Thunder and Lightning: Immunotherapy and Oncolytic Viruses Collide

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For the last several decades, the development of antitumor immune-based strategies and the engineering and testing of oncolytic viruses (OVs) has occurred largely in parallel tracks. Indeed, the immune system is often thought of as an impediment to successful oncolytic virus delivery and efficacy. More recently, however, both preclinical and clinical results have revealed potential synergy between these two promising therapeutic strategies. Here, we summarize some of the evidence that supports combining OVs with immuno-therapeutics and suggest new ways to mount a multipronged biological attack against cancers.

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INTRODUCTION

Advanced metastatic cancers are largely incurable and the last several decades of research into the biology of cancer has made it clear just why this is. Cancers have found multiple different ways to usurp signaling pathways to gain a growth advantage, making it unlikely that pharmacological attack on a single molecular target will significantly impact the long-term progression of the malignancy.[1] Furthermore, tumor cells become very heterogeneous (genetically and phenotypically) as they evolve under the selective pressure of their microenvironment.[2] The question becomes "how to deal with the chameleon-like behavior of evolving malignancies" that allows them to escape therapeutic intervention. We argue that what is required is a therapeutic strategy that can match the heterogeneity of the tumor and utilize the same activated pathways that drive tumor cell growth. Our immune systems have the capacity to rapidly respond and evolve to deal with a vast array of complex invading microorganisms and certainly have the potential to recognize the antigenic variations presented by malignant cells.[3] Viruses, on the other hand, have evolved to take advantage of many of the same pathways that cancer cells activate during their malignant progression and inherently activate both innate and adaptive immune responses.[4,5] Recent clinical and preclinical studies argue that there is significant interplay between viral and immune therapy approaches to cancer and that thoughtful partnering of these strategies could turn the tide on cancer.

STIMULATING ANTITUMOR IMMUNITY: HARNESSING BOTH INNATE AND ADAPTIVE RESPONSES

When tumor-associated antigens (TAAs), and the cytotoxic T cells (CTL) capable of recognizing them were identified and isolated toward the end of the last century, it seemed it would only be a matter of time before clinical strategies to activate specific, adaptive antitumor immunity would be improving patient outcomes.[6,7] Various approaches to present TAA to the immune system in an immuno-stimulatory context have been successfully piloted in preclinical animal models and early clinical trials; whole cells, cell lysates, proteins, single/multiple/long peptides, DNA and RNA were given with adjuvants or immune effector cells [particularly dendritic cells (DCs)], and shown to elicit CTL.[8] However, the final translational steps of proof of clinical benefit and adoption into routine clinical practice have proved elusive to date. For some time, the identification of TAA arguably led to a disproportionate focus on the adaptive arm of the antitumor immune response, to the exclusion of therapeutic strategies addressing nonspecific innate immune activation, despite its critical role in the early stages of adaptive priming. Significantly, clinical data show a correlation between improved outcome and infiltration into tumors of both innate natural killer and adaptive T cells, for example, in colorectal cancer,[9,10] and one of the few cancer immunotherapies in widespread clinical use—the intravesical administration of Bacillus Calmette–Guerin for superficial bladder cancer—is clearly innate and nonspecific in its action, utilizing antimicrobe immunity for antitumor effects.[11]

As the mechanisms underlying successful cancer immunotherapy were shown to include linked innate and adaptive effectors (for example cross-activation between natural killer cells and DC[12,13]), the importance of nonspecific as well as specific immune activation has become increasingly recognized, and both arms of the immune response have recently taken significant steps forward in the clinical arena (**Table 1**).[8–10,12,14–22] From a TAA-specific, adaptive perspective, the US Food and Drug Administration approval of sipuleucel-T (Provenge—a DC-based treatment for prostate cancer[23]) is encouraging, while the demonstration that ipilimumab (a nonspecific innate immunomodulatory antibody

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Table 1 The armory: antitumor immune mediators

Mediator	Role/mode of action	Clinical benefit/observation	References
CTL/TIL/CAR modified T cell	MHC Class I restricted tumor cell lysis	Enhanced tumor infiltration by responsive T lymphocytes corresponds with improved outcome of some cancers.	Galon *et al.* (2006)[10]; Hodi *et al.* (2010)[14]
NK cell	Antigen nonspecific tumor cell lysis	Enhanced infiltration of CRC tumors with NK corresponds with improved outcome.	Coca *et al.* (1997)[9]; Stagg *et al.* (2007)[15]
DC	Cross presentation of tumor antigen to CTL; NK activation	DC vaccination is an approved prostate cancer immunotherapy (Provenge)	Ilett *et al.* (2010)[8]; Ullrich *et al.* (2008)[16]; Degli-Esposti *et al.* (2005)[12]
Treg	Maintenance of peripheral tolerance; immune suppression	Impact of Treg depletion in cancer immunotherapy is inconclusive.	Mougiakakos *et al.* (2010)[17]
Antitumor antibody	Complement fixation/ADCC	Benefit in addressing minimal residual disease; used in treatment of non-Hodgkin's lymphoma	von Mensdorff-Pouilly *et al.* (2010)[18]
NKT cell	Tumor immunosurveillance; cytotoxicity	Abnormal number and function of NKT cells accompanied by poor clinical outcome.	Hong ***et al.*** (2007)[19]; Motohashi *et al.* (2009)[20]
Inflammatory cytokines	Polarize immune reactions; promote antitumor effector functions	GMCSF inclusion in anticancer vaccine strategies has shown clinical benefit in numerous clinical trials.	Müller-Hübenthal *et al.* (2009)[21]; Gupta *et al.* (2010)[22]

Abbreviations: ADCC, antibody dependent cellular cytotoxicity; CAR, chimeric antigen receptor; CRC, colorectal cancer; CTL, cytotoxic T lymphocyte; DC, dendritic cell; GM-CSF, granulocyte macrophage colony stimulating factor; MHC, major histocompatibility complex; NK, natural killer cell; TIL, tumor infiltrating lymphocyte; Treg, regulatory T cell.

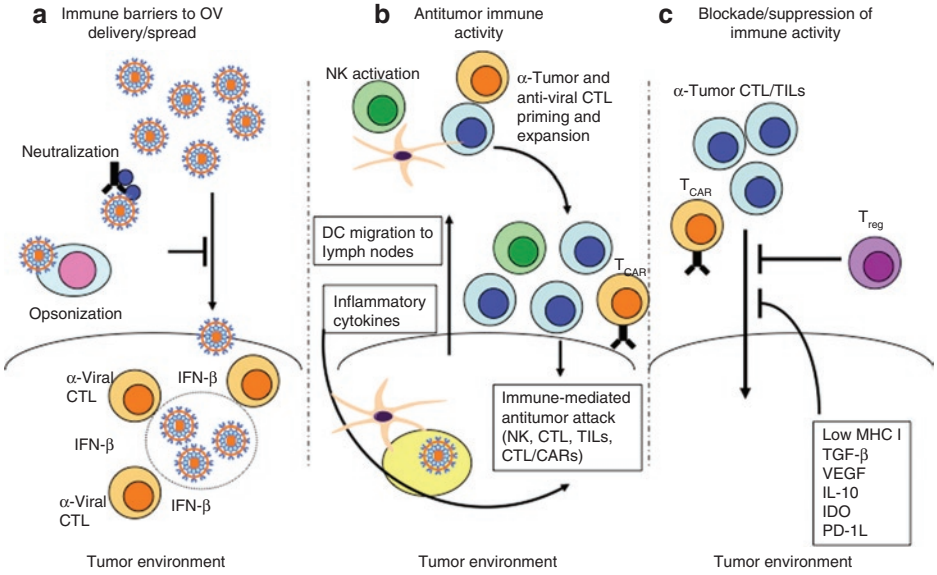

Figure 1 It takes two to tango: striking the balance between antitumor activity, and antivector immunity. (**a**) Immune barriers to oncolytic virus (OV) delivery/spread. (**b**) Antitumor immune activity. (**c**) Blockade/suppression of immune activity. CAR, chimeric antigen receptor; CTL, cytotoxic T lymphocyte; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer cell; TGF-β, transforming growth factor-β; TIL, tumor infiltrating lymphocyte; VEGF, vascular endothelial growth factor.

which blocks inhibitory CTLA-4) improves survival of patients with metastatic melanoma,[14,24] shows that specificity is not a prerequisite for therapeutic success. Hence, separate clinical progress with both specific (adaptive) and nonspecific (innate) cancer immunotherapy is now a reality; it would be ideal if the two could be harnessed together.

ONCOLYTIC VIRUSES: CREATING AN IMMUNE STORM WITHIN TUMORS

Oncolytic virus (OV) therapeutics are designed to rapidly and specifically grow in tumors with the primary objective of directly lysing cancer cells. However, it is becoming clear that their targeted infection of the tumor has the potential to create an "inflammatory storm" that arouses the innate and adaptive immune responses against tumors (**Figure 1b**). Indeed it appears that in some instances, during natural virus infections, an immune response can be generated that may protect from the onset of certain kinds of cancers.[25,26] Perhaps the transient expression of "neoantigens" during normal tissue repair in the context of a severe inflammatory reaction leads to the generation of an immune response with cancer surveillance properties.[25] There is increasing evidence that therapeutic virus mediated destruction or damage of tumors can lead to an antitumor immune response (**Figure 1b** and **Table 2**).[27–58] For instance, over a decade ago, Mastrangelo and his colleagues demonstrated that in advanced melanoma patients it was possible to use intralesional "vaccination" with an oncolytic

Table 2 The dream team: antitumor immune responses elicited by OVs

OV	Mediator	Mechanism of Action	References
HSV-1 (*ICP0* null)	Cytotoxic T Lymphocyte (CTL)	Antiviral and antitumor responses contribute to efficacy in murine breast cancer model	Sobol *et al.* (2011)[27]
HSV-1 (*ICP34.5* null)	CTL	Enhanced DC maturation, increased tumor infiltration of IFN-γ^+ CTL in murine ovarian cancer model	Benencia *et al.* (2008)[28]
HSV-1 (*ICP34.5* null)	NK/CTL	IT injection results in IFN, MIG, IP-10 production and subsequent infiltration of NK and $CD8^+$ T cells.	Benencia *et al* (2005)[29]
HSV (*α47* null)	CTL	Enhanced MHC I expression in human cells, enhanced stimulation of matched T cells.	Todo *et al.* (2001)[30]
HSV-2	T lymphocytes	Strong T cell responses against primary or metastatic tumors; variety of immune competent murine models.	Li *et al.* (2007)[31]; Li *et al.* (2007)[32]; Endo *et al.* (2002)[33]; Toda *et al.* (1999)[34]; Toda *et al.* (2002)[35]
HSV-*GMCSF*	Unspecified	OncoVEX undergoing phase 3 clinical trials for melanoma and squamous cell carcinoma of the head and neck; induction of adaptive antitumor immune responses.	Hu *et al.* (2006)[36]; Harrington *et al.* (2010)[37]
Reovirus	CTL	Generation of antitumor CTL against B16 tumors independent of oncolysis; enhanced priming of human CTL against Mel888 cells.	Prestwich *et al.* (2009)[38]
Reovirus	T/NK	Expansion of $CD3^+/CD4^+$ and $CD8^+$/perforin$^+$/granzyme$^+$ T cells, and enhanced circulating $CD3^-/CD56^+$ NK cells in patients on phase 1 trial with IV reovirus (T3D).	White *et al.* (2008)[40]
Reovirus	NK Cells	DCs loaded with reovirus infected melanoma cells results in NK activation and IFN-γ secretion.	Prestwich *et al.* (2009)[39]
Reovirus	NK/CTL	DCs activated upon reovirus infection; antigen non-restricted tumor cell killing by NK and T cells.	Errington *et al.* (2008)[41]
Measles	CTL	Measles virus infected mesothelioma cells activated DCs and primed autologous CTL.	Gauvrit *et al.* (2008)[42]
MV-*IFN-β*	$CD68^+$ cells	Infiltration of $CD68^+$ innate immune cells in murine mesothelioma improved survival.	Li *et al.* (2010)[43]
Measles	T lymphocytes	Phase 1 trial of cutaneous T cell lymphoma: increased IFN-γ^+ and $CD4^+/CD8^+$ T cell infiltration; overall expansion of $CD8^+$ T cells.	L Heinzerling *et al.* (2005)[44]
Adenovirus (Ad-*p53*T)	CTL	Induction of a therapeutically effective tumor-directed CTL response.	Gürlevik *et al.* (2010)[45]
Adenovirus (Ad-*GMCSF*)	Neutrophils	Oncolytic adenovirus-*GMCSF* induced neutrophil infiltration and inflammation.	Bristol *et al.* (2003)[46]
Adenovirus (Ad-*GMCSF*)	CTL	Phase 1 trial: IT treatment with Ad-*GMCSF* led to post-treatment enhancement in circulating antitumor IFN-γ secreting CTL.	Cerullo *et al.* (2010)[47]
Vaccinia (JX-594)	Multiple	Melanoma lesions treated IT with JX-594 showed immune infiltration and regression of untreated lesions (phase 1 trial).	Mastrangelo *et al.* (1999)[48]
Vaccinia (VV-*ova*)	T lymphocytes	Priming with OVA DNA vaccine and IT treatment with VV-ova enhanced CTL infiltration and killing of OVA-expressing tumors.	Chuang *et al.* (2009)[49]
Vaccinia	T lymphocytes	Heterologous prime-boost with VV and Semliki forest virus vectors elicits antitumor immunity against murine ovarian surface epithelial carcinomas.	Zhang *et al.* (2010)[50]
Vaccinia (*B18R* null)	IFN-β	Complete tumor response associated with protection from tumor rechallenge in CMT93 murine tumor model.	Kirn *et al.* (2007)[51]
VSV	CTL	CTL arose against viral and tumor epitopes; antitumor CTL are critical for efficacy of IT VSV	Diaz *et al.* (2007)[52]
VSV	NK/IL-28	IL-28 induced by VSV sensitized tumors to NK recognition and activation.	Wongthida *et al.* (2010)[53]
VSV	CTL/NK	Strong correlation between viral gene expression, proinflammatory reaction and therapeutic outcome in B16ova model.	Galivo *et al.* (2010)[54]
VSV-*IFN-β*	$CD8^+$ T cell	IFN-β potentiated $CD8^+$ T cell generalized reaction in AB12 murine mesothelioma model.	Willmon *et al.* (2009)[55]
VSV	CTL	Priming with Ad-tumor Ag prior to treatment with VSV-tumor Ag improved survival via antitumor CTL.	Bridle *et al.* (2010)[56]
VSV	T lymphocytes	Anti-B16 immunity contributes to purging metastases from spleen and lymph nodes and protected from long term metastatic disease.	Qiao *et al.* (2008)[57]
NDV	T lymphocytes	NDV expressing tumor antigen (+/− *IL-2*) enhanced tumor infiltration by T cells; therapeutic potential was T cell dependent.	Vigil *et al.* (2008)[58]

Abbreviations: Ad, Adenovirus; Ag, antigen; CTL, cytotoxic T lymphocyte; DC, dendritic cell; GM-CSF, granulocyte macrophage colony stimulating factor; HSV, Herpes simplex virus; IFN, interferon; IL, interleukin; IP-10, interferon-γ inducible protein 10; MIG, monokine induced by interferon-γ; NDV, Newcastle disease virus; NK, natural killer cell; OV, oncolytic virus; OVA, ovalbumin; T3D, type 3 Dearing; VSV, vesicular stomatitis virus; VV, vaccinia virus.

vaccinia virus expressing granulocyte macrophage colony stimulating factor (*GMCSF*) (trade-name JX-594[59]) to generate significant clinical responses that correlated with antitumor immune responses. Injected tumors became inflamed and infiltrated with a variety of immune cell types. Significantly, tumors that were not injected with JX-594 responded, suggesting that systemic antitumor immune responses had evolved during therapy.[48,60]

It has since become increasingly clear with other oncolytic virus platforms that the immune responses triggered by oncolytic virus infection is a critical component of the clinical benefit of these therapeutics. Reovirus, a naturally occurring, unmodified virus that has already completed significant clinical testing (as Reolysin), and has just entered phase 3 for head and neck cancer, can elicit antitumor immune activation.[61] In some models, reoviral replication and direct oncolysis are not necessarily required for therapy,[38] although the clinically relevant contribution of direct tumor killing and antitumor immune activation for any OV remains to be elucidated in patients. The antitumor immune effects of reovirus can be enhanced with the addition of interleukin-2 (IL-2),[62] and are associated with adaptive priming against TAA in tumor-draining lymph nodes,[61] illustrating that both innate and adaptive arms of the immune response can be exploited to improve therapy. This murine data is consistent with human *in vitro* systems, which show that reovirus activates DCs[41] to both stimulate natural killer cells and prime specific antitumor CTL. For viruses that can readily be genetically modified, the potential of antitumor immune activation after OV treatment has been further exploited to improve therapy. A range of genes has been incorporated into a number of viruses, although immuno-stimulatory modification of a virus does not inevitably enhance antitumor therapy. A vesicular stomatitis virus (VSV) encoding *CD40L* was no better than its unmodified equivalent on intratumoral injection, and indeed was less effective than a nonreplicating adenoviral vector expressing *CD40L*.[54] In this case, early nonspecific T-cell activation initiated by replicating VSV-*CD40L* distracted the immune response away from TAA, illustrating how important it is to compare, select, and optimize different viral and gene platforms in the context of innate and adaptive OV-associated antitumor immunity.

To date, *GMCSF* is the immune gene inserted most successfully into clinically advanced OV. This preference for GMCSF derives from its potent ability to generate systemic adaptive antitumor immunity *in vivo* after expression in tumor cells,[63] which is associated with the recruitment and differentiation of activating DC in the tumor microenvironment. As alluded to above, a replicating vaccinia virus expressing *GMCSF* (JX-594) has shown promise in preclinical[64,65] and clinical studies,[64] and is rapidly progressing toward phase 3 testing. A replicating herpes simplex virus type-1 expressing *GMCSF* caused tumor regression in mice,[66] a finding reproduced in a phase 2 trial in melanoma.[67] Currently, this virus (Oncovex) is being tested in a phase 3 clinical study, which is recruiting in both the United States and Europe. The other immunomodulatory gene which has been inserted most often into OV to date is interferon-β (*IFN-β*), although these viruses have not yet progressed as far in clinical testing as those expressing *GMCSF*. Interestingly, the initial aim of *IFN-β* expression by OV was to restrict viral replication in normal tissue, thus increasing direct oncolysis and the therapeutic index. However, IFN-β, despite its role in innate antiviral immune responses, can also support activation of antitumor immunity when expressed in vaccinia,[51] VSV,[55] and measles[43]. Hence genetic modifications which support both adaptive (*GMCSF*) and innate (*IFN-β*) antitumor immunity have been applied to improve OV therapy. Various other immunomodulatory molecules (including IL-12, IL-24, IL-4, RANTES, CD80, IL-18, and IFN-α), which impact on immunity via a range of effector pathways, have also been proposed for expression by OV.[68]

How can oncolytic virotherapy and cancer immunotherapy most effectively unite to improve potential treatment for patients? One key issue is how OV are delivered and access the cancer. In the largest, most promising published clinical trials to date, oncolytic viruses have been injected directly into the tumor, to initiate both local and distant regression.[64,67] Intratumoral delivery avoids the concern of virus neutralization by circulating antibodies (**Figure 1a**) and suits the paradigm whereby the mere presence of a virus within a tumor can act as a "danger signal" to alert and activate the immune system.[69] However, despite the acceptance of the intratumoral route used in the current phase 3 trial of Oncovex in melanoma, systemic intravenous delivery, if effective, is always likely to be more popular with clinicians. Moreover, there is currently no clinical evidence that the antiviral immune response to systemic OV impairs therapy in patients; indeed relatively late tumor regression can occur at a time when neutralizing antibody levels are known to be high. Indeed, in some preclinical models, the anticancer activity of oncolytic vaccinia was actually enhanced when animals were preimmunized against the virus.[70] More clinical experience will be required to determine the optimal mode of virus delivery to malignancies but, as discussed below with some viruses, systemic administration may be critical to maximize the immune boosting effects of some platforms.[71] In the meantime, as early OV clinical experience slowly accumulates, there is also a growing realization that apparently unrelated novel strategies to stimulate antitumor immunity, as well as the optimal application of traditional prime-boost immune vaccine sequencing, may have enormous potential in relation to OV, and it is to these that we turn next.

IMMUNOTHERAPY TO COMPLEMENT ONCOLYTIC VIROTHERAPY: ACTIVATING CELLULAR ASSASSINS TO KILL TUMORS

One of the exciting new strategies in immunotherapy is the adoptive T-cell therapy protocol developed by Rosenberg's group at the National Cancer Institute wherein tumor infiltrating lymphocytes (TILs) are isolated and expanded *ex vivo* before reinfusion back into the patient.[72,73] The successful application of this approach requires significant *in vivo* expansion of the infused cell product and this only occurs if the patient first undergoes chemotherapeutic or radiotherapeutic lymphodepletion.[74,75] While the response rates with this approach are breathtaking (objective tumor responses in up to 70% of cases[75]) patients experience sometimes lethal virus reactivation and other side effects of cytotoxic chemotherapy that reduce patients' quality of life.

Autologous T cells specific for Epstein–Barr virus (EBV) derived proteins have produced complete remission of disease in over 60% of patients with multiply relapsed or refractory EBV-associated lymphoma[76] while *ex vivo* expanded TILs have produced

complete remissions in patients with melanoma.[73] However, the extension of these successes to a broader range of tumors will require strategies to overcome many different mechanisms of immune evasion used by tumors to avoid immune elimination.[77] Perhaps, foremost of these mechanisms is poor presentation of tumor antigens to effector T cells. Not only do tumors downregulate molecules such as peptide transporter molecules,[78] endoplasmic reticulum aminopeptidases[79] and HLA class I molecules that are essential for antigen processing and presentation, but also they inhibit the maturation of local professional antigen-presenting cells by secreting IL-10 and transforming growth factor-β.[80,81] This inhibits their expression of costimulatory molecules, like CD80, CD86, and 41BB-ligand that are essential for the expansion of T cells activated by recognition of antigen through their T-cell receptor. Tumors also directly inhibit T cells and instead of costimulatory molecules, many tumors express coinhibitory molecules like PD-L1 and Caecam1 that signal through SHP1/2 phosphatases to dephosphorylate the kinases induced by T-cell receptor ligation and costimulation. Some tumors may not themselves express inhibitory molecules, but recruit inhibitory cell types that do. T-regulatory cells, myeloid suppressor cells, and tumor stroma secrete IL-10, vascular endothelial growth factor, and transforming growth factor-β and express arginase and indoleamine 2,3-dioxygenase that deplete amino acids from the tumor environment and induce metabolic stress in T cells (**Figure 1c**). Several clinical trials have indicated that *in vivo* expansion of adoptively transferred T cells is an absolute requirement for tumor-specific T-cell efficacy, so that ensuring T-cell expansion after infusion has emerged as the holy grail of T-cell immunotherapy.[77,82]

T-cell numbers in the body are maintained at a homeostatic steady state unless disturbed by infection or lymphopenia. Inflammatory responses to most pathogens result from the recognition of pathogen-associated molecular patterns by receptors on innate immune system cells like dendritic cells and natural killer cells. For example, toll-like receptors recognize structures unique to pathogens such as bacterial lipopolysaccharides, flagellins or double stranded RNAs, and toll-like receptor ligation signals the production of cytokines and chemokines that recruit and induce expansion of T cells specific for the infecting pathogen. Once the pathogen is eliminated the innate immune responses becomes quiescent and T-cell numbers return to their steady state. Unfortunately, even if tumor cells present tumor-specific antigens (TAs), they do not express pathogen-associated molecular patterns and therefore fail to activate the innate immune system. However, vaccines may be used to increase T-cell numbers and oncolytic viruses may encode several toll-like receptor ligands that effectively activate innate immunity.[83–86]

Another strategy that not only targets tumor antigens, but also enhances T-cell expansion in cases where tumor antigens are weak or unidentified, investigators have developed multifunctional CARs (chimeric antigen receptors) that can be expressed as transgenes in T cells and redirect T cells to tumor antigens, regardless of their native T-cell receptor specificity. CAR expressing T cells therefore can recognize and kill both tumor targets through their CAR and the natural target through their T-cell receptor. Each CAR is composed of single chain antibody variable regions that recognize whole antigens on a tumor cell surface, linked to the zeta ζ-chain of the T-cell receptor to trigger killing and to the intracellular endodomains of costimulatory molecules to trigger proliferation. Such receptors eliminate the requirement for antigen processing and presentation on HLA molecules and provide signals that induce T-cell cytotoxicity and proliferation upon antigen-receptor engagement, in principle eliminating the requirement for professional antigen presentation. In clinical practice, this strategy has yet to be optimized to produce antitumor effects without toxicity. The incorporation of a CD28 endodomain alone has so far been insufficient to induce extensive *in vivo* proliferation of transduced T cells, although a complete response of follicular lymphoma to a T cells expressing a CD19CAR encoding CD28 and zeta chain signaling domains infused after non-myeloablative conditioning has been described.[87] The addition of a 41BB endodomain to the CD28 endodomain onto a HER2-directed CAR to enhance T-cell proliferation produced a massive and fatal inflammatory response in a patient with metastatic colon cancer, who received a large dose (10^{10}) of cells after non-myeloablative chemotherapy.[88] Therefore, a strategy that balances *in vivo* proliferation and antitumor activity without toxicity is needed.

MARRYING ADOPTIVE CELL THERAPY WITH OVs: TIL(s) DEATH DO US PART?

Our group has evaluated the use of EBV-specific T cells as cellular hosts for CARs, with the idea that the *in vivo* presentation of EBV antigens by persistently infected B cells would ensure the correct stimulation of gene-modified EBV-specific T cells. We redirected EBV-specific T cells to the disialoganglioside, GD2 expressed by neuroblastoma using a GD2-specific CAR. Transduced EBV-specific T cells persisted for longer than similarly transduced CD3-activated T cells in an intra patient comparison in which three complete tumor remissions in 11 patients with relapsed disease were observed as well as tumor responses in 50%.[89] While EBV can produce potent antigenic stimulation *in vivo*, infused T cells compete with endogenous EBV-specific T cells that circulate with high frequency, and the degree of *in vivo* stimulation by EBV is uncontrollable. However, if T cells specific for oncolytic viruses could be produced from patients receiving virotherapy, then the T cells could be expanded, at will, using the OV as a vaccine. If the OV-specific T cells were modified to express a tumor-specific CAR, then virotherapy could be consolidated with tumor directed T-cell infusions (see **Figure 2**). The virotherapy would reduce the bulk of the tumor and modulate the immunosuppressive environment by activation of toll-like receptors and expression of transgenic immune enhancing cytokine like *GM-CSF*, while the T cells would eliminate residual and metastatic tumor cells that may be resistant to viral lysis. Additional modification of tumor cells with molecules that protect them from inhibitory ligands like transforming growth factor-β, may increase the potency of this approach.[90] Importantly, this strategy should have little toxicity, and should not require cytotoxic lymphodepletion.

OVs may also provide a solution to the problem of tumor antigen-specific T-cell anergy. While stimulation of peripheral blood T cells with viral antigens to which the donor has been exposed can reactivate polyclonal $CD4^+$ and $CD8^+$ T cells with specificity for multiple HLA class I and II epitopes in multiple viral antigens, this is rarely true for T cells specific for nonviral "self" antigens,

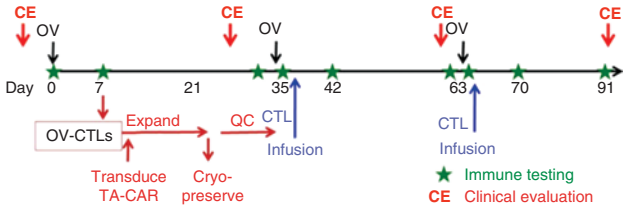

Figure 2 Combining oncolytic virotherapy with tumor-specific T cells. Oncolytic virus (OV)-specific T cells could be expanded *ex vivo* after the second vaccination. If the individual was already exposed to the OV by vaccination or prior infection, then the T cells could be manufactured earlier. After activation, the T cells could be transduced with a retroviral vector expressing a tumor-specific chimeric antigen receptor (CAR) to redirect their specificity to a tumor antigen. The transduced cytotoxic T lymphocytes (CTLs) could then be infused after virotherapy when the tumor load would be reduced. The OV could then be used as a vaccine to induce T-cell expansion and maintain function.

which are frequently tolerized during development and hence are weak and anergic to *in vitro* reactivation and expansion for use as T-cell therapy. While it is known that TILs have been successfully expanded from melanoma patients and retain their antitumor specificity, not all tumors have TILs and not all TILs can be successfully expanded *in vitro*. Enhanced reactivation of TA-specific T cells in patients who received an oncolytic adenovirus encoding human *GM-CSF* has been reported.[47] This characteristic of OVs may be exploited by the transgenic expression of tumor-encoded antigens, so that OV may be used not only to eliminate tumors, but to facilitate the *ex vivo* reactivation and expansion of TA-specific T cells that could subsequently be gene modified and infused as described above and further induced to expand by additional OV treatment.

CHOREOGRAPHING THE DANCE BETWEEN OVs AND THE IMMUNE SYSTEM—GETTING THE MOST OUT OF PRIMING AND BOOSTING

As discussed above, the ability of OVs to induce and express payloads of immune stimulating cytokines locally and to high levels within the tumor beds provides significant improvements in therapy both in animal models and in humans. Another strategy that is gaining support from several groups is to engineer OVs to encode and express TAAs. This has the advantage of expressing a relevant target antigen exactly at the time and site of an inflammatory reaction. Furthermore, the OV is likely to spread from the tumor bed and express the TAA in relevant immune organs (e.g., draining lymph nodes, spleen). Key to the success of this approach is selecting the correct/optimum tumor antigen. As one can imagine there are multiple parameters that could be considered in choosing a therapeutic target antigen and Cheever and colleagues have extensively reviewed a compendium of factors to be considered.[3]

Vigil and colleagues[58] have engineered an oncolytic NDV to express an artificial tumor antigen (β-galactosidase) and demonstrated that repeat intralesional administration of this virus into mice bearing tumors expressing the antigen was much more effective therapeutically. This approach would be especially useful in a situation where the tumor expresses a "foreign antigen" such as a viral protein (e.g., human papillomavirus) or a somatically mutated cellular protein. In a variation of this approach Chuang and colleagues vaccinated animals with a foreign antigen (ovalbumin or OVA) and then subsequently treated intratumorally with a vaccinia virus engineered to express *OVA*. This "prime boost" scenario is designed to educate or prime the immune system to recognize OVA and then locally boost this response by virus directed expression of the antigen at high levels within the tumor bed.[49] The observed increase in efficacy in this setting may reflect an epitope-spreading event within the tumor wherein new immune reactions against the tumor are generated. In principle by encoding *OVA* within the virus so that it is only expressed upon productive infection would allow systemic administration of the virus. This study demonstrated that it might be possible to design OVs to express antigens that the general population is already immunized against (e.g., diphtheria toxin) and then "boost" an already established immune response locally within the tumor through an oncolytic virus infection. Another prime boost strategy involves sequential treatment with two antigenically distinct oncolytic viruses expressing a common tumor associated antigen. Zhang and colleagues showed this is in principle possible by treating sequentially with oncolytic Semliki Forest Virus and Vaccinia Virus both encoding *OVA*.[50]

Bridle *et al.* have created a novel system that combines tumor-associated antigen immune stimulation with systemic oncolytic virus administration and may be the prime boost "pièce de résistance."[56,71] These authors reasoned that: (i) oncolytic destruction of tumors stimulates antitumor immunity, (ii) systemic administration of an OV is more likely to be effective against metastatic disease, (iii) OVs expressing tumor antigens increase immune response in infected tumors, (iv) prime:boost with heterologous expression systems is more likely to focus immunity on the tumor and not the vector.

To test their approach, they used a very aggressive and challenging tumor model which involved implanting the rapidly growing murine melanoma tumor (B16) in the brains of C57 mice.[91] The B16 tumor expresses an endogenous cellular antigen, dopachrome tautomerase (DCT) and so the authors engineered an oncolytic version of VSV that overexpresses *DCT* upon productive infection. To take advantage of the prime:boost strategy Bridle and colleagues vaccinated tumor bearing animals with an adenovirus vaccine vector expressing *DCT*. The Ad-*DCT* vaccine provided only modest improvement in animal survival although it did successfully generate a cellular anti-DCT response within the animals. They then showed that their replicating VSV-*DCT* oncolytic virus on its own could target brain tumors following intravenous administration and indeed demonstrated the virus caused substantive tumor destruction, but very limited impact on animal survival. What happens when a systemic oncolytic prime boost is used in this model? The results were quite remarkable: (i) ~40% of the circulating T cells in treated animals were now directed against DCT; (ii) there was substantive immunity generated against additional tumor antigens (epitope spreading); (iii) the immune response to VSV antigens was actually dampened (compared to treatment with VSV alone); and (iv) most importantly, some durable cures were observed. These results are striking considering the rapid growth of this tumor and its location within the brain. Furthermore the authors had broken tolerance to an endogenously expressed cellular antigen. So what are the

critical factors that lead to the impressive therapeutic outcomes observed by Bridle and colleagues. Sequential treatment with VSV-*DCT* (as both prime and boost) did not generate the impressive immune responses or improve animal survival arguing that a heterologous prime is required. Second, intravenous injection of the boosting vector is essential; intratumoral or subcutaneous VSV-*DCT* was ineffective. Perhaps a component of the activity requires that the VSV vector infects and expresses its TAA payload in a cell compartment that is only efficiently targeted by systemic administration. Other priming strategies are also effective with the oncolytic VSV-*DCT* boost suggesting that it may be effective with a number of vaccine platforms or perhaps in patients that have natural pre-existing anti-TAA immune responses that may just need a "jump-start".

WHAT'S NEXT?

The interplay between OVs and the immune system is at times a love–hate relationship. The ability to deliver OVs to tumors by systemic administration is a huge value of the platform but of course may be curtailed by the evolution of the immune response against the vector itself. When given appropriately in animal models, OVs are clearly capable of harnessing the innate and adaptive arms of the immune response they elicit, potentially bringing together both aspects of human cancer immunotherapy recently endorsed by the clinical success of sipuleucel-T[92] and ipilimumab.[93] The current consensus from the available preclinical data is that the immune response to OV is neither pure hindrance nor pure help, but something of both. The challenge is how best to manipulate the system to maximize benefit for clinical application. Additional factors which impact on the interface between oncolytic virotherapy and cancer immunotherapy are multiple and include the method as well as route of delivery ("neat" OV versus cell delivered[94]), co-treatment with other modalities (biotherapeutics,[95] small molecules,[96,97] chemotherapy,[98] and radiotherapy[99] and tumor-associated factors such as the vasculature and interstitial pressure.[100–102]

Old-fashioned vaccinology, as well as complementary advances in cancer immunotherapy which were not initially developed with OVs in mind, are now suggesting further rational strategies for improved viro-immunotherapy. Using OVs as vaccines to expand T cells which can be genetically modified with CAR, and protocols based on classic prime-boost immune priming are two examples in a field of united immune- and viral-therapies which is already blossoming in the laboratory. We believe that it is time to move toward more clinical testing of the ideas presented in this review, including extensive monitoring of the immune response against both virus and tumor in patients, to provide as much translational data as possible for continued iterative testing and optimization between laboratory and clinic.

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