell stimulators (MiTEs), and killer engagers (BiKEs and TriKEs). The armed OVs can target both cancer cells and tumor-associated stroma to promote anti-tumor immunity, resulting in enhanced therapeutic efficacy.

CONCLUDING REMARKS

Almost 30 years have passed since the beginning of the modern era of oncolytic virotherapy (i.e., specifically modifying a virus for the oncolytic purpose). Currently, there is one OV (T-VEC) that has received FDA approval for clinical use, and many others are at different stages of preclinical and clinical development. It is expected that more FDA approval on OV will come soon. It is likely that the new OV approved for clinical use will contain a component that can genuinely enhance antitumor immunity. As non-exhaustively mentioned in this review, many approaches and immune-stimulating molecules have been incorporated into various OVs with the intention to potentiate antitumor immunity. The question is which arming strategy for OV is the one that may produce the most desirable effect. The answer to this question is not straightforward, considering the complexity of the involving factors in this intricate process. For example, the intrinsic nature of the backbone virus (e.g., whether a DNA or RNA virus), the capability and speed of the virus in lysing tumor cells, and the impact on the immune cell landscape in TME are unique and different among the various OVs. Nevertheless, it is expected that the incorporated approach may need to have the combined capability in inducing a tumor-specific T cell response as well as impacting the immune landscape in favoring the infiltration and functionality of the induced antitumor immunity.

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AUTHOR CONTRIBUTIONS

Conceptualization, writing – original draft, and writing – review and editing, D.A.B.; conceptualization and writing – review and editing, D.R.; conceptualization, writing – review and editing, and supervision, S.X.Z.

DECLARATION OF INTERESTS

S.X.Z. is a co-founder of Tomahawk Therapeutics Inc., which is developing an oncolytic herpes simplex virus constructed in his lab for clinical application.

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