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Oncolytic viruses and antibodies: are they more successful when delivered separately or when engineered as a single agent?

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ABSTRACT

Oncolytic viruses (OVs) provide the promise of tumor-selective cytotoxicity coupled with amplification of the therapeutic agent (the virus) *in situ* within the tumor improving its therapeutic index. Despite this promise, however, single agent-treatments have not been as successful as combination therapies, particularly combining with checkpoint inhibitor antibodies. The antibodies may be delivered by two approaches, either encoded within the OV genome to restrict antibody production to sites of active virus infection or alternatively given alongside OVs as separate treatments. Both approaches have shown promising therapeutic outcomes, and this leads to an interesting question of whether one approach is potentially better than the other. In this review, we provide a brief summary of the combination OV-antibody therapies that target tumor cells, tumor microenvironment and immune cells to help define key parameters influencing which approach is superior, thereby improving insight into the rational design of OV treatment strategies.

INTRODUCTION

Oncolytic or 'cancer-lysing' viruses (OVs) are cytotoxic anticancer agents that preferentially replicate within and kill tumor cells, largely sparing normal tissues. Lytic death of cancer cells and the associated release of damage-associated or pathogen-associated molecular patterns (DAMPs, PAMPs) creates a proinflammatory tumor microenvironment (TME) that may lead to an anticancer immune response. Despite inducing antitumor responses in some patients, OVs used alone as directly cytolytic agents are usually not effective in conferring complete tumor clearance. Consequently, most contemporary clinical trials using OVs combine them with other therapeutic strategies or 'arm' the viruses by encoding biotherapeutic agents within them.

The combination of OVs with antibodies has been widely explored as a strategy to achieve synergistic antitumor activity. A range of antibody formats has been considered, including whole antibody molecules of different isotypes, fragments such as F(ab), single-chain variable fragment (scFv) and nanobodies and bispecific and trispecific agents that can mediate a variety of functions such as T-cell activation or binding and inactivation of pro-tumorigenic cytokines. Different platforms have been developed to deliver the antibodies locally and systemically. In this review, 'armed' OV encoding antibodies will be described as *cis* combination, while the OVs combined with stand-alone antibodies will be described as *trans* combination (figure 1). Considering challenges of efficacy, toxicity, pharmacokinetics and pharmacogenomics, we aim to define whether the *cis* or *trans* approach is emerging as a superior strategy for cancer treatment.

Cis combination—oncolytic viruses with antibodies encoded within them

Targeting the tumor cells with *cis* approach

Cancer cells often display various overexpressed proteins and tumor-associated antigens (TAA), some of which are present on the cell surface and accessible to extracellular antibodies. A number of antibodies against these TAAs have been used to arm OVs, usually with the intention of engaging immune cells to attack the cancer cells. In some instances, OVs have been armed with full-length antibodies, and these combine the immune-activating functionality of Fc tails with the potential for direct antagonism of cell surface receptors. For example, trastuzumab is a Food and Drug Administration-approved human epidermal growth factor receptor 2 (HER2) antagonist for $HER2^+$ breast and gastric cancer. When encoded within the adenovirus, Ad5/3-Δ24-tras, both heavy chain and light chain were preceded by a signal peptide. The virus-produced trastuzumab was

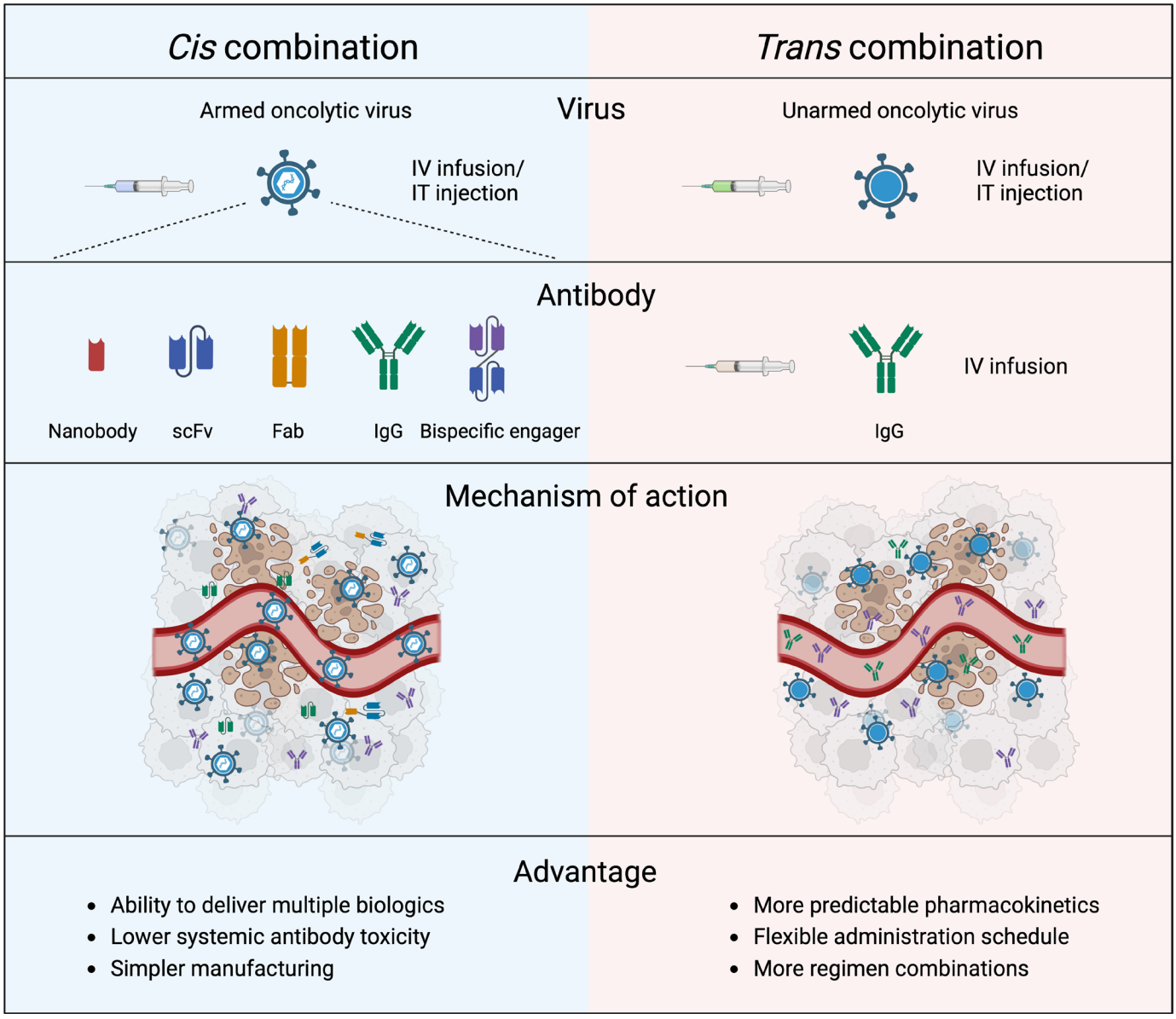

Figure 1 *cis* versus *trans* combination *Cis* combination involves the 'arming' of transgenes that encode antibodies or any anticancer agents in the oncolytic virus (OV) genome. As a single therapeutic agent, the OV expresses the armed biologics alongside its replication. The virus is delivered either by intratumoral (IT) injection or intravenous (IV) administration. Nanobody, single-chain variable fragment (scFv), fragment antigen-binding region (Fab), immunoglobulin G (IgG), and bispecific engager are the common types of antibody formats that are armed in the virus. Advantages of using the *cis* combination include the ability of delivering multiple biologics, lower systemic antibody toxicity and simpler manufacturing of therapeutic agents. *Trans* combination in OV therapy involves the use of OV and antibody as separate agents. IV infusion is a more common route for antibody administration, though IT injection might also be possible. IgG, compared with its smaller antibody fragments, is the most common form of antibody used. *Trans* combination offers a more predictable pharmacokinetic, flexible administration schedule and more regimen combinations.

shown to be folded correctly and was able to bind to HER2 antigen, confirming its biological functionality, though it is unclear whether the trastuzumab produced by the virus has the same potency as the commercially available one. Ad5/3-Δ24-tras showed potent growth inhibition of $HER2^+$ gastric cancer xenografts and induced antibody-dependent cellular cytotoxicity.[1] Importantly, this method of localized antibody expression was shown to achieve a higher tumor-to-systemic antibody concentration than that could be achieved using systemic antibody delivery, potentially obviating systemic toxicities.

Bispecific T-cell engagers (BiTEs) or trispecific T-cell engagers are popular antibody formats that are often encoded into OVs. Apart from their relatively small size (minimizing genetic disruption to the vector), one major advantage of these multispecific agents is their ability to direct potent T-cell cytotoxicity to any engaged target cells, independent of T-cell receptor specificity or human

leukocyte antigen (HLA) status of the cancer cells. Enadenotucirev (EnAd), an Ad3/Ad11p chimeric adenovirus (Ad) armed with a BiTE targeting epithelial cell adhesion molecule (EpCAM) showed that the BiTE could trigger T-cell proliferation, activation and cytotoxicity in normal media but also in immunosuppressive malignant ascites.[2] A signal peptide was inserted before the BiTE transgene to allow active secretion of biologics to the extracellular matrix (ECM). It is believed that even without the signal peptide, functional biologics may still be released to the ECM during oncolysis.

Similarly, an E1a-Δ24-based oncolytic Ad5, ICOVIR-15 also inhibited tumor growth when armed with a BiTE targeting epithelial growth factor receptor (EGFR) in severe combined immunodeficiency (SCID)/beige mice engrafted with human peripheral blood mononuclear cell.[3] An improved tumor response was also reported using measles virus (MV) armed with anti-carcinoembryonic antigen BiTE[4] and vaccinia virus (VV) armed with anti-tumor cell surface antigen (EphA2) BiTE.

Targeting the TME with *cis* approach

In recent years, several important roles of the TME in facilitating tumor growth have been elucidated, providing innovative new targets for therapeutic intervention. Many of these roles are undertaken by the three main TME cellular components, cancer-associated fibroblasts (CAFs), tumor-associated endothelial cells and immune cells. Antibody-OV combination strategies have focused particularly on inhibiting the provision of a blood supply and reversing immunosuppression. Most efforts to block angiogenesis of the tumor vasculature have targeted vascular endothelial growth factor (VEGF), which is considered a key mediator. Anti-VEGF scFv[5–7] and anti-VEGF-IgG (bevacizumab)[8] have been encoded within VVs and Ad, respectively, and mediated suppression of neovascularisation and tumor growth. Another proangiogenic signaling axis CXCR4/CXCL12 has also been targeted using a VV encoding a CXCR4 antagonist fused with the Fc domain, improving the antitumor effect and reducing the number of cancer-initiating cells in both syngeneic subcutaneous breast cancer and syngeneic orthotopic ovarian cancer mouse models.[9 10]

Tumor immunosuppression is emerging as a pivotal cancer hallmark, largely mediated by CAFs. Specific cell surface targets for CAFs are hard to identify. Currently, BiTE targeting fibroblast activation protein (FAP) encoded by Ads[11 12] and VV[13] is the only reported approach that effectively depleted CAFs, leading to a repolarisation of the TME towards a less immunosuppressive state. Another approach to reversing immunosuppression is to intervene directly in the immunosuppressive signaling mechanisms using checkpoint inhibitor antibodies. Encoding checkpoint inhibitors such as anti-programmed cell death protein-1 (PD-1), anti-programmed cell death ligand-1 (PD-L1)[14–16] and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[17–19] antibodies in OVs have been shown to synergise in mediating tumor regression and prolonging overall survival in syngeneic murine tumor models. More recently, enhancement of abscopal effects and regression of untreated distal tumors have been reported in herpes simplex virus (HSV) encoding anti-PD-1 scFv[15] and orthomyxovirus expressing anti-CTLA-4 scFv.[18] In addition, the PD-L1 BiTE expressed by HSV could deplete not only tumor cells but also M2 macrophages in human ascites. Intriguingly, the endogenous T-cell activation triggered by the BiTE in the immunosuppressive ascites was more significant than in tissue culture medium, thought to reflect a higher expression of PD-L1 on tumor cells.[20] The strategies of antibody-armed OVs in targeting the TME have been recently summarized by Wan *et al*.[21]

In a recently completed phase I clinical trial on EnAd expressing a fully human IgG agonistic anti-CD40 antibody, the treatment elevated the levels of Th1 cytokines including interleukin (IL)-12 and interferon (IFN)-γ in patients with advanced tumors.[22] There were more expanded T-cell clones with new T-cell receptor repertoires in blood and 50% of patients achieved stable disease. In another ongoing phase I trial on oncolytic HSV expressing anti-CTLA-4 antibody-like molecule, 33.3% of patients had an objective response with a patient with mucoepidermoid cancer having an ongoing complete remission for 19 months.[23] The treatment also led to an increase in tumor inflammation signature, expansion of existing T-cell clones and emergence of new T-cell clones. More recently, the trial on oncolytic HSV expressing three cytokines—IL-12, FLT3L, CCL4 and two antibodies—anti-PD-1 single variable heavy chain domain (VHH)-Fc and anti-CTLA-4 scFv-Fc (ONCR-177) in patients with injectable advanced tumors has launched.[24] The treatment induced T-cell infiltration and upregulated PD-L1 expression on T cells, macrophages and dendritic cells in the tumors. The clinical trials of combining OV and antibody in *cis* were summarized in table 1.

Trans combination—administering the antibody and OV independently

Targeting the tumor cells with *trans* approach

Combination treatment of anti-EGFR IgG, cetuximab, and an attenuated HSV-1 mutant variant, HF-10, has been shown to synergistically inhibit tumor growth in a human colorectal cancer xenograft mouse model. Unusually in this situation, the synergy was thought to reflect dual complementarity of both agents, whereby virus HF-10 led to reduced EGFR expression in cancer cells while cetuximab independently promoted more widespread intratumoral viral distribution.[25]

Targeting the TME with *trans* approach

Many antibodies have been reported to enhance the activity of oncolytic viruses by creating an environment that is favorable to viral propagation. For example, the anti-angiogenic antibody, bevacizumab, has been shown to disrupt vessel formation and promote hypoxia, creating an oxygen level that is favorable for HSV replication,

Table 1 Clinical trials of combining oncolytic viruses and antibodies in *cis* and *trans*

Virus	Antibody	Tumor	Phase/ no. of pts/ route	Clinical outcomes	Cellular outcomes	Trial ID	Ref
Cis combination							
NG-350A (Ad)	Agonist anti-CD40 IgG (*cis*)	Advanced epithelial tumors	1a/16/i.v.	SD: 50%	↑ IL-12, IFN-γ and IL-17a; ↑ T-cell clone in PBMC	NCT03852511	22
RP2 (HSV)	Anti-CTLA-4 antibody-like molecule (*cis*)	Advanced solid tumors	1/9/i.t.	ORR: 33.3%	↑ intratumoral T cell; ↑ inflammation gene signature (interim)	NCT04336241	23 107
ONCR-177 (HSV)	Anti-PD-1 antibody, anti-CTLA-4 antibody (*cis*)	Advanced solid tumors	1/14/i.t.	n.a.	↑ intratumoral T cell; ↑ IFN-γ, Ki67^{+} T cells in plasma	NCT04348916	24
Cis/trans combination							
RP2 (HSV)	Anti-CTLA-4 antibody-like molecule (*cis*); nivolumab (*trans*)	Advanced solid tumors	1/27/OV: i.t., Ab: i.v.	Anti-PD-1 failed cutaneous melanoma—PR: 40%; uveal melanoma—PR: 33%; SCCHN: PR: 33%	↑ intratumoral T-cell infiltration; ↑ inflammation signature; ↑ existing T-cell clone; ↑ new T-cell clone (interim)	NCT04336241	23
Trans combination							
ONCOS-102 (Ad)	Pembrolizumab (*trans*)	Melanoma	1/2/12/OV: i.t., Ab: i.v.	ORR: 33%	↑ abscopal antitumor response	NCT03003676	42
RP1 (HSV)	Nivolumab (*trans*)	Melanoma, NMSC	1/2/67/OV: i.t., Ab: i.v.	Melanoma—PR: 36.1% NMSC—CR: 61.3%	↑ intratumoral T cell; ↑ inflammation gene signature (interim)	NCT03767348	41
T-VEC (HSV)	Pembrolizumab (*trans*)	Melanoma stage IIIB–IV	Ib/ 21/OV: i.t., Ab: i.v.	CR: 33.3%; ORR: 61.9%; DCR: 76%; 18-month PFS: 67%. 18-month OS: 90%.	↑ intratumoral CD8^{+} T cell, GrB^{+} cell, CD45RO+memory T cell, Teff/Treg ratio ; ↑ proliferating CD8^{+} T cell in PBMC	NCT02263508	78
T-VEC (HSV)	Ipilimumab (*trans*)	Melanoma stage IIIB–IV	Ib/ II/ 18/OV: i.t., Ab: i.v.	ORR: 50% ; DRR: 44%; DCR: 72%; 18-month PFS: 50%. 18-month OS: 67%.	↑ total and activated CD8^{+}, ICOS^{+}CD4 T cell in PBMC	NCT01740297	108

Continued

Table 1 Continued

Virus	Antibody	Tumor	Phase/ no. of pts/ route	Clinical outcomes	Cellular outcomes	Trial ID	Ref
T-VEC (HSV)	Ipilimumab (*trans*)	Melanoma stage IIIB–IV	II/ 98/OV: i.t., Ab: i.v.	ORR: 39% (vs 18% without OV); median PFS: 8.2 months (vs 6.4 months without OV)	n.a.	NCT01740297	102
HF-10 (HSV)	Ipilimumab (*trans*)	Melanoma stage IIIB–IV	II/ 46/OV: i.t., Ab: i.v.	BORR (24 weeks): 41%; DSR: 68%; median PFS: 19 months; median OS: 26 months	↑ intratumoral $CD8^+$ T cell; ↓ $CD4^+$ T cell	NCT02272855	43
HF-10 (HSV)	Ipilimumab (*trans*)	Melanoma stage IIIB–IV	II/ 28/11OV: i.t., Ab: i.v.	DCR: 100%, median OS: 342 days with persistent infection (vs 33%, 251 days without persistent infection)	↑ ICOS on $CD4^+$ T cell; ↓ PD-L1 on monocyte in responders' PBMC	NCT03153085	109
V937 (Coxsackievirus A21)	Pembrolizumab (*trans*)	Melanoma stage IIIV–IV	Ib/ 36/OV: i.t., Ab: i.v.	CR: 22%; PR: 25%; PFS: 11.9 months; OS: 30.9 months	n.a. (Interim)	NCT02565992	110
V937 (Coxsackievirus A21)	Ipilimumab (*trans*)	Melanoma stage IIIB/C–IV	Ib/ 50/OV: i.t., Ab: i.v.	ORR: 30%; PFS: 6.2 months; OS: 45.1 months	↑ $CD4^+$, $CD8^+$, memory T cell in PBMC	NCT02307149	44 111
Pelareorep (Reo)	Pembrolizumab+chemotherapy: (i) gemcitabine or (ii) irinotecan or (iii) leucovorin with 5-fluorouracil (*trans*)	Relapsed metastatic PDAC	Ib/ 10/OV: i.v., Ab: i.v.	PR: 10%; SD: 20%; median PFS: 2 months; median OS: 3.1 months	↑ intratumoral $CD8^+$ T cell; ↑T cell clonality responders' PBMC; ↑ CXCL9, CXCL10, CXCL11 in PBMC	NCT02620423	112
Pelareorep (Reo)	leucovorin/5-fluorouracil/ oxaliplatin/ bevacizumab (*trans*)	Advanced RAS-activated CRC	II/ 51/OV: i.v., Ab: i.v.	ORR: 53%; DCR: 86%; median PFS: 7 months; median duration response of ORR: 5 months (vs 35%; 83%; 9 months; 9 months without OV)	n.a.	NCT01622543	113

Ab, antibody; Ad, adenovirus; BORR, best overall response rate; CR, complete response; CRC, colorectal cancer; CXCL9, C-X-C motif chemokine ligand 9; CXCL10, C-X-C motif chemokine ligand 10; CXCL11, C-X-C motif chemokine ligand 11; DCR, disease control rate; DRR, durable response rate (response lasting for ≥6 months); DSR, disease stability rate; Grb, granzyme B; HSV, herpes simplex virus; ICOS, inducible costimulatory; IFN, interferon; IL, interleukin; i.t., intratumoral; i.v., intravenous; NMSC, non-melanoma skin cancer; ORR, objective response rate; OS, overall survival; OV, oncolytic virus; PBMC, peripheral blood mononuclear cell; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed cell death ligand-1; PFS, progression-free survival; Pts, patients; Ras, rat sarcoma; Ref, reference; Reo, reovirus; SCCHN, squamous cell carcinoma of head and neck; SD, stable disease; Teff, effector T cell; Treg, regulatory T cell; T-VEC, talimogene laherparepvec.

persistence and distribution in a human breast cancer xenograft mouse model.[26] Building on this, the combination of bevacizumab and angiostatin-armed HSV was shown to abolish bevacizumab-induced collagen deposition and matrix metalloproteinases expression in glioma.[27] Interestingly, combining ICP-6-deleted rRp450 HSV-1 and bevacizumab showed that the improved antitumor response was not related to the intratumoral VEGF level in human Ewing sarcoma-bearing mice.[28] Instead, bevacizumab rescued themajor histocompatibility complex (MHC) class II-expressing macrophages that were selectively depleted by HSV, suggesting that VEGF blockade enhanced oncolytic virotherapy in part due to the modulation of the myeloid stroma.

In addition, many immunomodulators have recently been combined with unarmed or armed OVs. In preclinical studies, combining anti-PD-1 antibody with reovirus (Reo)[29] or CXCL11-armed VV[30] augmented both natural killer (NK) cell and $CD8^+$ T-cell responses in syngeneic tumor mouse models of melanoma, skin and ovarian cancer. VV-induced PD-$L1^+$ myeloid populations, Treg and exhausted T cells were also mitigated by the anti-PD-1 blockade.[30] Similar additive or synergistic antitumor effects has also been observed when combining PD-1/PD-L1 blockade in *trans* with EGFR-retargeted MV[31] and vesicular stomatitis virus encoding IFN-β[32] in syngeneic tumor mouse models of glioma and acute myeloid leukemia, respectively. Furthermore, the combination of PD-1 blockade with TILT-123, an adenovirus expressing tumor necrosis factor-alpha and IL-2 showed an encouraging antitumor response in a syngeneic Syrian hamster model.[33] Two clinical trials of TILT-123 combining with pembrolizumab for ovarian cancer (NCT05271318),[34] and with avelumab for advanced solid tumors refractory to or progressing after anti-PD-1/PD-L1 treatment (NCT05222932) are ongoing.[35]

The combinations of OVs with other immunomodulators have also been reported. Systemic anti-CTLA-4 antibody combined with Newcastle disease virus[36] and VV[37] induced anticancer immunity, mediating an abscopal effect in a syngeneic tumor mouse model. A triple combination of anti-CTLA4, anti-PD-1 and IL-12-armed HSV (G47Δ-mIL12) was shown to cure most glioblastoma-bearing mice without affecting viral spread and offered protective immunity after tumor rechallenge.[38] In addition, agonistic antibodies targeting the co-stimulatory receptor 4-1BB (CD137)[39] and glucocorticoid-induced tumor necrosis factor[40] combined with VV and HSV, respectively, demonstrated superior cytotoxicity than either agent alone in syngeneic tumor models. A list of *trans* combinations and their effect on the tumor cells, tumor vasculature and immune cells is summarized in tables 2–4, respectively.

Intermittent data from an ongoing clinical phase 1/2 trial (IGNYTE) of oncolytic HSV, RP1, which expresses granulocyte-macrophage colony-stimulating factor (GM-CSF) and the fusogenic protein GALV-GP R–, combined with nivolumab for skin cancer has been reported.[41] 62.5% of patients with anti-PD-1-naive cutaneous melanoma and 37.5% of patients with anti-PD-1/anti-PDL-1 plus anti-CTLA4-failed cutaneous melanoma had objective response to the treatment. For anti-PD-1-naive cutaneous squamous cell carcinoma, 47.1% and 17.6% of patients had complete remission and partial response, respectively. The treatment induced infiltration of $CD8^+$ T cells in the tumors, and the clinical responses are found to be independent of the baseline tumor PD-L1 expression status. In addition, the combination of ONCOS-102, a chimeric Ad expressing GM-CSF, with pembrolizumab demonstrated an enhanced shrinkage of non-injected lesions in patients with anti-PD-1 resistant/refractory melanoma.[42] In another phase II trial combining oncolytic HF-10, with ipilimumab, 18% and 23% of patients with advanced melanoma had complete remission or partial response.[43] There were more infiltrating $CD8^+$ T cells and fewer $CD4^+$ T cells in the tumors after the treatments. In addition, the combination of Coxsackievirus A21, V937, with ipilimumab in patients with advanced melanoma showed antitumor activity in local, visceral and non-visceral lesions, with an objective response rate of 50% as compared with 28% with V937 and 15–20% with ipilimumab.[44] The clinical trials of combining OV and antibody in *trans* were summarized in table 1.

Cis vs *trans* combination: which is a better option?

Until now, there have been more studies on the *trans* combination due to the readily available antibodies and simpler virus design. Indeed, the *trans* combination has been used as a standard approach for decades to deliver many combinations of anticancer agents, and clinical

Table 2 Combination therapy in *trans* targeting tumor cell

Target	Virus (species)	Antibody form	Effect ex vivo/in vivo	Delivery route (frequency)	Ref.
EGF/ EGFR	HF-10 (HSV)	Anti-EGFR IgG (cetuximab)	Colorectal cancer/ s.c. in BLAB/c nude mice: ↓ MVD (IHC, CD31, 13 dpi); ↑ viral replication (IHC, HSV-1 antigen, 2 dpi); ↓ tumor mass	OV: i.t. (3; 0, 3, 6 dpi) Ab: i.p. (5; –1, 2, 5, 8, 11 dpi)	25

Ab, antibody; dpi, days post-infection; EGF, endothelial growth factor; EGFR, vascular endothelial growth factor receptor; HSV, herpes simplex virus; IgG, immunoglobulin G; IHC, immunohistochemistry; i.p., intraperitoneal; i.t., intratumoral; MVD, micro-vessel density; OV, oncolytic virus; Ref, reference; s.c., subcutaneous.

Table 3 Combination therapies in *trans* targeting tumor vasculature

Target	Virus (species)	Antibody form	Effect ex vivo/in vivo	Delivery route (frequency)	Ref.
VEGF/ VEGFR	HF-10 (HSV)	Anti-VEGF IgG (bevacizumab)	Breast cancer/ s.c. in BLAB/c nude mice: ↓ MVD (IHC, CD31, 35 dpi); ↑ viral replication (IHC, HSV-1 antigen, 2, 35 dpi); ↑ hypoxia (IHC, HIF-α, 35 dpi)	OV: i.t. (1) Ab: i.p. (4; twice a week from –1dpi to 13dpi)	26
VEGF/ VEGFR	hrR3 (HSV)	Anti-VEGF IgG (bevacizumab)	Gastric cancer/ s.c. in BALB/c nude mice: ↓ tumor mass; ↓ MVD (IHC, CD31, 12 dpi); ↑ viral replication (CSLM, LacZ, 12 dpi)	OV: i.t. (4; 0, 3, 7, 10 dpi) Ab: IC (4; 0, 3, 7, 10 dpi)	114
VEGF/ VEGFR	rRp450 (HSV ICP-6 mutant)	Anti-VEGF IgG (bevacizumab)	Ewing sarcoma/ s.c. in nude mice: ↓ tumor mass; ↑ survival; ↓ MVD (IHC; CD31, 3 dpi); ↓ viral replication (plaque assay; 3 dpi); ↑ rescue of OV-depleted MHC class II TAM (FC; 3dpi)	OV: i.t. (1) Ab: i.p. (1; 0 dpi)	28
VEGF/ VEGFR	CRAd-S-pk7 (Ad)	Anti-VEGF IgG (bevacizumab)	Glioma/ i.c. in nude mice: ↓ tumor mass; ↑ viral replication, distribution (IHC, hexon, 3 dpi)	OV: i.c. (1) Ab: i.p. (2; –5 to –2 dpi)	115
VEGF/ VEGFR	dl992/947 (Ad)	Anti-VEGF IgG (bevacizumab)	Thyroid cancer/ s.c. in nude mice: ↓ tumor mass; ↓MVD (IHC, CD31, 2 dpi); ↑viral replication (IHC, Ad-GFP, 2 dpi)	OV: i.t. (1) Ab: i.p. (2; –6 to –1 dpi)	116
VEGF/ VEGFR	GLV-1h68 (VV)	Anti-VEGF IgG (bevacizumab)	Lung, prostate cancer/ s.c. in nude mice: ↓ tumor mass; ↓ MVD (IHC, CD31, 21dpi)	i.v. (1) i.p. (10; 2 times/week for 5 weeks from 13 dpi)	5
VEGF/ VEGFR	G47δ-mAngio (HSV armed with angiostatin)	Anti-VEGF IgG (bevacizumab)	Glioma/ s.t. in nude mice: ↓ tumor mass; ↑ survival; ↓ MVD (IHC; CD31; 3 dpi); ↓ viral replication, distribution (IHC, LacZ, 3 dpi); ↑ macrophage (IHC, F4/F80, 3dpi)	OV: i.t. (1) Ab: i.v. (1; –2 dpi)	27

Ab, antibody; Ad, adenovirus; CSLM, confocal laser scanning microscopy; dpi, days post-infection; EMH, extramedullary hematopoiesis; FC, flow cytometry; HER, HIF response elements; HIF, hypoxia inducible factor; HSV, herpes simplex virus-1; HUVEC, human umbilical vein endothelial cells; i.c., intracranial; IFN-γ, interferon-γ; IgG, immunoglobulin G; IHC, immunohistochemistry; i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous; MHC, major histocompatibility complex; MVD, micro-vessel density; OV, oncolytic virus; Ref, reference; s.c., subcutaneous; s.t., stereotactic; TK, thymidine kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VV, vaccinia virus.

protocols can be readily developed. Nonetheless, the *cis* combination approach may provide important advantages, particularly where systemic delivery of antibodies may cause toxicity. In the following sections, we highlight specific challenges where switching from *cis* to *trans*, or vice versa, may allow strategic improvements in anticancer efficacy.

Localized expression mitigates the systemic toxicity of checkpoint inhibitors

An acceptable safety profile is essential during the selection of treatment paradigms.

Though immune checkpoint inhibitors have revolutionized cancer treatment by inducing robust immune responses, they have been associated with a variety of adverse events or even lethality. Treatment with systemic anti-CTLA-4 antibody (ipilimumab) is closely associated with adverse events in the skin, gastrointestinal tract, liver and endocrine system, as reported in different clinical trials.[45] Meanwhile, treatment with systemic PD-1 antibody (pembrolizumab or nivolumab) is more frequently associated with thyroid disorders such as hypothyroidism and hyperthyroidism. These toxicities often affect patient compliance and treatment outcome.

Armed OVs become a potential approach to address this toxicity by allowing expression of checkpoint inhibitor antibodies selectively within cancer cells, followed by secretion into the TME. This minimizes systemic exposure and hence reduces inflammatory and auto-immune side effects while simultaneously maximizing local concentrations within the tumor and draining lymph nodes. Locally expressed PD-1 by myxoma virus in *cis* has been shown to reduce the severity of alopecia, prolong

Table 4 Combination therapies in *trans* restoring or activating immune cell functions

Target	Virus (species)	Antibody form	Effect ex vivo/in vivo	Delivery route (frequency)	Ref.
PD-1/PD-L1	vvDD (VV)	Anti-PD-1 IgG	Fibrosarcoma/ s.c. in C57BL/6 mice: ↓tumor mass; ↑survival	OV: i.t. (2; 0, 3 dpi) Ab: i.v. (3; 0, 3, 6 dpi)	[14]
PD-1/PD-L1	MV vaccine strain	Anti-PD-L1 IgG	Melanoma/ s.c. in C57BL/6 ↑survival; ↓tumor mass	OV: i.t. (4; 4 consecutive days) Ab: i.p. (4; every third day)	16
PD-1/PD-L1	Pelareorep (Reo serotype 3-Dearing strain)	Anti-PD-1 IgG	Melanoma/ s.c. in C57BL/6 mice: ↓ tumor mass; ↑ survival; ↑ IFN-γ (ELISA); ↓ Treg activity	OV: i.t. (3; day 0, 3, 5 dpi) Ab: i.v. (8; 7, 9, 11, 13, 15, 17, 19, 21 dpi)	29
PD-1/PD-L1	VSV-mIFN-β-NIS (VSV Indiana strain; encode IFN-β, NIS)	Anti-PD-L1 IgG	AML s.c./ in C57/B6 mice: ↓tumor mass in blood, bone marrow, spleen; ↑survival; ↑IFN-γ^{+}CD8^{+} T cell (FC; 7 dpi)	OV: i.v. (1) Ab i.p. (3; 3, 6, 9 dpi)	32
PD-1/PD-L1	MV-EGFR (MV vaccine strain; redirect viral entry via EGFR)	Anti-PD-1-IgG	Giloma o.t./ in C57BL/6 mice: ↓tumor mass in blood, bone marrow, spleen ↑survival; ↑intratumoral T cell (FC, MRI, 6 dpi); ↑ granzyme B^{+}CD8^{+} T cell, CD8^{+}/ Treg ratio (FC, 6 dpi)	OV: i.t. (4; day 0, 3, 7, 10) Ab: i.p. (3; day 1, 3, 9)	31
PD-1/PD-L1	GM-CSF/Reo/VSV-ASMEL (GM-CSF/REO for prime-boost; VSV expresses cDNA library of melanoma antigens)	Anti PD-1 IgG	Melanoma s.c./ in C57BL/6 mice: ↑ survival; ↑ IFN-γ, IL-17 (ELISA); ↑Th1, Th17 response	GM-CSF/REO: i.p./i.v. (2 cycle; −14 to −7 dpi) VSV-ASMEL: i.v. (3; day 0, 2, 4 dpi) Ab: i.v. (6; 0, 2. 4, 7, 9, 11 dpi)	117
PD-1/PD-L1	vvDD-IL-2-RG (VV; encodes IL-2)	Anti-PD-1 IgG; Anti-PD-L1	Colon cancer/ i.p. in C57BL/6 mice: ↑ survival; ↑ abscopal effect; ↓ s.c. distant tumor mass	OV: i.p. (1) Ab: i.p. (0; 0, 2, 4, 6 dpi)	118
PD-1/PD-L1	vvDD-CXCL11 (VV; encodes CXCL11)	Anti-PD-L1 IgG	Colon cancer/ i.p. in C57BL/6 mice: ↓ tumor mass; ↑ survival ; ↓ PD-L1 in intratumoral TAM, MDSC, DC, ↑ CD8^{+} T cell activation, CD8^{+}/Treg ratio; ↓ LAG3^{+}PD-1^{+}CD8^{+} T cells (FC, 5 dpi); ↓ s.c. rechallenged tumor; ↑ systemic antitumor immunity	OV: i.t. (1) Ab: i.p. (4; 0, 2, 4, 6 dpi)	30
PD-1/PD-L1	Ad5-CMV-mIL-2 and Ad5-CMV-mTNF α(2 Ad clones expressing IL-2 and TNF-α)	Anti-PD-1 IgG	Melanoma/ s.c. in C57BL/6JOlaHsd mice: ↓tumor mass; ↑survival; ↑ intratumoral CD4^{+}, CD8^{+} T cells Melanoma/ s.c. in C57BL/6JOlaHsd mice: ↓tumor mass; ↑survival; ↑ intratumoral CD8^{+}/ CD4^{+} T-cell ratio	OV: i.t. (1) Ab: i.p. (4; 0, 3, 6, 9 dpi) OV: i.t. (4; 0, 1, 3, 6 dpi) Ab: i.p. (4; 0, 1, 3, 6 dpi)	119 120
PD-1/PD-L1	TILT-123 (Ad; encodes TNF-α and IL-2)	Anti-PD-1 IgG	PDAC/ s.c. in Syrian golden hamsters: ↓tumor mass; ↑survival Urological tumor biopsies: ↑ IFN-γ, granzyme B, CXCL10	OV: i.t. (8; 0, 3, 6, 9, 12, 15, 18, 21 dpi) Ab: i.p. (8; 0, 3, 6, 9, 12, 15, 18, 21 dpi) OV: (1) Ab: (1)	33 120
PD-1/PD-L1	DP-TRP2-PeptiCRAd (Ad; coated with MHC-II–restricted Diphtheria–Pertussis peptides and MHC-I–restricted TRP2 peptides)	Anti-PD-1 IgG	Melanoma/ s.c. in C57BL/6 mice: ↓tumor mass; ↓ naive T cells in lymph nodes; ↑ CD4^{+} memory T cells in lymph nodes	OV: i.t. (4; 0, 2, 4, 6 dpi) Ab: i.p. (3; 0, 4, 8 dpi)	121
CTLA-4	MV vaccine strain	Anti-CTLA-4 IgG	Melanoma/ s.c. in C57BL/6 mice: ↓tumor mass; ↑survival	OV: i.t. (four consecutive days) Ab: i.p. (4; every third day)	16

Continued

Table 4 Continued

Target	Virus (species)	Antibody form	Effect ex vivo/in vivo	Delivery route (frequency)	Ref.
CTLA-4	NDV LaSota strain	Anti-CTLA-4 IgG	Melanoma/ i.d. in C57BL/6 mice: ↓ local, distant tumor mass; ↑ survival; ↑ distant CD45$^+$ cells, CD8$^+$/Treg ratio, granzyme B$^+$CD8, Ki67$^+$CD8 T cell (FC, 8 dpi) ↑ systemic antitumor immunity	OV: i.t. (4; 0, 3, 7, 9 dpi) Ab: i.p.: (4; 0, 3, 7, 9 dpi)	36
PD-1/PD-L1; CTLA-4	VV$_{WR}$/TK$^-$RR$^-$/FCU1 (WR; TK, RR deletion)	Anti-PD-1 IgG; Anti-CTLA-4	Fibrosarcoma/ s.c. in C57BL/6 mice: ↓ local, distant tumor mass; ↑ survival; ↑ abscopal effect	OV: i.t. (2; 0, 3 dpi) Ab: i.p. (3; 6, 9, 12 dpi)	37
PD-1/PD-L1, CTLA-4	VSV-HIF2α+VSV-SOX-10+VSV-c-Myc (3 VSV clones expressing HIF2α, SOX-10, c-Myc)	Anti-PD-1 IgG, anti-CTLA-4 IgG (co-use)	Glioma/ i.c. in C57BL/6 mice: ↑ survival; ↑ IFN-γ, IL-17 (ELISA); ↑Th1, Th17 response	OV: i.v. (6; 0, 2, 4, 7, 9, 11 dpi) Ab: i.v. (3; 7, 9, 11 dpi)	122
PD-1/PD-L1, CTLA-4	G47Δ-mIL12 (HSV; encodes IL-12)	Anti-PD-1 IgG, anti CTLA4 IgG (co-use)	Giloma i.c./ in C57BL/6 mice: ↑ survival; ↑ intratumoral CD11b$^+$CD45^{hi} peripheral macrophage, CD8$^+$/Treg ratio (FC, 7 dpi); ↑ T-cell activation (IHC, CD3, Ki67, 7 dpi); ↑ M1-like macrophage repolarization (IHC, CD68, pSTAT1, 7 dpi)	OV: i.t. (1) Ab: i.p.: (3; day 0, 3, 6 dpi)	38
PD-1/PD-L1, CTLA-4	rAd.GM (Ad; encodes GM-CSF)	Soluble TGF-βRII-Fc (E1B promoter), anti-PD-1 IgG, anti-CTLA-4 IgG (co-use)	Breast cancer o.t./ in BALB/c mice: ↓ tumor mass; ↑ survival; ↑ intratumoral CD8$^+$ T, memory T cells, ↓Treg; ↑ M1-like macrophage repolarization (IHC, 17 dpi); ↑ Th1 cytokine genes (qPCR, 17 dpi)	OV: i.t. (2; 0. 3 dpi) Ab: i.p.: (3; day 1, 4, 7 dpi)	123
PD-1/PD-L1, CTLA-4, TGF-β/ TGF-βR	rAd.sT (Ad; encodes soluble TGF-βRII-Fc)	Soluble TGF-βRII-Fc (TERT promoter), anti-PD-1 IgG, anti-CTLA-4 IgG (co-use)	Breast, colon cancer/ s.c. in BALB/c mice: ↓ tumor mass, metastasis	OV: i.t. (2; (0, 2 dpi) Ab: i.p. (4; day 1, 3, 5, 9 dpi)	124
4-1BB	vvDD (VV)	Anti-4-1BB IgG	Breast cancer/ s.c. B6 mice: ↓ tumor mass, metastasis; ↑ intratumoral CD11b$^+$, CD11c$^+$ myeloid cells, CD8$^+$ T cells, CD11b$^+$Ly6G$^+$ neutrophils (nine dpi)	OV: i.t. (2; 0, 2 dpi) Ab: i.p. (2; 4, 6 dpi)	39
GITR	HF-10 (HSV)	Anti-GITR IgG	Colon cancer, fibrosarcoma/ s.c. in BALB/c mice: ↓ tumor mass; ↓ intratumoral Tregs (FC, 5 dpi); ↑ IFN-γ (FC, 7 dpi); ↑ antiviral CD8$^+$ T cell	OV: i.t. (3; 0, 1, 2 dpi) Ab: i.t. (1; two dpi)	40

Ab, antibody; Ad, adenovirus; AML, acute myeloid leukemia; CMV, cytomegalovirus; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; dpi, day post-infection; FC, flow cytometry; FRβ, folate receptor-beta; GITR, glucocorticoid-induced tumor necrosis factor receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HER2, human epidermal growth factor receptor 2; HIF2α, hypoxia-inducible factor-2 alpha; HSV, herpes simplex virus; i.c., intracranially; i.d., intradermally; IFN-γ, interferon-γ; IgG, immunoglobulin G; IHC, immunohistochemistry; IL-2, interleukin 2; i.p., intraperitoneal; i.t., intraturmoral; i.v., intravenous; IVA, influenza A virus; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; MV, Measles virus; NDV, Newcastle disease virus; o.t., orthotopic; OV, oncolytic virus; PD-1, programmed cell death protein-1; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed cell death ligand-1; Ref, reference; Reo, reovirus; RR, ribonucleotide reductase; Sox10, Sry-related HMg-Box gene 10; TAM, tissue-associated macrophage; TGF-β, transforming growth factor beta receptor II; TGF-βRII, transforming growth factor beta receptor; TK, thymidine kinase; Treg, regulatory T cell; Treg, regulatory T cell; VEGFA, vascular endothelial growth factor A; VEGFR, vascular endothelial growth factor receptor; VSV, vesicular stomatitis virus; VV, vaccinia virus; vvDD, vaccinia virus Western Reserve strain.

the overall survival and increase the completed response rate when compared with the *trans* combination in a syngeneic melanoma mouse model.[46] Besides, no overt immune-mediated toxicity was observed in immunocompetent mice injected with MV armed with anti-PD-L1 scFv or anti-CTLA-4 scFv.[16] In addition, local injection with low dose anti-CTLA-4 antibody in slow-release delivery formulation Montanide ISA-51 reduced the toxicity but was still able to confer a strong antitumor response as the high dose systemic antibody treatment.[47 48] Increasingly, it appears the site of action of anti-CTLA4 may be in the tumor-draining lymph nodes, which is a site thought to be accessed by tissue fluid drainage following intratumoral injection of macromolecules and also by proteins secreted from OV into the TME.

Other immunomodulatory antibodies, such as agonistic CD40 antibody, can also lead to systemic toxicities when given intravenously, in this case, cytokine storm and hepatotoxicity.[49] Direct intratumoral injection of anti-CD40 antibody[50] or slow-release delivery formulation[51] has been demonstrated to regress both local and distal antitumor response with minimal side effects in syngeneic tumor

mouse models. Furthermore, systemic administration of antibodies targeting pan-transforming growth factor-beta (TGF-β), an inhibitory cytokine, was associated with mortality caused by serious cardiovascular toxicity and acute hemorrhage in mice and cynomolgus monkeys.[52] Such toxicities were not observed in tumor-bearing mice treated with Ad expressing soluble TGF-β receptor II-Fc fusion protein.[53 54] These studies suggest that the *cis* combination using armed OVs could potentially overcome one of the biggest immunotherapy challenges, namely the toxicity associated with systemic delivery of immunomodulatory proteins, including checkpoint inhibitors.

Armed OVs open a new avenue for the delivery of multiple anticancer agents

Given the dynamic nature of the immune response and the complexity of immune checkpoint regulation, it is difficult to rely on any single immunotherapeutic to boost the antitumor response. For example, monotherapies against PD-1 or CTLA-4 have both been associated with a low response rate in patients, with anti-PD-1 antibody showing an objective response rate (ORR) of 10–40% and anti-CTLA-4 antibody 10–20% in solid tumors.[55] Targeting several inhibitory receptors simultaneously could potentially boost this response rate. In a phase I dose-escalating study for previously-untreated melanoma, an ORR of 61% was seen in the group receiving both ipilimumab and nivolumab in *trans* versus 11% in the group receiving ipilimumab monotherapy. However, the combinational use of antibodies resulted in a higher level of toxicity, with 54% of patients in the combination group developing grade 3 to 4 drug-related adverse events as compared with 24% of patients who received monotherapy.[56] Although the toxicity of the combination treatment was seen as acceptable, comparable treatment with better safety profiles would always be preferable. On the contrary, no grade ≥3 treatment-related adverse event was reported in a phase I clinical trial on oncolytic HSV, ONCR-177 that expresses IL-12, FLT3L, CCL4, anti-PD-1 and anti-CTLA-4 antibodies. However, the phase I trial has recently been discontinued. Whether the relatively well-tolerated profile was due to the localized expression of biologics or the lack of efficacy of the *cis* combination remains unclear.[24]

To date, clinical trials exploring localized delivery of multiple antibodies for cancer treatment are very limited, yet the advantages have been clearly exemplified in some preclinical studies. For example, Palazón *et al* demonstrated that intratumoral injection of low dose anti-CD137 monoclonal antibody with systemic PD-L1 blockade gave a synergistic antitumor effect in mice bearing syngeneic colorectal cancer, simultaneously avoiding any liver toxicity[57] which is a commonly-reported side effect in patients receiving systemic anti-CD137 antibody.[58] Though only CD137 antibody but not PD-L1 antibody was locally delivered, the overall toxicity was dramatically reduced. This study suggested that systemic toxicity caused by immunomodulators might add up in a synergistic manner, and the *cis* combination becomes particularly desirable when multiple agents are used. In other words, the more systemic toxicity associated with a treatment, the stronger the case of local or intratumoral delivery.[59] Just a note of caution, the delivery of more antibodies does not necessarily give better outcomes. The addition of anti-PD-1 antibody in *trans* showed no additional benefit to the combination treatment of CTLA-4-armed VV and radiation, possibly due to the immune activation ceiling.[60]

Armed OVs avoid systemic toxicities associated with tumor stroma depletion and angiogenesis inhibition

The selectivity of oncolytic viruses allows the use of antibodies that would otherwise not be suitable for systemic administration. Localized expression of antibodies can improve the biodistribution of antibodies targeting not only tumor antigens but also stromal and tumor vasculature antigens. For example, despite the fact that EpCAM is upregulated on most cancer cells, it is also present on normal epithelial cells. Targeting cancer cells with the EpCAM BiTE might mediate significant on-target off-tumor toxicity if it is systemically administrated. In a phase I trial of EpCAM BiTE in patients with relapsed/refractory solid tumor, 95% of patients had treatment-related adverse events grade 3 or above, primarily diarrhea, elevated liver parameters, and elevated lipase.[61] Similarly, although trastuzumab is a well-tolerated drug, a low level of therapeutic antibodies in the circulation is considered to be desirable to avoid potential toxicities.[62]

An additional advantage of this strategy is that localized expression of the biologic may achieve far higher concentrations within the tumor than that could be achieved following intravenous administration of the free protein. For example, the development of Ad5/3-Δ24 expressing trastuzumab in *cis* was found to significantly lower the concentration of trastuzumab in blood and showed a promising tumor-to-systemic ratio of trastuzumab of 1.17 as compared with 0.009 in tumor-bearing mice that received systemic trastuzumab administration.[1]

FAP being a cell surface marker of CAFs, has long been a target for tumor stroma depletion. An early attempt by Tran *et al* to kill FAP^+ cells using adoptive transfer of FAP-reactive CAR T cells was reported to cause cachexia and anemia in mice due to the FAP expression on bone marrow stromal cells.[63] In addition, Roberts *et al* showed that FAP^+ cells are widely distributed in most tissues of adult mice. Ablation of FAP^+ cells caused atrophic muscle response and impaired erythropoiesis and B lymphopoiesis, suggesting that systemic depletion of FAP causes adverse biological consequences.[64] Approaches to localize FAP-targeting antibodies by OVs were then explored. Mice treated with FAP BiTE-encoding OVs showed no overt toxicity, and their body weight and the number of total bone marrow cells remained stable throughout the experiment.[12] Freedman *et al* also showed that adding FAP BiTE-expressing oncolytic virus to samples of the primary bone marrow showed no anti-fibroblast activity,

presumably because there are no tumor cells present to allow the production of the BiTE by the virus.[11] Recent evidence has also demonstrated the use of tumor protease-cleavable polypeptide to mask the antibody–antigen interaction for adding another level of safety to the antibodies.[65 66] Epitopes or binding motifs are only free from masking in the tumor site, minimizing any on-target off-tumor effect.

The ability to temporally regulate transgene expression is another advantage of armed OVs. In *cis* combination, OVs offer a tight regulatory system that enables the expression of the encoded biologic only in certain conditions. For instance, transgenes in EnAd could be designed to be expressed only in cancer cells or cells that are permissive to viral replication when the transgenes are placed under the major late promoter.[2] In addition, tumor-specific or tissue-specific promoters such as prostate-specific antigen[67] and telomerase reverse transcriptase promoter[68] have also been used to regulate transgene expression, restricting the expression of biologics in the tumor site or the designated regions. Furthermore, a recent study demonstrated the use of inducible promoter systems to regulate transgene expression. Three chemogenetic switches, that is, the rapamycin-inducible ST7 RNA polymerase expression system, Dox-inducible expression, and cumate-inducible expression (CymR/CuO) either being used alone or together were shown to control replication and transgene expression in VV, highlighting the potential of expanding the use of armed OVs in the clinic.[69] Without any regulation on transgene expression, a high dose of the transgene may be produced systemically. For example, constitutive expression of soluble vascular endothelial growth factor receptor 1 (VEGFR1) by Ad vector[70] or a high dose of VEGFR-1-Ig-armed VV[71] showed lethal toxicity in mice, even though VEGF-inhibiting molecules are considered relatively well-tolerated agents. In contrast, inducible expression of soluble VEGFR1[70] and a lower dosage of VEGFR-1-Ig-VV[71] showed no sign of toxicity and did not compromise the antitumor activity.

In addition, toxicities may develop when a well-tolerated drug is combined with other treatments. For instance, anti-VEGF bevacizumab is generally known as a relatively safe drug, yet 'bevacizumab-associated' toxicities develop when it is combined with other standard chemotherapies—for example, carboplatin plus paclitaxel, resulting in hypertension and thromboembolic events in phase III clinical trials of metastatic breast cancer and ovarian cancer.[72 73] Nonetheless, no additional toxicity was observed when anti-VEGF scFv was co-armed with anti-EGFR nanobody or anti-FAP scFv in VV as compared with anti-VEGF scFV-armed VV or the parental virus alone in a tumor xenograft mouse model.[74] Taken together, armed OVs allow the delivery of biologics that would otherwise not be possible by systemic administration, particularly when multiple agents have to be delivered.

Armed OVs 'convert' tumor-infiltrating antivirus T cells into antitumor T cells

OVs are capable of turning the TME from 'cold' to 'hot' by remodeling the cytokine environment that promotes the maturation of immune cells. The lysis of tumor cells releases not only TAAs but also PAMPs and DAMPs. The antigens and patterns are recognized by the immune system, and trigger the release of inflammatory cytokines, which promote the recruitment and activation of different immune cells.[75] Since dendritic cells and macrophages present both TAAs and viral elements, primed T cells can recognize TAA but also viral antigens. In general concomitant antiviral immune responses are undesirable as they likely hinder the virus persistence and spreading in the TME, though any bystander killing effect of the antiviral killing may also kill a portion of the cancer cells.[76]

The *cis* combination provides an advantage of 'converting' the activated antiviral T cells to become antitumor by encoding bispecific antibodies that engage immune cells to the cancer cells. Bispecific antibodies such as BiTE may engage tumor-infiltrating antiviral T cells and redirect their cytotoxicity towards tumor cells. NK cell engagers also possess the ability to redirect the cytotoxicity to eliminate tumors, and perhaps some of the antitumor activity is converted from the early antiviral innate response.[77] Despite being promising, it is still unclear whether the T cells still possess the antiviral response or how potent the antiviral response would be after the dissociation of the bispecific antibodies. Immunomodulatory antibodies as single-agent therapies are often not enough to drive potent immune response due to the lack of the TILs, but with the combination approach, these antibodies can exploit the antiviral response to augment the antitumor response.

Comparative efficacy of *cis* and *trans* combination strategies

One fundamental difference between the *cis* and *trans* combination is the administration schedule and administration concentration of the therapeutic antibodies. In the *cis* combination, *in situ* amplification and expression of transgenes by OVs are defined by the rate and efficiency of virus infection, duration of life cycle and ease of viral spread from tumor cell to tumor cell and across the TME. In contrast, the *trans* combination allows a more flexible administration program, and clinicians may choose to modulate the doses of antibody used depending on patient response or even change treatment altogether. One important question is whether the *cis* combination approach is disadvantaged by the flexible administration schedule offered by the *trans* combination or whether they perform equally well.

The synergy of OVs and checkpoint inhibitors in *trans* has been demonstrated in several clinical trials. The ORR increased from 35–40% in patients treated with pembrolizumab or nivolumab alone to 62% in patients treated with talimogene laherparepvec (T-VEC) with pembrolizumab in clinical trials of metastatic melanoma.[78–80] Since OVs are thought to prime the tumor immune system for

checkpoint blockade, the administration schedule of the agents could be critical in inducing optimal immune responses. Anti-PD-1[37] and anti-PD-L1[30] antibodies have been shown to achieve maximal antitumor response and confer survival advantage when they were administrated simultaneously or shortly after the OV injection in syngeneic tumor mouse models. The synergy was lost when the first dose of anti-PD-L1 antibody was delivered late—4 days after OV treatment. Besides, the anti-CTLA-4 antibody achieved the greatest therapeutic outcome when its administration was shortly (1 day) after the OV treatment. Conversely, the benefit of CTLA-4 blockade was attenuated when it was applied before OV treatment and was completely abolished when it was applied late—7 days after the OV treatment.[37] These suggest that blockade of PD-1 and CTLA-4 functioned the best when the antibodies were injected shortly after OV treatment. Is it possible to achieve this comparative timing of administration using the *cis* approach?

The time between OV infection and the release of antibodies to the TME depends on the life cycle of the virus and the model system used, but typically this process appears to take 18–36 hours. Coincidently, this timing, which reflects the mechanism of action, matches the administration schedule of the *trans* combination that gives the best therapeutic outcome. Indeed, many armed viruses, including anti-PD-1 scFv-armed or IgG-armed VV,[14] anti-PD-1 scFV-armed HSV[15] and anti-PD-L1 scFv-Fc-armed MV[16] performed equally well as the *trans* combination and showed a comparable antitumor response in syngeneic tumor mouse models.

Nonetheless, although anti-CTLA-4 scFv-Fc-armed MV significantly inhibited tumor growth in a syngeneic melanoma model, treated mice showed slightly inferior overall survival than those who received the *trans* combination.[16] There could be many contributory factors, but one possibility is that the location difference between antibody production (within the tumor) and where the antibody acts on its target might attenuate the antitumor response because anti-CTLA-4 antibodies are increasingly thought to regulate T-cell activity primarily in lymph nodes. Interestingly, Newcastle disease virus expressing anti-CTLA-4 scFv plus radiotherapy-induced potent antitumor response and abscopal effect that were comparable to the *trans* combination with irradiation.[60] This suggests that, at least sometimes, localized delivery of anti-CTLA-4 antibodies could be as potent as systemic delivery. Though OVs do not infect lymph nodes, antibodies may be able to drain from the TME to the tumor-draining lymph nodes via the lymphatic vessels to target immunological events that occur there.

Similar arguments apply to anti-angiogenic and antivascular strategies. For example, VV expressing anti-VEGF scFv was able to inhibit angiogenesis in both virus-infected and uninfected areas of the human xenograft tumor and achieved a comparable antitumor effect as the *trans* combination, in which the first dose of bevacizumab was administrated 10 days after OV.[5 74]

Nonetheless, the *cis* combination may not be suitable when better therapeutic effects are observed when the antibody administration is prior to the OV injection. For example, administration of cetuximab 1 day before HF-10 in a human colorectal cancer xenograft mouse model has been shown to confer a stronger antitumor effect than the administration of cetuximab 1 day after HF-10, which only elicited antitumor response comparable to HF-10 alone.[25] These could potentially be the limitations of the *cis* combination, particularly when the antibodies have to be delivered for an extended period. Repeated administration of the armed OVs is likely not possible due to the presence of antivirus antibodies. In addition, it is speculated that OVs may act as adjuvants to induce anti-drug antibodies against therapeutic proteins, though there is no evidence about this. The use of humanized or fully human antibodies should minimize this risk.

Trans combination offers flexible administrations

Trans combination provides a flexible administration schedule, where OVs and antibodies can be administered at different times and through different routes. One key difference between *cis* and *trans* combination is that the latter supports the exposure of antibodies to the systemic circulation, allowing the antibodies to reach the disseminated disease readily. Although intravenous infusion is possible for some viruses, the efficiency of delivery to disseminated disease following intravenous injection of OV is not well documented. Similarly, even for the intratumor injection of OVs in *cis* combination, the amount of antibodies that can reach the distal lesion is less predictable as compared with the *trans* combination, thereby affecting the antitumor effect of the non-infected lesions. This is particularly significant when the disseminated tumors are difficult to reach or when there are several metastatic lesions. For instance, a recent clinical trial of ONCR-177, which already encodes an anti-PD-1 antibody in *cis* was planned to combine with the anti-PD-1 monoclonal antibody pembrolizumab *in trans*.[81] The co-use of virally encoded and systemically administrated antibodies was apparently considered non-redundant. Indeed, preclinical studies demonstrated that combination with systemic anti-PD-1 blockade enhanced the abscopal antitumor effect,[82] and this may reflect that the amount of VHH produced *in situ* was not enough to cause optimal abscopal effect. The use of systemic antibodies targeting the same antigens as the virally encoded one may highlight a better strategy to regress distal tumors.

Challenges of OV delivery

Systemic delivery is desirable for oncolytic virotherapy as it not only allows the OVs to reach disseminated regions but also supports *in situ* expression of antibodies in the tumor sites. Nonetheless, systemic delivery of OVs remains a major challenge because of the usually poor stability of OVs in human blood, coupled with challenges of extravasation and limited duration of persistence.[83] For instance, the complement system can neutralize many

virus particles[84] and human erythrocytes can function as 'virus traps' by sequestering virus particles and targeting them for rapid elimination.[85]

The physical properties of OVs that support systemic delivery have recently received much attention. For instance, EnAd has been safely delivered via intravenous injection because of its stability in whole human blood and a low level of pre-existing neutralizing antibodies to type 11 adenovirus.[83] In addition, oncolytic poxvirus[86] and measles viruses[87] have been shown to selectively replicate and express transgenes in cancer tissue after intravenous infusion. Finally, Reo has been routinely administered intravenously, although the virus is probably too small for stable encoding of transgenes.[88]

Intratumor injection apparently is an alternative to intravenous infusion. It is also currently the most common route of virus administration. Accurately controlled concentration of OVs and their evasion of systemic exposure are the obvious theoretical advantages.[89] Nonetheless, this route is more suitable for superficial tumors than deep or visceral tumors, which generally requires sophisticated imaging or difficult operations for delivery. Regardless of intravenous or intratumor delivery, the persistence of the OVs is another concern, which can be relatively short, and this could pose a limitation to the antibody expression in the tumor bed. Perhaps increasing OV persistence within tumors is the key area that should be addressed to potentiate this aspect.

Armed OVs allow better distribution of antibodies in the TME

Antibody format, which defines their molecular size and stability, affects therapeutic efficacy and duration of responses. When given in *trans* combination, most of the antibodies are delivered as IgG (~150 kDa) because the Fc domain confers better stability and a longer half-life to antibodies in the circulation. Systemic administration is the standard route of delivery as it provides a more predictable pharmacokinetic.[59] Full receptor occupancy is also considered to be more achievable by systemic administration, although penetration into solid tumors is notoriously difficult and incomplete receptor occupancy of T cells in the TME has been reported in patients showing complete receptor occupancy in circulating T cells after a full dose of anti-PD-1 antibody.[90] Along with other macromolecules, antibodies that can extravasate into tumor tissue will be concentrated predominantly in the subendothelial layers, which are typically rich in the stroma, because the TME provides a challenging barrier to the intratumoral convection of macromolecules.

Armed OVs offer an alternative approach to delivering antibodies to the TME, in which the antibodies are expressed *in situ*, and the antibody concentration builds up alongside viral replication. Though antibodies would meet the same barriers to intratumoral convection as when given systemically, the spread of the virus through layers of parenchymal cells would give a proportionately better distribution of their encoded antibody. Delivery of smaller antibody formats such as scFv and nanobodies also becomes much more realistic by encoding them within OVs because the relatively small molecular size, which promotes rapid renal excretion following intravenous administration, may afford improved intratumoral convection following their local expression. For example, the whole IgG is estimated to take 54 hours to move 1 mm within the solid tumor, whereas a Fab fragment takes only 16 hours for the same distance.[91] Apart from improving efficacy, antibody fragments would also be expected to decrease systemic toxicity since any OV-encoded agent that does leak back from the tumor into the circulation would be expected to be cleared rapidly by renal excretion, limiting systemic exposure.

In addition, antibody affinity and avidity affect the efficiency of tissue penetration. While it is commonly assumed that a tighter binding is always better, a strong binding hinders the antibody from penetrating deeper into the tumor core until the antigens at the peripheral regions become saturated. The 'binding site barrier effect' describes the phenomenon of high-affinity antibodies being 'stuck' at the tumor periphery.[92] Adams *et al* reported that antibody penetration inside the tumor was optimal when the antibody binding affinity is lower (10^{-7} M) and antibodies with higher affinity (10^{-9} M) had better retention and selectivity.[93] However, most of the monoclonal antibodies targeting tumor antigens have higher affinity ranging from 10^{-9} M to 10^{-10} M (cetuximab: 0.38 nM[94]; trastuzumab: 7 nM[95]). A study on the affinity of EGFR antibodies showed that when the scFv format has an affinity of 10^{-7}, the affinity of the corresponding IgG is 10^{-9}, implying that the structure and bivalency of IgG have dominant roles in defining its affinity and hence penetration ability.[96] Monovalent antibody fragments may therefore penetrate better within the TME, particularly when affinity is high.

Nonetheless, using smaller antibody fragments does not necessarily associate with a better antitumor effect because small molecular size may sacrifice the retention ability. VV expressing anti-PD-1 scFv induced a similar but not superior antitumor response as the counterpart expressing the IgG format in syngeneic mice bearing fibrosarcoma.[14] The right balance between affinity, penetration and retention of the antibodies is essential for targeting both the tumor cells and the TME. One important thing to note is that, because of the competitive binding, the affinity of an antibody to its target should ideally be higher than that of its natural binding ligand.

Armed OVs may provide better delivery of antibodies into immune-privileged regions

One final aspect of the *cis* combination to consider concerns access of antibodies into immune-privileged sites, which is notoriously difficult. For example, the efficacy of antibody therapy against tumors in the brain is primarily dampened by the blood-brain barriers, which limits the extravasation of antibodies and immune cells from the blood.[97] Intratumoral injection of OVs has been evaluated in patients with glioma, yet this administration

route requires challenging surgery and might not be feasible for repetitive injections.[98 99] Remarkably, a recent study by Samson *et al* showed that oncolytic Reo delivered by intravenous delivery was able to infect glioma and brain metastases, proving that at least some OVs are able to cross the blood-brain barrier.[100] The infection was also associated with the infiltration of cytotoxic T cells, showing a pharmacodynamic effect. This approach may help to circumvent a major limitation imposed by the blood-blood barrier and provides the possibility of localized expression of antibodies within brain tumors following systemic administration of OVs.

Acquired resistance to antibody therapy

The development of resistance to antibody therapy is a potential challenge for armed OVs as the target cells would lose their sensitivity to the encoded payloads. For example, anti-PD-1 therapy has been shown to upregulate the expression of T-cell immunoglobulin mucin-3 (TIM-3), and subsequent administration of anti-TIM-3 antibody demonstrated a survival advantage after the failure of PD-1 blockade in mice.[101] This highlights an advantage of the *trans* combination, in which switching therapeutic antibodies is possible during treatment based on the patient's response. Of course, sequential injection of two OVs armed with different antibodies may be an alternative to the administration of unarmed OVs and two standalone antibodies, but this would incur considerable costs of manufacturing more than one therapeutic OV. A superior approach could be to express both antibodies within the same OV, effectively trying to resist provoking resistance by targeting multiple pathways from the beginning.

CONCLUSIONS

The recent phase II study on OVs and ipilimumab has demonstrated the synergy between these two agents.[102] Combining T-VEC in *trans* increased the ORR of ipilimumab from 18% to 39% in patients with advanced melanoma, yet the incidence of grade ≥3 adverse events also increases from 35% to 45%. Despite the tolerable safety profile, the *cis* combination might reduce the ipilimumab-associated toxicities. In an interim analysis of a phase I solid cancer trial of RP2, an oncolytic HSV expressing anti-CTLA4 antibody-like molecule, fusogenic protein (GALV-GP R−) and GM-CSF, only approximate 10% of patients developed grade ≥3 adverse effects.[23] Though RP2 and T-VEC are different HSVs and the trials might not be completely comparable, a significant reduction in the adverse events in the RP2 trial (*cis* combination) highlighted the advantage of *cis* combination to minimize the antibody-associated toxicity. However, more early-phase clinical trials are needed to demonstrate this. To date, there are only a few completed trials on antibody-armed OVs, but several trials have been scheduled. EnAd armed with CXCL9, CXCL10, IFN-α and anti-CD3/FAP BiTE[103] and VV armed with GM-CSF and Treg-depleting anti-CTLA4 antibody[104] are both currently in phase I studies. Combining OVs with antibodies has been shown to effectively modulate immune responses and remodel the TME. A better understanding of the interaction between OVs, antibodies and immune cells in both spatial (site of action) and temporal (sequence of the biological events) manner is essential for designing optional combinational regimes.

Given the huge variation in the packaging capacity of different virus types, antibody formats such as IgG are rather large in size compared with some small viruses. However, some viral genes are often removed from the genome to confer the virus cancer-selectivity. For instance, EnAd has a large deletion in E3, and a small deletion in E4, providing an extra 2.5–3 kb space for the insertion of therapeutic transgenes into the genome. Inserting the BiTE of 1.6 kb large did not affect the replication nor oncolytic ability of the virus as compared with the parental virus.[2 11] In addition, it is found that Ad can generally tolerate up to 105% but not more of its parental genome size, without affecting its replication and stability.[105] When the total transgene size exceeds the viral packaging capacity, potency or replication of the virus, at least for Ad, would be hindered, though the exact mechanism is still not well studied. To express multiple antibodies, it really requires larger viruses such as HSV, VV or helper-dependent adenovirus (which would not be oncolytic). Replimune is currently developing an oncolytic HSV (RP2) containing three transgenes (GM-CSF, GALV-GP R− and anti-CTLA-4 antibody)[23] and Akamis Bio, an adenovirus with four transgenes (a fibroblast-targeting bispecific, alpha interferon, CXCL9 and CXCL10).[106] Provided the packaging capacity is not exceeded, there is no indication of deleterious virus performance due to encoded antibodies.

Antibody-armed OVs have recently been gaining more attention because of their safer delivery route for potent biologics and their ability to amplify and secrete therapeutic antibodies locally in the tumor. Besides, some OVs are able to cross the blood-brain barrier, potentially facilitating the expression of antibodies within immune-privileged sites. In addition, the choices of antibodies encoded in OVs are versatile, allowing more combination regimens and novel therapeutic strategies. Given the heterogeneity of the tumors, armed oncolytic virotherapy is a promising strategy for cancer immunotherapy.

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