

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 OV, 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 OV 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, OV 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 OV display intrinsic tumor tropism (naturally occurring OV), 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, OV 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 eIF2 α and improved anticancer efficacy (21). The key point is that some OV 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 OV 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).

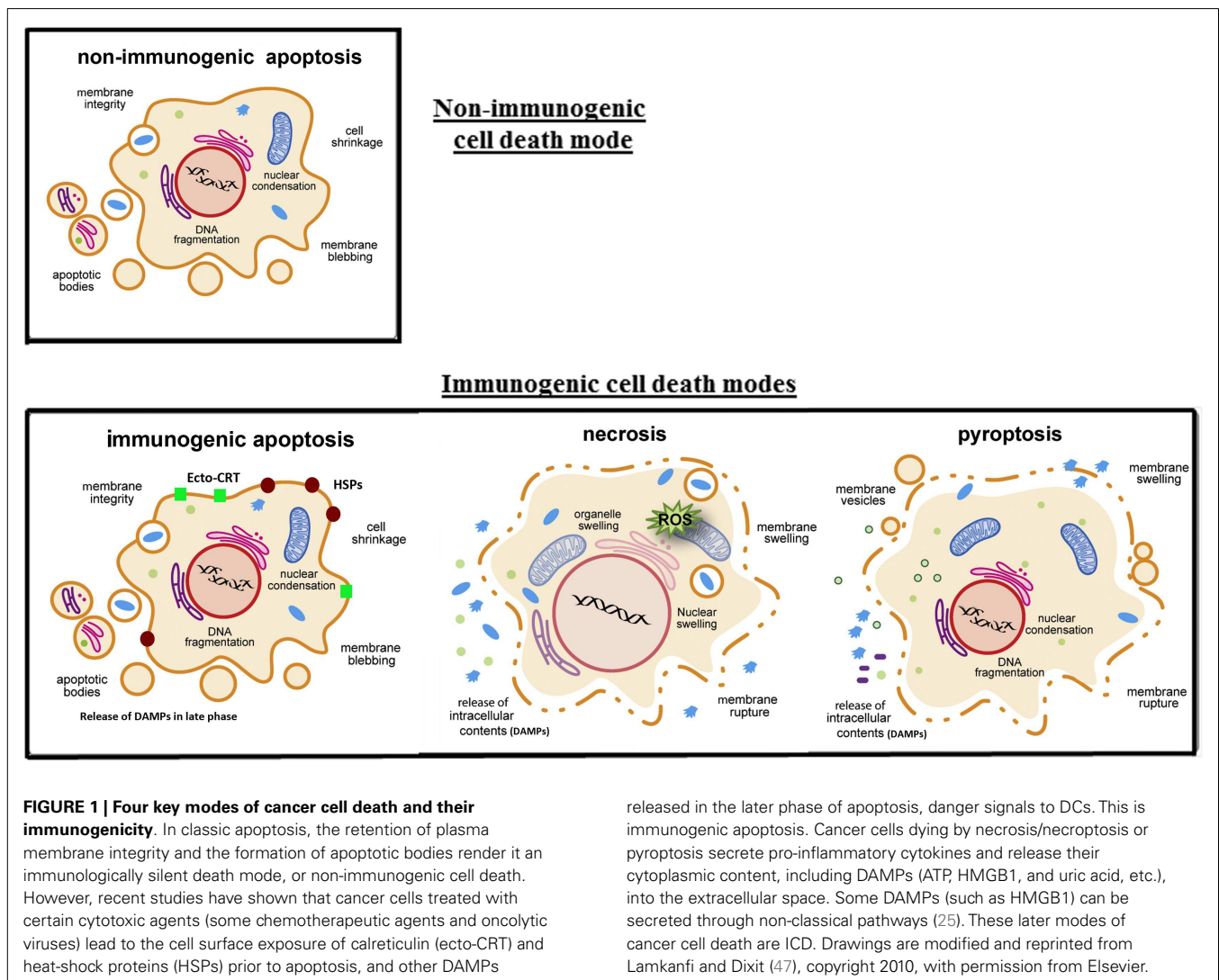
Damage-associated molecular patterns are molecules derived from normal cells that can initiate and perpetuate immunity in response to cell stress/tissue damage in the absence of pathogenic infection. DAMPs vary greatly depending on the type of cell and injured tissue. They can be proteins, DNA, RNA, 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 Tesnière, 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.



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-1 β 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 elicitation 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- β 1 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 <i>in vivo</i> 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-encoded 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-2 α 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 γ 34.5 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 OV, 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.

ACKNOWLEDGMENTS

This work has been supported by the grants P01CA132714 and R01CA155925 from the National Institutes of Health. Additional

Support was provided by David C. Koch Regional Therapy Cancer Center. We would like to thank Ms. Roshni Ravindranathan for critical reading and comments on the manuscript.

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Conflict of Interest Statement: David L. Bartlett is a scientific advisor for and has financial interest with Jennerex Biotherapeutics, a biopharmaceutical company developing oncolytic virotherapy. The other authors declare no conflict of interest.

Received: 07 February 2014; accepted: 24 March 2014; published online: 10 April 2014.

Citation: Guo ZS, Liu Z and Bartlett DL (2014) Oncolytic immunotherapy: dying the right way is a key to eliciting potent antitumor immunity. *Front. Oncol.* **4**:74. doi:10.3389/fonc.2014.00074

This article was submitted to *Tumor Immunity*, a section of the journal *Frontiers in Oncology*.

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