Molecular Therapy
Review

Oncolytic Virus Combination Therapy: Killing One Bird with Two Stones

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Over the last 60 years an eclectic collection of microbes has been tested in a variety of pre-clinical models as anti-cancer agents. At the forefront of this research are a number of virus-based platforms that have shown exciting activity in a variety of pre-clinical models and are collectively referred to as oncolytic viruses. Our true understanding of the potential and limitations of this therapeutic modality has been substantially advanced through clinical studies carried out over the last 25 years. Perhaps not surprising, as with all other cancer therapeutics, it has become clear that current oncolytic virus therapeutics on their own are unlikely to be effective in the majority of patients. The greatest therapeutic gains will therefore be made through thoughtful combination strategies built upon an understanding of cancer biology.

Oncolytic or "cancer-lysing" viruses (OVs) have been designed or selected to kill cancer cells but have their replication blunted in normal tissues (extensively reviewed elsewhere[1–3]). Several interesting OV platforms are being evaluated pre-clinically and clinically including, but not limited to, adenovirus (AdV),[4] coxsackievirus,[5] herpes simplex virus (HSV),[6–8] Maraba virus,[9,10] measles virus,[11,12] Newcastle disease virus,[13] parvovirus,[14] reovirus,[15] vaccinia virus (VACV),[16] and vesicular stomatitis virus (VSV).[17,18] The mechanism of tumor selectivity of these viruses is variable, ranging from control of expression of viral genes by transcriptional elements that are uniquely/primarily used in cancer cells,[19–21] to cancer-specific virus receptor expression (e.g. Martin et al.,[22] Petrovic et al.,[23] and Bhatia et al.[24]), metabolic over-activity of tumor cells,[25] tumor-specific defects in antiviral responses,[26] and combinations thereof. In theory, OVs are the ultimate cancer-targeted therapeutic because they are able to selectively amplify themselves within the tumor milieu increasing therapeutic dose over time. However, when considered as stand-alone, purely oncolytic agents, there is a clash between theory and clinical reality. For an OV to be curative, as a *purely cytolytic* agent, it would have to infect and kill the vast majority, if not all, cancer cells within the tumor. Human tumors are well known to be genetically[27] and phenotypically[28] heterogeneous and built of mixtures of normal and malignant cell types, making it unlikely that an OV could find, infect, and completely kill widespread disseminated disease in the majority of patients. However, the therapeutic activity of OVs is not limited to their tumor oncolytic activity but rather is multi-faceted, including interactions within the stromal cells of the tumor microenvironment (TME),[29] as well as the vascular[30] and immune system[31] within the patients. Each of these points of interaction provides an opportunity for combining orthogonal therapeutics to complement OVs and improve outcomes for cancer patients. Furthermore, many of the OV platforms under development can be engineered to express transgenic payloads vastly expanding their therapeutic potential.[32] For the purposes of this review, we borrow from the genetics world and use *cis* combinations to describe approaches that involve encoding of transgenes within the virus backbone and *trans* combinations to describe the coupling of an OV with another stand-alone therapeutic (e.g., drugs, antibodies, cells). Advantages and limitations of *cis* and *trans* combinations are depicted in Figure 1.

OVs Mount a Multi-pronged Attack on the Tumor

Although the primary target of all OV products is the tumor cell, it has become clear that much like many natural virus infections of normal tissues, OVs cause collateral damage as they multiply within the TME. For instance, certain OVs can attack and destroy tumor neo-vasculature, creating areas of tumor hypoxia that culminate in extensive bystander killing of uninfected cells.[30,33] In addition, because of the cytokines expressed within the TME, stromal cells that support cancer cell growth can be infected and destroyed.[29] However, it has become evident that perhaps the most impactful therapeutic byproduct of OV infection is stimulation of the host's immune system. OVs have been shown to initiate immunogenic cell death (ICD), and thus releasing damage-associated molecular patterns (DAMPs) such as HMGB1, heat shock proteins, and ATP, as well as virus-derived pathogen-associated molecular patterns (PAMPs). ICD culminates in the recruitment/activation of both the innate and adaptive immune systems within the TME, usually a site of immune tolerance, and in combination with proteasome blockade can sensitize tumors to adjuvant NK cell therapy by enhancing necroptosis and autophagy.[34] Additionally, by inducing an antiviral immune response both through Fc-fragment-dependent NK cell activation[35] as well as pre-existing immunity against the OV, a systemic antitumor immune response can be observed.[36] This "adjuvant effect" of virus infection is recognized to be a key component of the long-term efficacy of OV therapy (reviewed in Kaufman et al.,[3] Aurelian,[37] Aitken et al.,[38] Guo et al.,[39] De Munck et al.,[40]

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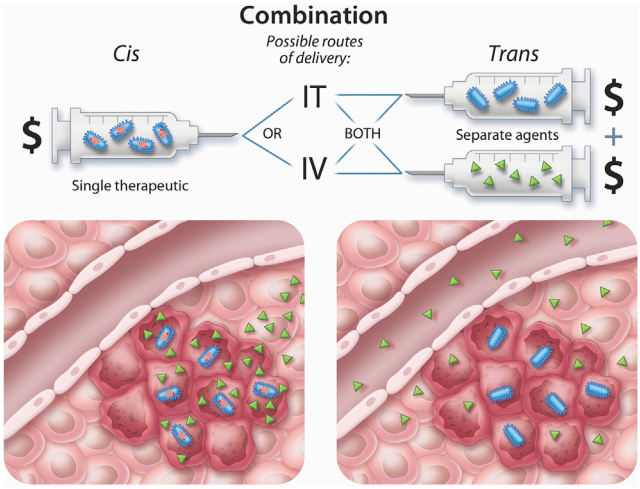

Figure 1. *cis* versus *trans* Combination: Advantages and Limitations
OV therapy in *cis* (left) involves encoding a gene product or therapeutic payload (green) directly in the virus backbone (blue). When a productive infection takes place within cancer cells, the payload is locally expressed at high levels with minimal systemic exposure. This has the advantage of avoiding systemic toxicities and reducing costs because only a single therapeutic (i.e., OV) is required. The disadvantage of this approach is that the expression of the therapeutic payload is linked to the longevity of the virus replication within the tumor. If a therapeutic requires sustained expression over several months, then this approach may be suboptimal. Additionally, if a virus can only be administered by direct intratumoral injection (IT), then the payload will not be optimally expressed in disseminated tumors. Providing a therapeutic in *trans* with OV administration is required if the complementary treatment is a chemical, small molecule, or form of radiation. It may also be desirable if the therapeutic needs to be dosed for a shorter or longer period than the virus that is found within the tumor. In the setting of *trans* administration, the costs of using therapeutics together is additive. In order to achieve therapeutically effective doses within the tumor, systemic administration of large amounts of therapeutic with associated toxicities will be required.

and van Vloten et al.[41]). Despite the multiple OV platforms that are in development, to date only one has been approved as a monotherapy by the US Food and Drug Administration (FDA) and the European Commission. The HSV-based talimogene laherparepvec (T-Vec) was approved in 2015 for the treatment of inoperable malignant melanoma[42] and represented a tremendous breakthrough for the field overall. However, as discussed below, the ultimate clinical application of T-Vec is likely to be in the setting of combination therapy.

cis and *trans* Combinations to Increase Oncolytic Activity

An initial critical step in OV therapy is the ability of virus to infect and kill cancer cells. It has been well documented that there is heterogeneity in the ability of a range of OVs to infect a broad spectrum of cancer cell lines and patient samples. Jean-Simon Diallo has championed the identification and mechanism of action of small molecules and chemotherapeutics that selectively improve virus replication within cancer cells. The *trans* combination of OV therapy with organic or inorganic molecules (so-called viral sensitizers) to potentiate virus infection and spreading within the tumor as well as antitumor immune response has been successfully demonstrated in several syngeneic and xenograft tumor models.[43–45] For example, Selman et al.[46] demonstrated that dimethyl fumarate inhibits type I interferon (IFN) production and response by blocking nuclear translocation of NF-κB, thus potentiating oncolytic virotherapy. Similarly, Arulanandam et al.[33] showed an improved bystander killing effect caused by reduction of IFN expression and secretion when combining VSV with microtubule-destabilizing agents. VSV as well as VACV have also been synergistically combined with histone deacetylase inhibitors[47,48] as well as second mitochondrial activator of caspase (Smac)-mimetics.[49] The chemotherapeutic cyclophosphamide has been shown to enhance oncolytic HSV efficacy by inhibiting the innate immune response when combined in *trans*;[50] therefore, Currier et al.[51] have successfully constructed an oncolytic HSV encoding a prodrug converting enzyme for cyclophosphamide and demonstrated the safety and efficacy of this *cis* combination in xenograft models. Another important aspect of the innate immunity is the activation of macrophages and microglia, causing the expression and secretion of tumor necrosis factor alpha (TNF-α), which in turn induces both intrinsic and extrinsic apoptosis and necroptosis as demonstrated for Newcastle disease virus (NDV)-infected (tumor) cells.[52] The effect of TNF-α on OV therapy is being investigated because although it enhances the bystander effect, especially when encoded under a late promoter in HSV,[53] it can also inhibit virus replication by inducing apoptosis of infected cells. This effect, however, can be counteracted by transient TNF-α blockade.[54] The innate antiviral defense is not the only factor diminishing OV efficacy: as already discussed, a tumor is usually highly heterogenic, comprising fibrotic barriers, extracellular matrix, and other physical barriers inhibiting OV spread and infection of tumor cells.[55] Several groups have pursued a variety of *cis* combinations to enhance virus replication, e.g., encoding fusogenic glycoproteins to enhance virus spread via syncytia formation as demonstrated for HSV,[56,57] as well as VSV and measles virus,[58] or by encoding enzymes actively remodeling the extracellular matrix as demonstrated by AdV encoding relaxin[59] or hyaluronidase,[60] the latter demonstrating improved efficacy over the parental virus in orthotopic murine models for osteosarcoma,[61] as well as glioma.[62] Several groups have also successfully combined antiangiogenic therapies with OVs, both in *trans* as well as in *cis*. Tan et al.,[63] for example, showed synergistic effects, i.e., improved viral distribution throughout the tumor, as well as enhanced tumor hypoxia of HSV *trans*-combined with the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab in a human breast cancer xenograft mouse model. Similarly, Buckel et al.[64] successfully *cis*-combined VACV with a single-chain variant of bevacizumab together with radiotherapy for the treatment of a subcutaneous patient-derived glioma xenograft model. Using a comparable model of glioblastoma, Zhang et al.[65] demonstrated that treatment with bevacizumab is augmented by *trans* combination with an HSV armed with angiostatin, an antiangiogenic polypeptide. The observed synergistic effects of this combination are only partly caused by the antiangiogenic effect, but also by modulation of the innate immune response as demonstrated for an HSV encoding different anti-VEGF antibodies.[66] Not pursuing an antibody-based approach, Bolyard et al.[67] showed significant antitumor efficacy of a

cis-combination of HSV and vasculostatin, a secreted angiogenesis inhibitor, in an ovarian cancer xenograft mouse model, additionally combined in *trans* with the approved chemotherapeutic doxorubicin.

The combination of OVs with approved chemotherapeutics holds several benefits, foremost the known safety profile and indications of an approved drug. Also, when it comes from bench to bedside, patients may have already been treated with chemotherapeutics, and it is therefore important to determine whether OVs can work in combination or not. Several pre-clinical and clinical studies have been conducted investigating the combination of OVs with approved chemotherapeutics, and also a few reviews have been written especially on this topic (e.g. Simpson et al.[68]). Recently, Binz et al.[69] demonstrated the successful combination of the two chemotherapeutics nab-paclitaxel and gemcitabine with oncolytic VACV GLV-1h68 for the treatment of pancreatic cancer *in vitro*, Tanaka et al.[70] investigated a combination therapy with oncolytic HSV and dacarbazine in a mouse melanoma model, and Bourgeois-Daigneault et al.[71] were able to show synergistic cytopathic activity, and therefore reduced tumor growth and prolonged survival, in a murine breast cancer model treated with a combination of Maraba MG1 and paclitaxel.

Priming and Boosting T Cell Responses with OV Vaccine Combinations

cis *Approaches*

Our immune systems have evolved to recognize and vigorously respond to virus infection, and thus in many ways a virus is the perfect immunological adjuvant. With this in mind, investigators have created OVs that encode and express tumor-associated antigens (TAAs) with the goal of coupling oncolytic activity with targeted T cell stimulation. In early iterations of this approach, OVs encoding TAAs (e.g., Her2) were directly injected into tumor beds to locally stimulate immune responses against the TAA in a milieu where cancer cells are being lysed and releasing inflammatory cytokines.[72] As a further advancement on this strategy, OVs expressing a TAA can be used to synergize with OVs armed with cytokines to enhance systemic antitumor immunity either with *cis* encoding of cytokines and TAAs or by *trans* combinations (e.g., VACV-granulocyte-macrophage colony-stimulating factor [GM-CSF] with VACV armed with HER2/neu[16,73]). Although this approach shows promise, it may be limited because multiple treatments with the same virus encoding a tumor antigen are likely to skew the immune response toward the vector and away from the TAA. More recently, Bridle et al.[74] introduced the concept of *heterologous prime-boost* regimens to focus immune responses on the TAA and away from the OV backbone. In their initial studies they used a non-replicating AdV vector expressing a TAA "self-antigen," namely dopachrome tautomerase or DCT to prime animals and generate a central memory (T_{CM}) response. This was followed 2 weeks later by systemic administration of an oncolytic rhabdovirus also encoding DCT.[74] This approach provided outstanding systemic T cell responses against DCT while simultaneously recruiting anti-DCT immune cells into the TME. The unprecedented T cell responses appear to be related to the natural tropism of rhabdoviruses for follicular B cells that leads ultimately to boosting of central memory T cells harbored within the splenic follicle.[75] Since the first description of this concept, several studies investigating possible antigens and combinations have been conducted and recently summarized in two comprehensive reviews by Aitken et al.[38] and Meyers et al.[76] The promising results of prime-boost regimens in pre-clinical experiments resulted in two clinical phase I/II studies (NCT02285816 and NCT02879760), both employing a prime with a non-replicative AdV encoding the antigen MAGE-A3 followed by a boost with MG1 Maraba/MAGE-A3. Notably, NCT02879760 also includes the combination with the immune checkpoint inhibitor (ICI) pembrolizumab.

An alternative approach to OV vaccination has been described by Lemay et al.,[77] who generated "infected cell" vaccines (ICV) through *ex vivo* infection of γ-irradiated tumor cells with oncolytic VSV. This approach can generate potent antitumor immune response in immunocompetent mouse models and provide long-term protection against future tumor challenges. Recently, Alkayyal et al.[78] described potent NK cell activation induced by an Maraba MG1-interleukin-12 (IL-12) ICV for the treatment of peritoneal carcinomatosis.

trans *Approaches*

So far, there is only one approach described that combines TAAs with OV therapy in *trans*. Capasso et al.[79] coated an oncolytic AdV with tumor-specific major histocompatibility (MHC) class I peptides and observed an increase in antitumor cytotoxic T cells, as well as epitope-specific dendritic cells (DCs), and consequently an improved therapeutic efficacy of this peptide-coated conditionally replicating AdV (PeptiCRAd) in a humanized mouse melanoma model.

Combinatorial Application of Microbes

In contrast with the described prime-boost regimen where two viruses encode the same antigen, it is also possible to combine viruses with different immune profiles to overcome antiviral immune responses and exert synergistic effects, even when applied sequentially. For example, Le Boeuf et al.[80] demonstrated improved antitumor response of VSV combined with VACV in immunodeficient and immunocompetent mouse tumor models, as well as *ex vivo* studies in primary human cancer samples compared with each virus alone; Tysome et al.[81] successfully applied a sequential treatment regimen of oncolytic AdV followed by VACV for the treatment of pancreatic cancer in a Syrian hamster model; and recently Nistal-Villan et al.[82] demonstrated the feasibility of sequential intratumor administrations of AdV and NDV, the latter in *cis* combination with the immunostimulatory cytokine, oncostatin M. Recently, Ilett et al.[83] demonstrated the feasibility of the combination of a heterologous prime-boost model using reovirus and VSV together with anti-programmed cell death protein 1 (anti-PD1) checkpoint blockade. To date there have been no clinical studies testing the sequential administration of two distinct OVs; however, there is plenty of potential for using rationally engineered OV combinations.

Similar to the previously discussed *trans* combination of OVs with other viruses, it has been demonstrated that bacteria can synergize

with OVs. For example, Cronin et al.[84] showed that intravenous (i.v.) application of nonpathogenic *E. coli* expressing the vaccinia type 1 IFN antagonist B18R augments subsequent therapy with oncolytic VSV in an athymic nude mouse model by overcoming innate immunity against the OV. Recently, Aitken et al.[85] demonstrated the feasibility of a heterologous prime-boost regimen in immunocompetent mice. In this proof-of-concept study the intracellular bacteria *Listeria monocytogenes* was used as a priming agent in an exemplary prime-boost regimen together with Maraba virus. This study demonstrated that *L. monocytogenes* not only directly lyses tumor cells, but also primes an immune response comparable with the commonly used adenoviral vectors. Because bacteria are well-described (cancer) vaccination agents,[86] the use of other bacteria, e.g., *Salmonella typhimurium*,[87,88] warrants further investigation.

Teaching OVs to Drive a CAR

Probably one of the most substantial advancements in cancer immunotherapy was the description of chimeric antigen receptor (CAR) T cells. In this approach, cytotoxic T cells are genetically modified to express an engineered recombinant antigen receptor, usually comprising a single-chain variable fragment of an antibody, directed against a surface antigen on malignant cells, thus inducing cell death.[89] Given the promising results of this technique so far, it makes sense to combine OVs with CAR T cells (for a review, see Ajina and Maher[90]). Nishio et al.[91] demonstrated that an AdV armed with the chemokine RANTES and the cytokine IL-15 not only exerts oncolytic function on its own, but also enhances migration and proliferation of CAR T cells specific for the TAA GD2 in a xenograft human neuroblastoma mouse model. As a result, tumor burden was reduced and overall survival was improved compared with each treatment alone, encouraging further combination approaches of OVs and CAR T cells. Tanoue et al.[92] demonstrated the feasibility of *trans*-combining an AdV armed with an anti-programmed death ligand 1 (PD-L1) minibody with HER2/neu CAR-T cells in a xenograft model in nude mice. A similar concept of complementing HER2/neu-specific CAR T cells with oncolytic Ad expressing PD-L1 blockade and combining cytokine (IL-12) expression in *cis* for the treatment of head and neck squamous cell carcinoma in a human xenograft mouse model was recently described.[93] These few studies so far demonstrate the feasibility and the promising potential of combining CAR T cell therapy with OVs, and they encourage further studies.

Strategic Combinations of Immune Modulators with OVs

Since the description of costimulatory receptors and their ligands acting as immune checkpoints, envisioned as "brakes" during an immune response, several of these "brakes" have been identified and antibodies blocking them have been described (for a review, see Pardoll[94]). These ICIs have proven themselves very promising tools in the therapeutic toolbox, even when used as monotherapies (for a review, see Wilson et al.[95]). So far, the monoclonal antibodies blocking the Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and PD1, as well as the corresponding ligand, PD-L1, were the first ICIs to be approved by the US FDA. The blockade of CTLA-4 enhances T cell responses, whereas the blockage of PD1/PD-L1 interaction dismantles the tumor defense; therefore, it is obvious that these ICIs can be synergistically combined in *trans* with OVs[96,97] or even combined in *cis* as a transgene for localized expression within the TME, as demonstrated for CTLA-4,[98] CTLA-4 and PD-L1,[99] the ICOS ligand (combined in *trans* with anti-CTLA-4),[100] and soluble PD1.[101,102] It has also been demonstrated that influenza A virus can be engineered to express an ICI (single-chain anti-CTLA-4 antibody) within the infected tumor tissue in a mouse melanoma model.[103] Recently, Saha et al.[104] demonstrated the treatment of glioblastoma with a triple combination of CTLA-4 and PD-L1 blockade together with an IL-12 *cis*-armed HSV. In their study they also confirmed that the observed enhanced therapeutic efficacy is caused by $CD8^+$ and $CD4^+$ T cells, as well as macrophage polarization within the tumor. In a neo-adjuvant setting, two groups reported that OVs (reovirus and Maraba MG1, respectively) can precondition tumors and metastases to ICI therapy and synergize with surgery in breast cancer and glioblastoma models in mouse.[105,106] In the clinical setting, Ribas et al.[107] *trans*-combined the oncolytic HSV T-Vec with pembrolizumab and demonstrated increased infiltration of the tumor by $CD8^+$ cytotoxic T cells and elevated levels of PD-L1 and IFNγ, leading to a synergistic improvement in therapeutic efficacy with a complete response in a third of the patients according to immune-related response criteria. Similarly, the oncolytic Coxsackievirus A21 is being *trans*-combined with ipilimumab (anti-CTLA-4),[108] as well as pembrolizumab,[109] in phase 1b clinical trials for the treatment of advanced melanoma. Both trials demonstrate high overall response rates. Taken together, it is clear that OVs and ICIs can work synergistically together when combined both in *cis* and in *trans*.

Taking a BiTE out of Cancer with OVs

In 2014, the US FDA approved the use of a novel type of immune modulator for the treatment of acute lymphoblastic leukemia, the CD3/CD19 bispecific T cell engager (BiTE) blinatumomab. This new family of very promising immune modulators, which exert direct effect on T cells, are artificial fusion proteins composed of two single-chain variable fragments designed to bind CD3 on T cells and a known antigen on the target cell, effectively overruling TCR specificity, MHC expression, and costimulatory signals, forming a so-called pseudoimmunological synapse and triggering CTL activation. Upon activation, CTLs release perforins and granzymes, effectively inducing apoptosis of the target cell. Several BiTEs targeting different TAAs such as the epithelial cell adhesion molecule EpCAM (solitomab or AMG 110, MT110), the carcinoembryonic antigen CEA (AMG 211, MT111), and prostate-specific membrane antigen PSMA (AMG 212) are currently being evaluated in clinical trials (for a review, see Krishnamurthy and Jimeno[110]). A major shortcoming of BiTEs, however, is the need for continuous i.v. perfusion due to their rather short half-life in circulation (approximately 1.25 hr for blinatumomab[111]). A way to overcome this is to encode the BiTE as a transgene within an OV to sustain production, as described in several pre-clinical studies (see Scott et al.[112]). In a pioneering approach, Yu et al.[113] constructed a thymidine kinase (TK)-deleted VACV encoding a BiTE specific for ephrin type-A receptor 2 (EphA2) and

observed not only oncolytic activity and BiTE secretion from infected cells, but also potent CTL activation and a bystander killing effect on uninfected cells both in *in vitro* co-culture assays and *in vivo* in an A549 lung carcinoma xenograft mouse model. In a xenograft model, Fajardo et al.[114] made supporting observations with an oncolytic AdV encoding an epidermal growth factor receptor (EGFR)-specific BiTE. Freedman et al.[115] observed potent oncolysis and CTL mediated anti-tumor effect both *in vitro* and in primary human tumor samples using the oncolytic group B AdV EnAdenotucirev (EnAd) encoding a BiTE targeted to EpCAM. These findings together suggest that the combination of the novel immune modulator BiTE with an OV, especially in a transgenic approach, can work in a synergistic fashion. Although both CAR T cells and BiTEs are yielding very promising results *in vitro* and *in vivo*, especially in combination with OVs, and undoubtedly are the most powerful tools in the immune modulator toolbox so far, they share the common shortcoming of being dependent on known TAAs.

Arming OVs with Cytokines or Chemokines

One of the first *cis*-combination therapies with any OV involved encoding cytokines, most notably GM-CSF, which has been introduced as a transgene in several different backbones. GM-CSF promotes the maturation of monocytes into DCs and increases antigen presentation on DCs, and therefore enhances the activation of NK cells and CD8-mediated T cell response downstream.[116–118] So far, multiple OVs have been armed with different cytokines, all following the rationale to "heat up" the TME, either by attracting immune cells to the tumor bed or by activating cells already present within the tumor. The arming of OVs with cytokines is a well-established method[119] to improve therapeutic outcome. Notable examples apart from GM-CSF are VSV-IL-15,[120] VSV-IFNγ,[121] VACV expressing CCL5,[122] and MG1-IL-12.[31] Viruses armed with two cytokines are possible, for example, AdV in *cis* combination with TNF-α and IL-2 and in *trans* combination with adoptive cell therapy.[123] Notably, OVs can also be successfully armed with cytokines not specifically targeted toward cells of the innate or adaptive immune system: by *cis*-combining oncolytic Maraba MG1 with fibroblast growth factor 2 (FGF-2), Ilkow et al.[29] showed improved therapeutic efficacy in tumor-bearing mice by exploiting the crosstalk between cancer-associated fibroblasts (CAFs) and malignant cells, thus rendering them more susceptible to OV infection. These studies demonstrate that OV platforms can work synergistically in combination with other immune effectors.

cis/trans Combination Therapies

Currently, most clinical trials already use a combination of OVs with other therapeutic agents in *cis* and in *trans*. For example, T-Vec already is *cis*-combined with GM-CSF and can be synergistically *trans*-combined with ICIs.[107] However, the idea of *cis/trans*-combination therapies becomes clearer when the OV is armed with a prodrug converting enzyme and then *trans*-combined with the prodrug, allowing for localized action of the drug and an improved bystander effect. Notable examples for this *cis/trans* strategy apart from the already discussed HSV encoding a prodrug converting enzyme for cyclophosphamide[51] are OVs *cis*-armed with the HSV TK and *trans*-combined with ganciclovir.[124] Also feasible is the *cis* combination of two prodrug converting enzymes in a single OV as demonstrated by Gibson et al.[125] for an AdV armed with GM-CSF, HSV TK, and cytosine deaminase (CD) converting 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) (notably, this virus did not show significant improvement over AdV-GM-CSF in a mouse mammary tumor model) and also demonstrated by Tyminski et al.[126] for oncolytic HSV armed with two enzymes, YP2B1 and secreted human intestinal carboxylesterase, *trans*-combined with cyclophosphamide and irinotecan in a xenograft glioma mouse model. On the clinical side, a phase 1b trial is investigating the therapeutic efficacy and safety of a nonlytic replicating retrovirus encoding yeast CD, converting the prodrug Toca-FC (extended-release 5-FC) into 5-FU for the treatment of recurrent or progressive high-grade glioma.[127] OVs can also be *cis/trans*-combined with radiotherapy. Radiotherapy can cause an anti-tumor immune response by inducing ICD similar to OVs (reviewed in Levy et al.[128]). It is therefore logical to investigate synergistic effects[129,130] of this established therapy with novel approaches like ICIs. The combination of radiotherapy with OVs, so-called radiovirotherapy, has been well described. For example, oncolytic AdV can be used as radiosensitizers,[131] and other OVs can be armed with the gene for the sodium iodide symporter (NIS). This method has been demonstrated to augment localized radiotherapy and imaging by actively transporting radioactive iodine into infected (cancer) cells.[132] Dingli et al.[133] employed an oncolytic measles virus encoding NIS for the treatment of multiple myeloma in a mouse xenograft model, and Gholami et al.[134] demonstrated therapeutic efficacy in triple-negative breast cancer using VACV encoding NIS in combination with radioactive iodide. Pursuing a combination approach, Markert et al.[135] demonstrated safety and efficacy of oncolytic HSV in combination with radiotherapy of glioblastoma in a phase 1 clinical trial, and an ongoing phase 2 clinical trial (NCT02819843) is investigating the efficacy of *trans*-combining T-Vec with radiotherapy for the treatment of melanoma, Merkel cell carcinoma, and other solid tumors.

Conclusions

In summary, OV therapy, and even more so the combination of OVs and immunotherapy, is a rapidly expanding field. There is considerable reason for optimism because combinations of OVs with innovative immune modulators like ICIs, CAR T cells, and BiTEs are accelerating the field toward our long-term goal of achieving lasting cures for cancer patients. To take advantage of these exciting opportunities, there are some clear hurdles and knowledge gaps we need to overcome. For instance, we need a more comprehensive understanding of the multi-mechanistic actions of OVs and how they interact with the finely tuned regulatory pathways of the immune system. At the molecular level, unraveling of the details of the interactions between OVs, the TME, and the immune system are crucial to optimization of combination strategies. One important example is understanding the molecular aspects of "epitope spreading" in the context of OV therapy and how to maximize this phenomena in order to potentiate long-term immunotherapeutic attack on the tumor. Coupling this with a

thorough analysis of the many ways cancer cells evade immune surveillance will help in the design of multiplexed immunotherapeutic approaches to target the heterogeneity of cancer. Finally translating this knowledge into the clinic remains a challenge. Pre-clinical animal models are of limited value when using therapeutics that have species-specific interactions. The field needs more rationally designed, high-content clinical trials exploring thoughtful therapeutic combinations. Substantial benefit is likely to be gleaned with a better understanding of the optimal doses and timing of therapeutic combinations.

As multiple studies demonstrate, for the majority of patients with systemic disease, the only way to fight cancer is through combinations with multiple therapeutics. It seems that it is not only important to hit the enemy hard and early,[136] but also necessary to throw more than two stones to finally kill the bird.

CONFLICTS OF INTEREST

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