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REVIEW

Viro-antibody therapy: engineering oncolytic viruses for genetic delivery of diverse antibody-based biotherapeutics

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ABSTRACT

Cancer therapeutics approved for clinical application include oncolytic viruses and antibodies, which evolved by nature, but were improved by molecular engineering. Both facilitate outstanding tumor selectivity and pleiotropic activities, but also face challenges, such as tumor heterogeneity and limited tumor penetration. An innovative strategy to address these challenges combines both agents in a single, multitasking therapeutic, i.e., an oncolytic virus engineered to express therapeutic antibodies. Such viro-antibody therapies genetically deliver antibodies to tumors from amplified virus genomes, thereby complementing viral oncolysis with antibody-defined therapeutic action. Here, we review the strategies of viro-antibody therapy that have been pursued exploiting diverse virus platforms, antibody formats, and antibody-mediated modes of action. We provide a comprehensive overview of reported antibody-encoding oncolytic viruses and highlight the achievements of 13 years of viro-antibody research. It has been shown that functional therapeutic antibodies of different formats can be expressed in and released from cancer cells infected with different oncolytic viruses. Virus-encoded antibodies have implemented direct tumor cell killing, anti-angiogenesis, or activation of adaptive immune responses to kill tumor cells, tumor stroma cells or inhibitory immune cells. Importantly, numerous reports have shown therapeutic activity complementary to viral oncolysis for these modalities. Also, challenges for future research have been revealed. Established engineering technologies for both oncolytic viruses and antibodies will enable researchers to address these challenges, facilitating the development of effective viro-antibody therapeutics.

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Introduction

Antibodies of various formats are widely approved as cancer therapeutics, including monoclonal antibodies (mAbs) for targeted therapy or immune checkpoint inhibition, antibody-drug conjugates, and recombinant bispecific antibodies that target endogenous T cells to tumors.[1] Virotherapy with oncolytic viruses (OVs) is an emerging modality for treatment of cancer, with marketing approval in 2015 of the first OV in the Western hemisphere.[2–4] Preclinical and clinical data have revealed that the therapeutic activity of OVs depends on viral oncolysis-triggered antitumoral immune activation, thus encouraging combination immunotherapy with OVs.[5–9] Both therapeutic modalities can be combined in a single, multi-effector agent by insertion of antibody-encoding transgenes into OV genomes, thereby facilitating genetic delivery and expression of antibodies in tumors, i.e., *viro-antibody therapy*. Thus, this approach facilitates higher local antibody concentrations while reducing systemic side effects, and complements viral oncolysis with selected therapeutic modes of action dependent on the choice of antibody.

Both OVs and antibodies evolved by nature, but therapeutic versions have been improved by sophisticated molecular engineering technology. Both facilitate outstanding tumor selectivity and pleiotropic activities, and both can be used to deliver therapeutic payloads into tumors, for example, by insertion of therapeutic genes into the virus or by linkage of drugs to antibodies. Treatment with OVs, i.e., virotherapy, features a unique amplification mechanism by intratumoral virus replication, cell lysis and spread, and thereby triggers *in situ* tumor vaccination by release of tumor antigens in the context of virus-induced inflammation. Antibodies implement an unparalleled binding specificity and technologies are in place to rapidly obtain antibodies for virtually any target structure.[10,11] In cancer therapy, antibodies targeting cell surface markers of cancer cells or cancer-supportive stroma cells (targeted therapy), of immune cells (checkpoint inhibition), or both (bispecific antibodies) are well established. However, the success of both kinds of biotherapeutics is limited by several barriers, including tumor heterogeneity, structural and immunological barriers to OV spread, and poor tumor accumulation and penetration of antibodies. However, as mentioned above, both modalities are amenable to sophisticated engineering approaches that can be exploited toward overcoming these barriers. One innovative engineering approach is to merge both therapeutics into a viro-antibody therapy, i.e., the genetic delivery of recombinant antibodies with accordingly "armed" OVs that are still able to replicate and lyse tumor cells. From the OV perspective, this strategy allows the killing

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of bystander cells that are not reached or infected by the OVs themselves. To this end, antibodies might better penetrate the extracellular matrix (ECM) than OVs, implement an additional, but independent level of tumor targeting and allow for the selection and tailoring of effector mechanisms. The latter may include the targeting of cells of the tumor microenvironment (TME), i.e., stroma and immune cells, in addition to tumor cells. From the antibody perspective, genetic delivery allows local and extended expression, thus enabling the accumulation of smaller antibody formats that better penetrate the tumor at higher concentrations than is feasible by systemic application. Moreover, viro-antibody therapy combines different modes of direct and indirect cancer cell killing for enhanced therapeutic potency and reduced chances for development of resistance.

In light of the therapeutic potential and opportunities of viro-antibody therapy, we first provide an overview of OVs, recombinant antibody therapeutics and genetic delivery approaches for therapeutic antibodies. Then, we highlight applied antibody formats, strategies for their expression by OVs, implemented therapeutic modes of action by delivered antibodies, and examples for resulting therapeutic activities. A comprehensive overview of reported antibody-encoding OVs with key results of each report is provided in Table **5**. Finally, we discuss the major conclusions and challenges resulting from viro-antibody therapy to date and look ahead on future opportunities that further underline the potential of this combination biotherapy.

Oncolytic viruses, virus engineering, and insertion of therapeutic genes

Virotherapy is an emerging modality of cancer treatment with OVs that selectively infect and lyse cancer cells, thereby replicating and spreading in the tumor.[2] Tumor selectivity of OVs is based on intrinsic features of cancer cells, such as unrestricted proliferative signaling, increased nucleotide synthesis, defects of apoptosis pathways and deficient antiviral defense mechanisms.[12] OV development has exploited a wide repertoire of viruses with or without envelope, with DNA or RNA genomes, and of a wide spectrum of genome and particle sizes.[13] This includes wild-type viruses – usually of animal hosts, such as vesicular stomatitis virus (VSV), Newcastle disease virus (NDV) or parvovirus (PV) – or vaccine strains, such as measles viruses (MeV), that are highly sensitive to cellular antiviral defenses in normal human cells, but replicate and kill cancer cells deficient in antiviral defenses.[12] Designer OVs, in contrast, are generated by genetic engineering of viral genomes for tumor-specific cell entry or post-entry replication.[13–16] Tumor-specific cell entry of OVs has been achieved particularly for enveloped viruses by fusion of ligands to the cell-binding glycoproteins that are further mutated to avoid binding to natural viral receptors. Especially tumor-specific single-chain antibody fragments (scFvs) have been successfully exploited as ligands for entry targeting of MeV, herpes simplex viruses (HSV), and VSV.[13,15] Enhanced entry of non-enveloped OVs, especially adenoviruses (Ads), into cancer cells has been achieved by genetic modification of the viral protein capsid, for example, by insertion of an integrin-binding RGD peptide or switching of cell-binding domains between related viruses binding to different receptors.[17,18] Post-entry targeting has been achieved by deletion of viral genes or domains responsible for functions redundant in cancer cells (e.g., induction of S phase or apoptosis inhibition) or by tumor-specific expression of viral genes using cellular promoters (DNA viruses only) or target sites for micro-RNAs overexpressed in healthy tissues.[14,16]

It is now well established that the therapeutic activity of OVs, at least in part, depends on oncolysis-triggered, tumor-directed innate and adaptive immune responses. This oncolytic vaccination effect results from the release of tumor antigens, as well as pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), during OV-induced immunogenic cell lysis.[5–9,19] Importantly, it has been shown that local oncolysis, e.g., after intratumoral OV application, can trigger systemic antitumor immunity in animals and patients, thereby mediating the destruction of metastatic lesions not reached by the OV ("abscopal effect"). This oncolytic vaccination effect also suggests virotherapy as a promising modality for combination immunotherapies. As such, preclinical studies have shown that viral oncolysis can sensitize tumors to immune checkpoint inhibitors (ICIs), which are therapeutic antibodies that block inhibitory immune signaling.[20] In the clinic, ICIs have shown unprecedented durable responses in subsets of patients suffering from selected inflamed tumor types; however, most patients do not respond.[21] This is especially the case for tumors that are immunologically "cold", i.e., lacking signatures of immune activity, including infiltrating immune cells. Virotherapy has the potential to switch such tumors to "hot", i.e., stimulating, via an inflammatory response, the infiltration of immune cells, including tumor antigen-specific T cells, that can be unleashed by ICIs.[5–9,19] Preliminary results of ongoing clinical studies exploring the combination of OVs with ICIs are promising.[22,23] One interesting approach to viro-antibody therapy described in more detail below is the genetic delivery by OVs of antibodies with ICI activity.

With marketing approval of the first OV in the United States and European Union in 2015, the engineered HSV-1 T-Vec encoding granulocyte-macrophage colony-stimulating factor, virotherapy has reached routine clinical oncology.[3] A wide panel of other OVs with various genetic modifications are being investigated in numerous clinical Phase 1–3 studies.[4,8] While safety of OVs and therapeutic activity in individual patients has been demonstrated, it is clearly necessary to develop OVs with improved potency in order to realize the full potential of virotherapy in clinical oncology. To this end, established technologies to engineer virus genomes are a clear advantage of the OV drug platform, as they facilitate efforts to overcome barriers, e.g., by improving tumor cell infectivity, or to implement additional modes of action. A prominent example for the latter is the insertion of transgenes into OV genomes in order to express proteins or small regulatory RNAs that improve viral oncolysis or secrete biotherapeutics for complementing oncolysis with the destruction of tumor cells not reached by the OVs themselves.[8,13,24] Compared with replication-deficient vectors, such "armed" OVs facilitate dramatically increased expression of biotherapeutics restricted to tumors, where the OV genomes are amplified and OV infections spread.[25] Recombinant therapeutic

antibodies are powerful candidates for such "armed" OVs for at least four reasons: (1) they implement a paracrine mode of action to target noninfected cancer cells, (2) they possess an additional level of tumor selectivity, (3) structurally nearly identical molecules targeting basically any desired cancer surface marker are available or can be rapidly generated, and (4) they allow customization by adapting the antibody format to make it fit for purpose, for example, by modifying valencies or by fusing effector proteins, all of which have been pursued and reported in viro-antibody therapy, as discussed below.

Therapeutic antibodies

With more than 40 molecules approved for cancer therapy (www.antibodysociety.org/antibody-therapeutics-product-data), antibodies are established treatment options for more than 30 different tumor entities, including numerous hematologic malignancies and solid tumors.[1,26] Many more are in different stages of preclinical and clinical development.[27] Most of these antibodies recognize tumor-associated antigens on tumor cells. Prominent examples are IgG molecules targeting CD20 to treat B-cell malignancies and antibodies targeting epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) approved for the treatment of different types of solid tumors, e.g., colorectal cancer, gastric cancer, lung cancer, and breast cancer. These antibodies utilize the natural functions of IgG molecules to destroy tumor cells by blocking the activity of target structures, by inducing apoptosis, and/or via Fc-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement fixation.[28] These activities, however, are not always sufficient to efficiently induce a long-lasting tumor regression. Therefore, in a second generation of developments, the mode of action was extended by using the antibody as a targeting module to deliver cytotoxic compounds, such as radionuclides, chemotherapeutics and toxins, to tumor cells.[29]

A growing number of molecules are used as antibody drug-conjugates (ADCs) to delivery cytotoxic compounds to and into tumor cells.[30] More recently, a first recombinant immunotoxin composed of a disulfide-stabilized Fv fragment directed against CD22 fused to a *Pseudomonas* exotoxin A fragment was approved for the treatment of hairy cell leukemia.[31] Conceptually, this delivery approach offers a multitude of possibilities by combining the antigen-binding site of an antibody as a targeting unit with an effector moiety. For example, various so-called immunocytokines are being developed to locally induce and enhance an antitumor immune response by binding to tumor cells or targets expressed by cells of the tumor microenvironment.[32] Besides immunoregulatory fusion partners, other proteins such as death ligands (e.g., TRAIL, TNF) and enzymes (RNases) have been used to generate antibody fusion proteins.[33,34]

The application of antibodies in tumor therapy was also extended by targeting and inhibiting ligands and receptors involved in tumor angiogenesis.[35,36] For example, antibodies targeting vascular endothelial growth factor (VEGF) or VEGFR2 are approved for the treatment of different types of solid tumors.

A rapidly expanding area for use of therapeutic antibodies is in the field of immuno-oncology. Here, antibodies are developed to initiate or foster an antitumor immune response.[37] This can be achieved, for example, with bispecific antibodies capable of redirecting immune effector cells, especially T-cells, to tumor cells by binding with one arm to a surface antigen on tumor cells and with the second arm to a trigger molecule on the immune effector cell, e.g., CD3 on T-cells. Here, new tools to genetically engineer molecules with the desired composition have emerged in recent years.[38] The first genetically engineered bispecific antibody, blinatumomab, is a tandem scFv molecule (bispecific T cell engager, BiTE) directed against CD19 and CD3 for the treatment of acute lymphoblastic leukemia approved in 2014. Many more are in clinical development, most of them differing from this small BiTE format by comprising an Fc region (quite often with silenced effector functions) as a half-life extension module.

As a further immune-oncology approach, already introduced above, several antibodies are approved as ICIs to block inhibitory signals, for example, through binding of CTLA-4 or PD-1 on T-cells, or PD-L1 on tumor cells, to restore a potent antitumor T-cell response through recognition of MHC-displayed peptides on tumor cells.[20] Other antibodies targeting next-generation immune checkpoint molecules, such as LAG-3, TIGIT, TIM-3, NKG2A, and CD47 are in clinical development.[39,40]

In summary, the field of therapeutic antibodies has rapidly expanded during the past two decades into different directions with the aim to enhance and employ novel modes of action for the development of novel antibodies and antibody derivatives following a "fit for purpose" strategy. However, antibodies still face various hurdles to be efficacious and safe therapeutics.[41] One of the main limitations of antibodies, especially for the treatment of solid tumors, lies in their often rather poor and heterogeneous tumor penetration after systemic application,[42] which is a result of physical barriers and a high interstitial fluid pressure limiting extravasation and interstitial diffusion of large antibody molecules. This can be improved by using smaller antibody fragments, such as single-domain antibodies, scFv and Fabs,[43,44] but removal of the Fc region eliminates Fc-mediated effector functions and FcRn-mediated recycling response for the long half-life of IgGs.[45] Additionally, biochemical properties intrinsic to the antibody, such as surface charge, can influence the pharmacokinetic properties and their tissue distribution.[46,47] Furthermore, affinity for the target antigen has been shown to limit penetration of antibodies into the tumor tissue.[48,49] Thus, antibodies with high affinity accumulate preferably around the blood vessels, known as binding-site barrier effect.[50,51] A further limitation results from "on target, off tumor" activities, due to the lack of tumor-specific targets, which can lead to adverse effects as shown for bispecific T-cell engagers and CAR-T therapeutics, which can induce T-cell reactivities against normal tissues.[52,53] Several strategies have been developed to increase tumor selectivity. This includes the introduction of locks into the antigen-binding site, which can be conditionally removed through proteolytic cleavage within the tumor or as a response to hypoxia or low pH.[54] Alternatively, intratumoral injections of antibody drugs are used to increase local accumulation.[55] A promising approach toward addressing some of the mentioned limitations of antibody therapy of cancer is genetic antibody delivery, especially when targeted to solid tumors.

Genetic antibody delivery – lessons from gene therapy

Antibody gene transfer was initially pursued using replication-deficient viral vectors as passive immunization against infectious diseases or for delivery of therapeutic tumor-targeted antibodies,[56–65] and more recently (transient) delivery of antibodies by mRNA has also been pursued.[66] Gene therapy studies showed that the kinetics of gene expression were determined by the choice of vector. Specifically, Ad vectors mediate fast, strong, but, due to immune-mediated virus elimination, transient expression in animal models after intravenous (i.v.) injection. In contrast, adeno-associated virus (AAV) vectors enabled delayed, but persistent expression,[59,61,65] but clinical studies revealed that AAV vectors are immunogenic in humans.[66] Furthermore, gene expression could be strongly increased by choosing appropriate promoters, posttranscriptional stabilization and codon optimization.[67] As discussed below, control of antibody gene expression is also a critical parameter for viro-antibody therapy, for which gene expression strength and kinetics are primarily determined by virus replication. Importantly, antibody gene therapy addressed a problem also of relevance for viro-antibody therapy: the genetic delivery of full-length antibodies requires the co-expression of immunoglobulin (Ig) heavy and light chains, ideally at similar concentrations to ensure optimal efficiency and minimize adverse effects. Initial approaches explored the expression of both Ig chains as separate transcription units using two promoters and polyadenylation sequences (see, e.g., refs57, 58, 60). Such expression cassettes depend on extensive genomic space, which may compromise virus titers due to packaging limits, as observed for AAV vectors.[60] Alternative approaches link heavy- and light-chain genes in one bicistronic mRNA via an internal ribosome entry site (IRES)[56] or in a single open reading frame (ORF) using viral 2A peptides mediating a "ribosomal skip", i.e., a specific peptide bond is not formed during translation.[63] The former approach has the disadvantage that IRESs are also quite long sequences and IRES-mediated translation of the downstream Ig chain is less efficient than cap-dependent translation of the upstream chain.[68] The 2A approach ensures an expression ratio of close to 1:1 but is hampered by immunogenicity and/or structural constraints resulting from retained amino acids of the 2A sequence. To overcome this limitation, the insertion of a cleavage site for endogenous proteases has been explored.[63] Of note, the expression of recombinant single-chain or homodimeric antibody derivatives, including derived fusion proteins, circumvents problems related to co-expression.

Strongly attenuated derivatives of vaccinia viruses (VVs), which are also in development as OVs (see below), have been explored as gene transfer vectors for antibody delivery. One report explored the expression of a membrane-bound IgG (with two separate transcription units encoding heavy and light chains) or an scFv in macrophages or T cells, resulting in antibody-mediated binding and subsequent killing of cancer cells.[57,58] As an alternative approach, polyclonal stimulation of T cells by VV gene transfer vectors encoding membrane-bound, T-cell receptor (TCR)-specific IgGs was investigated, demonstrating strong expression in tumor cells mediating recruitment and activation of T cells and tumor rejection.[69]

The combination of antibody gene transfer and virotherapy has been explored by applying two separate Ad-derived viruses, namely a replication-deficient antibody-encoding Ad vector and an oncolytic Ad.[70–73] Thus, in co-infected cancer cells, the antibody-encoding Ad vector can replicate via complementation by the oncolytic Ad. This co-infection approach allows for flexibility in the combination of OVs with antibody-delivering vectors, e.g., for testing different combinations of OVs and (vector-encoded) antibodies. Also, oncolytic Ads were combined with Ad vectors encoding two different antibodies or antibodies in combination with cytokines in order to implement different antibody- and cytokine-mediated modes of action.[71,72] In contrast to the single agent viro-immunotherapy approaches discussed below, this combination approach, especially its clinical translation, is hampered by the necessity to produce and apply two separate virus products and the expected rarity of co-infections *in vivo*.

Therapeutic antibody-encoding OVs

Viro-antibody therapy has been pursued with increasing intensity in the past decade, with studies investigating different OVs, antibody formats and antibody-mediated modes of action (Figure 1). The following sections highlight the principles and key achievements of viro-antibody therapy research according to the major parameters, i.e., the explored viruses, antibody expression strategies, antibody formats, therapeutic targets and effector mechanisms. The accompanying Tables 1 – 5 provide a comprehensive overview of the developed therapeutic antibody-encoding OVs with key results.

When exploring viro-antibody therapy in immunocompetent syngeneic models, it is important to consider that these models face limitations with respect to OV transduction, replication and oncolysis. This results from the overall species-specificity of virus replication, meaning that human-infecting OVs are usually strongly attenuated in murine cells, including murine cancer cells. Thus, studies of viro-antibody therapy in syngeneic mouse models underestimate the oncolysis effect. In this light, xenograft models with additionally applied human immune cells are being explored (see viro-BiTE therapy, Table 2). Of note, patient-derived *ex vivo* models, for example, pleural effusion and peritoneal ascites, containing cancer cells, stroma cells and immune cells, are increasingly valued for analysis of OVs including immune effects, as reported for studies exploring oncolytic Ad-encoded BiTEs.[74–76]

We note that we do not discuss the insertion of recombinant antibody genes into OV genomes pursued to target virus cell entry to tumor cells, either by genetically fusing tumor-specific scFvs to viral glycoproteins[77–79] or by virus-encoded, antibody-based bispecific adapter proteins.[80,81] Also not covered are fusion proteins containing IgG Fc domains, but not antigen-binding antibody domains. Such approaches aim at Fc-mediated protein multimerization and stabilization,

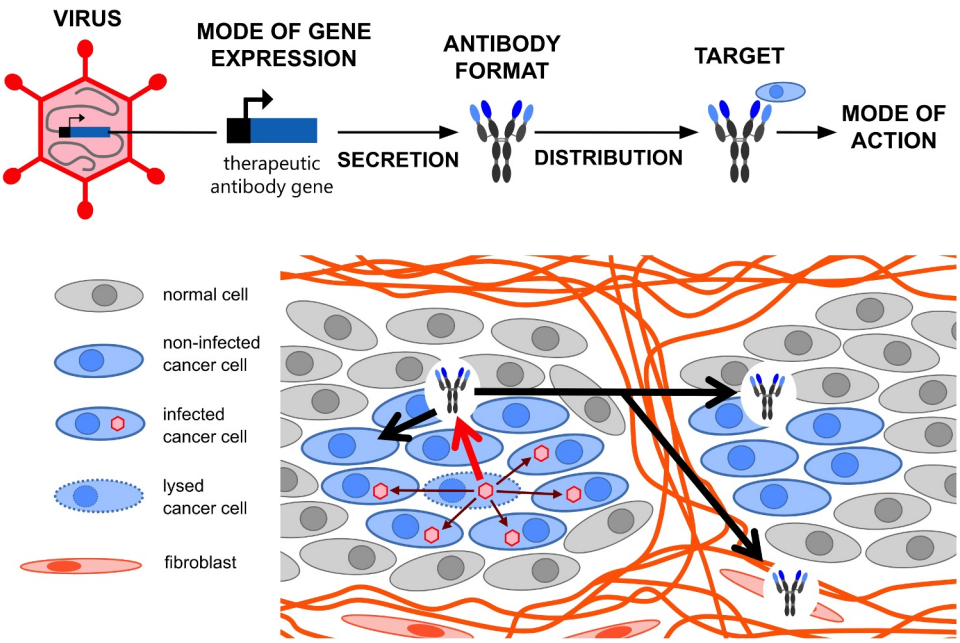

Figure 1. Viro-antibody therapy – an overview. Transgenes encoding recombinant therapeutic antibodies are inserted into the genome of oncolytic viruses (OVs) by exploitation of diverse strategies for transgene expression (see Figure 2). OV-encoded antibodies are produced locally in the tumor by infected cancer cells and expression lasts as long as active OV infection and spread is ongoing. The produced antibodies, dependent on their format, valency and size (see Figure 3), perfuse the tumor, bind their target on (noninfected) cancer cells or cancer-associated cells, and trigger their direct or indirect killing via diverse modes of action (see Figure 4).

modulation of protein size and thus biodistribution, and/or Fc-mediated effector functions.[82] Applications include the expression of decoy receptors consisting of extracellular receptor domains fused to Fcs, e.g., TGF-β inhibitors[83–86] and recombinant checkpoint inhibitors, such as the Fc-fused extracellular domain PD-1.[87]

Therapeutic antibody-encoding OVs: viruses and expression strategies

Of the established repertoire of different viruses used for virotherapy, most allow the insertion of therapeutic antibody genes, as they fulfill the requirements of available genomic space, knowledge of viral genome organization and replication, and established technology for genome engineering (including reverse genetics for RNA viruses). Thus, it is not surprising that a panel of OVs have been explored for application in viro-antibody therapy: Ads, VV, HSV, MeV, VSV, NDV, and influenza virus (Figure 2). The properties of the chosen virus (or of the specific strain or mutant), i.e., infection efficiency, mode of tumor-targeting, the kinetics of genome amplification and of viral spread, determine the kinetics, strength and tumor-selectivity of antibody expression. However, direct comparisons of different viruses, as for Ad and AAV vectors in gene therapy, remain to be performed for viro-antibody therapy. The choice of OV moreover determines both the feasible gene expression strategy and the genomic space available for transgene insertion, the latter being restricted by the packaging capacity of the virus particle (Figure 2). Powerful viral gene expression mechanisms can be exploited for efficient and/or replication-dependent, thus tumor-specific antibody expression, as discussed below.

Most viro-antibody therapy studies explored OVs with DNA genomes: Ads, VVs and HSVs (Figure 2). They allow for the insertion of large heterologous DNA sequences and the utilization of diverse transgene expression strategies. To this end, the most straightforward strategy is to insert additional transcription units with promoter/enhancer, antibody ORF and polyadenylation sequences. For strong but constitutive expression, heterologous viral promoters of cytomegalovirus (CMV), respiratory syncytial virus (RSV) or Moloney murine leukemia virus have been used in OVs derived from Ad or HSV.[74–76,88–93] In VV-derived OVs, natural or synthetic VV promoters were used, including late, i.e., replication-dependent, promoters.[94–105] The expression of two scFvs by a single OV has been reported for a VV using two viral promoters and insertion sites.[102] For Ads, besides separate transcription units with CMV promoter, for which the orientation might be critical,[74–76] the insertion of antibody genes into endogenous viral transcription units have been established. To this end, antibodies are expressed either from ORFs replacing viral ORFs[106–108] or by alternative splicing using splice acceptor sites upstream of the antibody ORF.[74,75,109–114] These approaches need less genomic space. Moreover, insertion of transgenes into late transcription units enables replication-dependent gene expression,[25] which is tumor-restricted dependent on the level of tumor-selectivity of the OV.[115]

Replication-dependent antibody expression from late viral promoters, directly, as established for oncolytic VV, or via alternative splicing, as established for oncolytic Ads, might affect therapeutic activity and/or side effects of viro-antibody therapy (or of therapeutic gene-expressing OVs in general). Constitutive promoters can mediate a faster onset

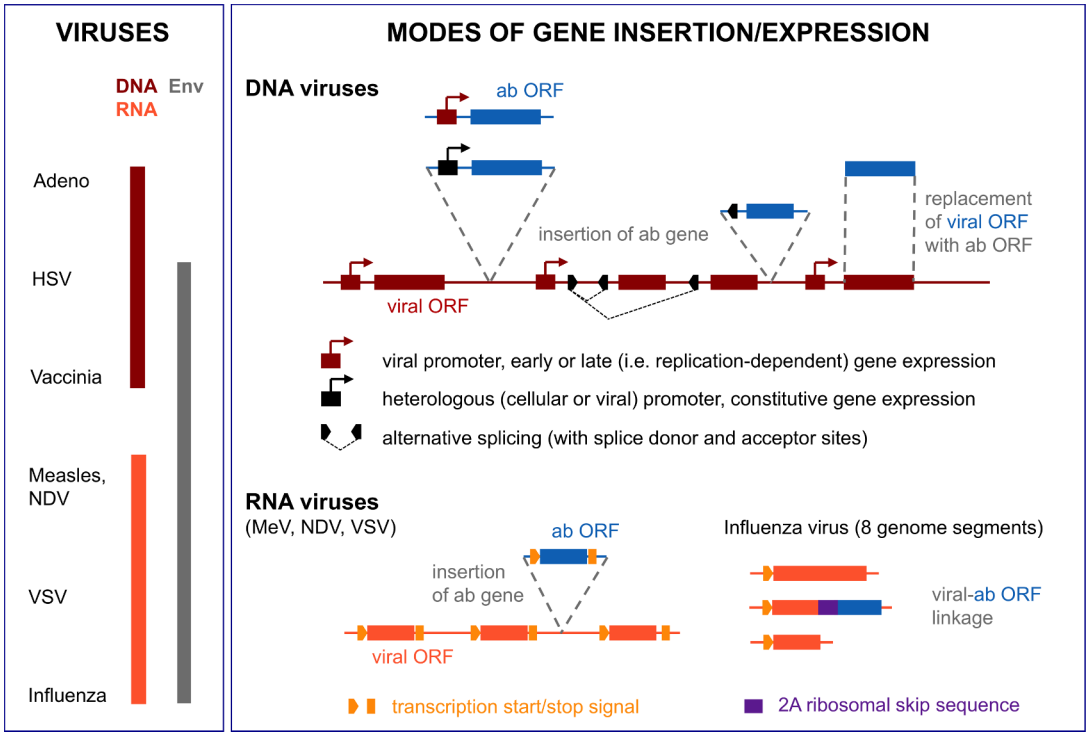

Figure 2. Viruses and modes of antibody expression exploited for viro-antibody therapy. A panel of viruses with or without envelope and with DNA (double-strand) or RNA genomes (negative strand, continuous for MeV, NDV, VSV or segmented for influenza virus) have been exploited for viro-antibody therapy (left panel). Dependent on the virus chosen, different strategies have been pursued for the insertion of antibody genes into the viral genomes toward efficient and/or replication-dependent gene expression and minimizing the required genomic space (right panel). ab, antibody; Env, envelope; HSV, herpes simplex virus; MeV, measles virus; NDV, Newcastle disease virus; ORF, open reading frame; VSV, vesicular stomatitis virus.

of effector functions, as results for CMV promoter-driven versus replication-coupled expression by oncolytic Ads indicate: the former approach resulted in a more rapid T cell activation and target cell killing by virus-encoded BiTEs in co-cultures.[74] On the other hand, replication-dependent antibody expression might be an advantage when expression in normal cells needs to be avoided, i.e., especially after systemic OV application. This is because constitutive promoters mediate transgene expression also in healthy cells transduced by OVs (if they are not entry-targeted), even if they do not replicate. For example, the above study with oncolytic Ads reported GFP expression in macrophages of malignant exudates with CMV promoter-driven GFP expression, but not with replication-coupled GFP expression. A study investigating the same oncolytic Ad format for CMV-driven or replication-dependent expression of a fibroblast-targeted BiTE, directed against the fibroblast-activating protein (FAP) and CD3, observed toxicity for normal fibroblasts in co-cultures with T cells (no tumor cells) only for the CMV promoter virus.[75] In this setting, the oncolytic Ad can enter normal fibroblasts, but cannot replicate and lyse these cells. Thus, fibroblast killing results from BiTE expression, which is active only for the CMV virus, and BiTE-mediated T cell cytotoxicity. This result is in so far relevant, as systemically applied conventional FAP-targeted therapeutics have been reported to induce toxicity in FAP+ cells of the bone marrow.[116] In conclusion, the opportunity to implement replication-dependent versus immediate antibody expression for some OVs has functional implications, but more studies are needed to exploit and optimize this mechanism for improving the therapeutic window of viro-antibody therapy.

Late expression during the OV replication cycle might also be necessary for therapeutic antibodies that otherwise interfere with viral replication, thereby impairing virus manufacturing as well as oncolysis *in situ*. As such, high-quality virus preparations of oncolytic Ads encoding an immunoRNase for targeted cancer cell killing required tight replication-dependent expression, as strong expression, both by replication-deficient vectors or OVs, produced viruses of low infectivity.[109] Still, the immunoRNase was expressed by the optimized Ad at levels sufficient to mediate enhanced therapeutic efficacy *in vitro* and *in vivo*.

Antibody expression by OVs is not restricted to DNA viruses but has been demonstrated also for RNA viruses MeV, VSV, NDVs, and influenza virus (Figure 2). For viruses with non-segmented genomes (MeV, VSV, NDV), the expression strategy is to insert additional transcription cassettes including viral gene start and stop elements into the RNA genome. Gene expression strength is determined by the insertion site/position within the virus genome considering expression gradients from the 3′ to the 5′ genome terminus. Influenza viruses possess a segmented genome of eight segments. ORFs encoding heavy and light chains of an IgG were fused via a “ribosomal skip” 2A sequence to viral ORFs of different segments.[117]

Considering that antibodies are naturally produced by specific and highly differentiated plasma cells, it is worth mentioning that reported viro-antibody therapy studies clearly show that functional therapeutic antibodies can

also be produced in cancer cells of diverse tissue origin *in vitro* and in tumors of animal models *in vivo*. One study suggested that antibody production by tumor cells requires active cell lysis, as antibodies were not released from cancer cells after transduction with a replication-deficient, and thus not lytic Ad vector.[108] However, another study detected antibodies in supernatants after transduction of a different tumor cell line with a replication-deficient Ad[109]; thus, viral cell lysis might not be a requirement for antibody release from cancer cells. It remains to be investigated in more detail how cell lysis affects biosynthesis and release of antibody from infected tumor cells and whether modulation of cell lysis kinetics by OV engineering, as reported for transgene-encoding Ads,[118] allows for further increasing antibody production.

When inserting transgenes into OV genomes, adverse effects to virus replication kinetics or integrity must be considered. These might result from the disruption of viral gene regulation circuits, from surpassing the genome packaging limit of virus particles, or from the activity of the encoded therapeutic protein. Note that most of the reported viro-antibody studies demonstrate the expression and function of recombinant antibodies *in vitro* and show that OV replication and tumor cell lysis are not affected by insertion of antibody genes and their expression. Only exemptions to the latter aspect will be discussed in the following sections or are mentioned in Tables 1–5. Interestingly, a study exploring an oncolytic Ad encoding a FAP-specific BiTE was actually reported to increase infectious virus particle production *in vivo*.[104] This is likely a consequence of the BiTE-mediated killing of cancer-associated fibroblasts and resulting destruction of the extracellular matrix, thereby eliminating a barrier to intratumoral spread of viral infection. This hypothesis is supported by reports of enhanced intratumoral infection of oncolytic Ads encoding matrix-degrading enzymes, such as relaxin or hyaluronidase.[119,120]

Therapeutic antibody-encoding OVs: antibody formats, targets and therapeutic strategies

Various viro-antibody therapy approaches for expression of therapeutic antibodies and derivatives have been reported. These have explored OVs encoding full-length IgGs, Fabs, scFvs, bispecific tandem scFvs (BiTEs), nanobodies, or scFv fusion proteins (Figure 3) that directly target tumor cells (tumor surface antigens), tumor supporting fibroblasts (α-FAP), tumor angiogenesis (α-VEGF), or immune cells (ICIs, BiTEs) (Figure 4). In the following, we highlight key properties and results of reported viro-antibody therapy studies beginning with OVs encoding full-length IgGs/mAbs (see Table 1 for an overview of all reported studies) and continuing with different virus formats according to effector strategies, i.e., T cell engagement, immune checkpoint inhibition, anti-angiogenesis and direct tumor cell killing.

Expression of recombinant monoclonal antibodies (viro-MAb therapy)

As highlighted above, a panel of mAbs has been approved and is widely used in clinical oncology in order to directly target and kill cancer cells, de-block immune cells for

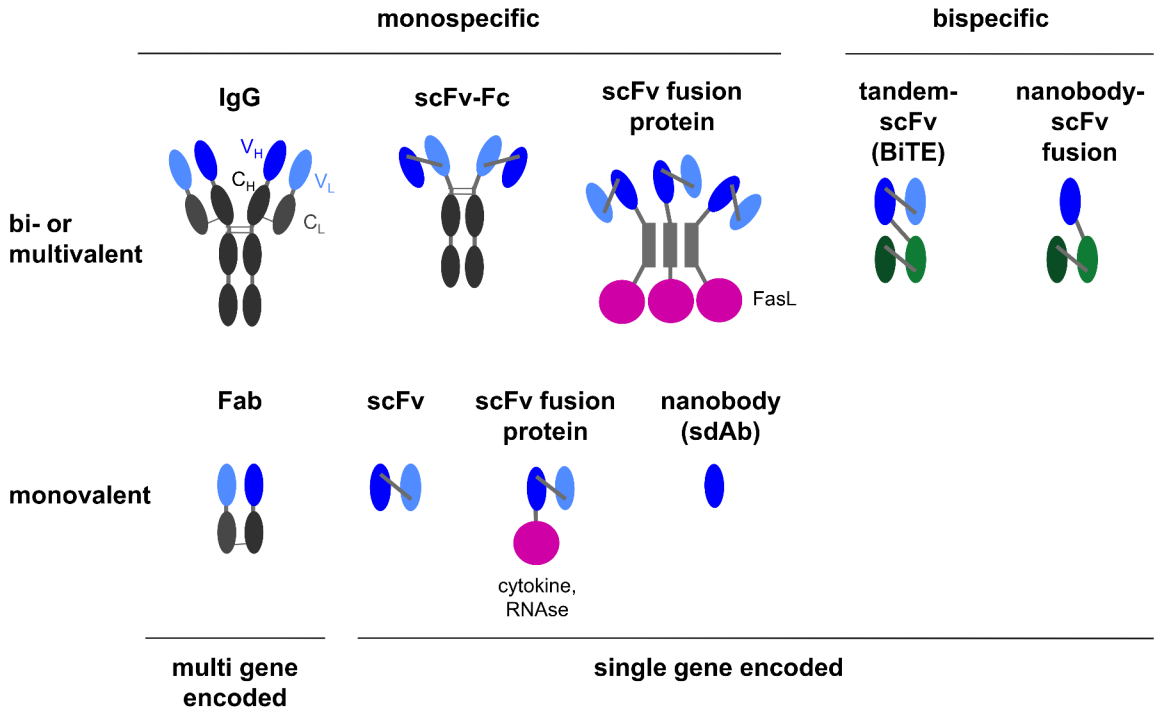

Figure 3. Antibody formats utilized in viro-antibody therapy. BiTE, bispecific T cell engager; C_H/C_L, constant domain of heavy/light chain; Fab, antigen-binding fragment; Fc, constant fragment of antibody; IgG, immunoglobulin G; RNase, ribonuclease; scFv, single chain variable fragment; sdAb, single domain antibody (variable fragment of camelid antibody heavy chain); V_H/V_L, variable domain of heavy/light chain. For the scFv fusion proteins, proteins might be fused at the C-terminus of the scFv (cytokine, trimerization peptide and FasL) or at the N-terminus (RNase); scFvs might be in V_H-V_L or V_L-V_H configuration.

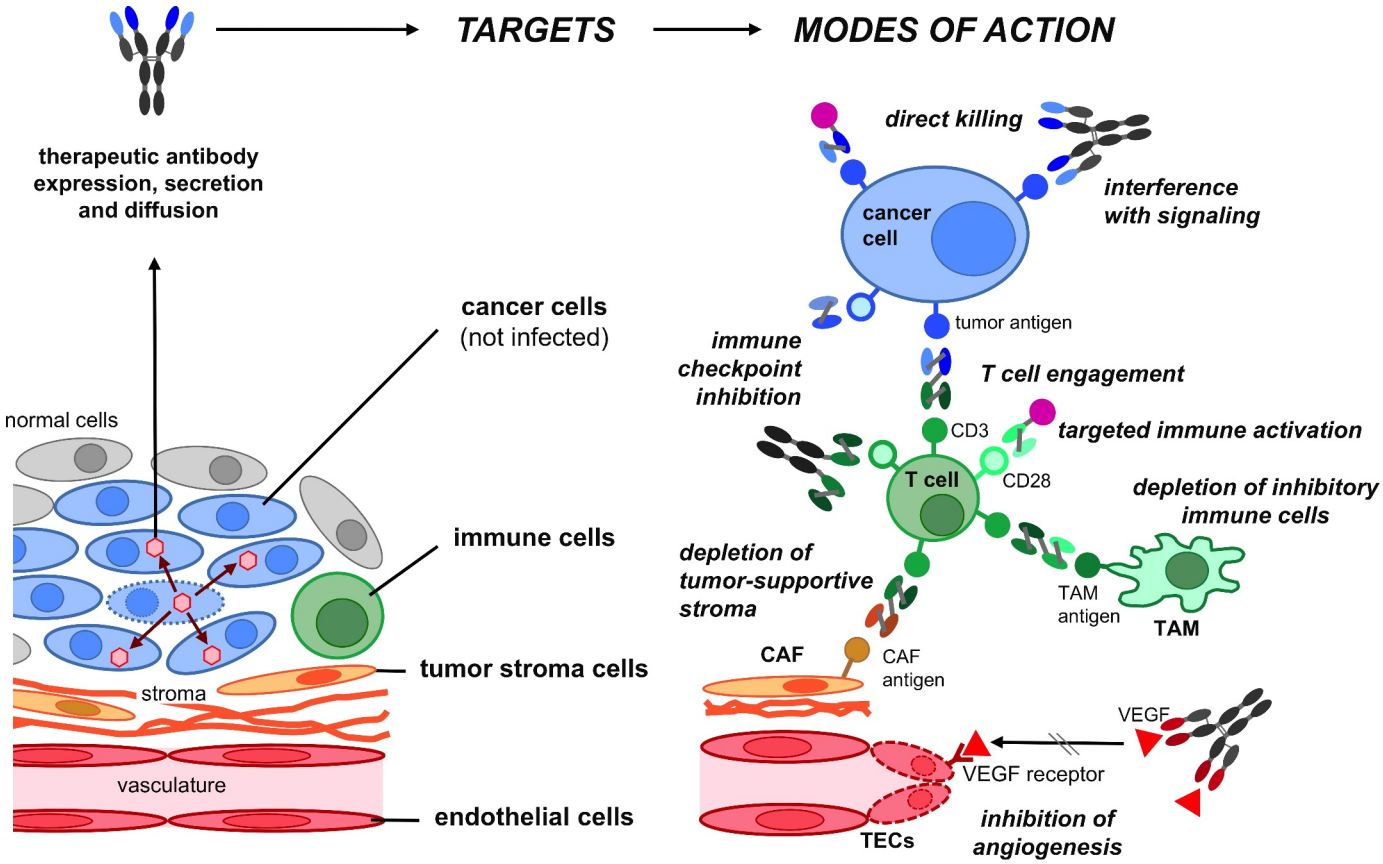

Figure 4. Target cells and modes of action of OV-encoded antibodies. The depicted cancer targets and direct or immune- or stroma-mediated modes of action have been reported. For some of the depicted modes of action only examples of the explored antibody formats are depicted (see Tables 1 – 5 for a comprehensive list). CAF, cancer-associated fibroblast; TAM, tumor-associated macrophage; TECs, tumor endothelial cells.

antitumor immunity (checkpoint inhibition), or deprive growth factors, such as VEGF for inhibiting tumor angiogenesis. Several reported viro-antibody studies aimed at establishing the genetic delivery of full-length IgGs by OVs exploring these different modes of action (***viro-MAb therapy***). These studies included a model antibody to demonstrate proof-of-principle,[117] an experimental therapeutic antibody targeting the tumor vascular antigen fibronectin extradomain B,[121] IgGs with specificity for tumor targets (CD147),[122] or targets of approved mAbs (CTLA-4, PD-1, VEGF),[92,103,106,111] and the approved antibody trastuzumab.[108] Overall, the reports established that the genetic delivery of full-length IgGs is feasible for both RNA viruses, as reported for NDV[121,122] and influenza A,[117] and for DNA viruses, reported for Ad,[106–108,111] VV,[103,105] and HSV.[92] Strategies for the required co-expression of IgG heavy and light chains were the insertion of two separate transcription units for NDV and VV, fusion to two viral ORFs of different gene segments using viral 2A sequences for influenza A virus or co-expression via an IRES or a 2A sequence for Ads. Co-expression in VV via separate transcription units was improved with optimized promoter choice, which avoided misassembled by-products that were considered to be homodimers of (over-) expressed Ig light chains.[103]

Viro-MAb therapy studies demonstrated that genetic delivery by OVs enables selective, strong and prolonged expression of active IgGs in tumors. Tumor-selective antibody expression was shown for oncolytic NDV and Ad after *in vitro* infection of tumor versus normal cells, with a more than 2000-fold window of tumor-specificity observed for the oncolytic Ad.[111,121] In particular, strong antibody expression mediated by the replicative nature of OVs was confirmed in a xenograft model allowing for efficient Ad replication that showed an 81-fold higher antibody expression after i.t. injection of an oncolytic Ad compared with a matching replication-deficient Ad vector.[106] IgG concentrations were 43-fold higher in the tumor than in plasma. A follow-up study with the same oncolytic Ad and expression strategy to deliver a recombinant derivative of trastuzumab allowed a comparison of a recombinant OV-encoded IgG, purified using a standard laboratory procedure, with the matching commercial mAb,[108] revealing that the activity of the oncolytic Ad-encoded antibody was similar regarding direct growth inhibition or ~30% regarding ADCC. Equivalent or even superior activity of OV-encoded compared with a matching hybridoma-produced or commercial mAb were also reported in studies using VV or influenza virus.[103,117] The oncolytic Ad-encoded trastuzumab, after i.t. injection in a mouse xenograft model, produced higher antibody concentrations in tumors and dramatically higher tumor-to-blood antibody concentration ratios compared with

Table 1. Overview of developed therapeutic antibody-encoding OVs: OVs expressing recombinant IgGs/monoclonal antibodies (Viro-MAb therapy) sorted according to targets and publication date.

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
NDV (wt mesogenic strain)	IgG heavy and light chains as separate, adjacent additional transcription cassettes with gene stop and gene start signal for viral transcription	IgG	Fibronectin extradomain B (tumor-specific vascular marker)	• OV-encoded antibody is produced and binds to antigen after infection of tumor cells *in vitro* and after intratumoral injection in a xenograft mouse model	[121]
Ad (Ad5/3-E1AΔ24)	Replacement of early genes (E3) by Ig chains linked via IRES	IgG2	Human CTLA-4	• Subcutaneous xenograft mouse tumor model/intratumoral virus injection: ○ OV-encoded antibody detected in xenografts; 43-fold higher antibody concentration in tumor versus plasma ○ 81-fold higher antibody concentration detected in tumors after injection of antibody-encoding OV compared with antibody-encoding replication-deficient control virus • OV-encoded antibody activates T cells from cancer patients, which are more susceptible than T cells from healthy donors	[106]
Ad (E2F promoter driving viral E1A)	Replacement of early genes (E3) by Ig chains linked via 2A	IgG2	Murine CTLA-4	• Antibody-encoding OV results in tumor-specific antibody expression in a panel of cell cultures *in vitro* • Subcutaneous syngeneic mouse tumor model/intratumoral OV injection: tumor growth inhibition by antibody-encoding OV	[107]
Influenza A virus (IAV)	Heavy chain in PB1 segment downstream of PB1 gene via 2A; light chain in PA segment downstream of PA gene via 2A; scFv cloned into both segments	IgG and scFv	Proof-of-concept (IgG) and murine CTLA-4 (scFv)	• Antibody insertion reduces titer, replication and *in vivo* morbidity and mortality of IAV • Functions of OV-produced IgG similar to hybridoma-produced ab • Subcutaneous syngeneic bilateral mouse tumor model/intratumoral OV application: scFv-encoding OV shows superior tumor growth inhibition (both flanks) and prolonged survival compared with parental virus	[117]
NDV (wt velogenic Italien strain)	IgG heavy and light chains as separate, adjacent additional transcription cassettes with gene stop and gene start signal for viral transcription	IgG	CD147 (metuximab)	• Orthotopic xenograft mouse tumor model/intravenous OV application: antibody-encoding OV results in ○ Antibody expression in tumors and tumor necrosis, ○ Reduced intrahepatic metastasis and prolonged survival compared with parental virus	[122]
Vaccinia virus (Western Reserve strain, TK^- and RR^-)	Separate transcription unit with different viral promoters at TK locus	IgG, Fab, scFv	murine PD-1	• Optimal promoter choice is required to avoid imbalance in expression of light versus heavy chain (IgG, Fab) • OV-encoded IgG, Fab, and scFv are expressed and functional • Subcutaneous syngeneic mouse tumor model/intratumoral virus application: ○ OV-encoded IgG peaks at d 5 in tumor and serum mirroring virus replication ○ IgG concentration and tumor/serum ratio higher for antibody-encoding OV than after i.t. injection of 10 µg mAb ○ Therapeutic activity of IgG- and scFv-encoding OV in one of two tumor models superior to parental virus and similar to parental virus plus repeated large dose systemic antibody therapy	[103]
HSV-2 (ICP34.5$^-$ and ICP47$^-$)	Expression of heavy and light chains as separate transcription units from CMV and RSV promoters, respectively	IgG	Human PD-1	• Syngeneic mouse tumor model with humanized PD-1 mouse and tumor cells with recombinant HSV receptor/intratumoral OV injection: ○ Antibody-encoding OV compared with parental OV: superior tumor growth inhibition, numbers and activation of T cells and induction of tumor-specific T cells in spleen ○ Tumor growth inhibition by antibody-encoding OV superior to systemic antibody application and similar to combined treatment with parental virus and systemic antibody application • Induction of memory response (protection from tumor cell rechallenge) by antibody-encoding OV	[92]

(*Continued*)

Table 1. (Continued).

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Ad (Ad5/3-E1AΔ24)	Replacement of early genes (E3) by Ig chains linked via IRES	IgG1	Human HER2 (Trastuzumab)	• OV-encoded antibody shows direct antitumor activity and triggers ADCC *in vitro* • Subcutaneous xenograft mouse tumor model/intratumoral virus injection: ○ Enhanced antitumor efficacy of antibody-encoding OV compared with parental virus or trastuzumab for Her2-positive xenografts ○ Higher tumor-to-blood antibody concentrations (for endpoint tumors) by antibody-encoding OV compared with conventional antibody application • NK cell–dependent DC activation in draining lymph nodes by antibody-encoding OV	[108]
Ad (EnAd, chimeric type B Ad)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter), Ig chains linked via IRES	IgG1	Human VEGF	• tumor cell-specific replication and antibody production *in vitro* with >2000-fold window of selectivity • orthotopic xenograft mouse tumor model/intravenous OV application: strongly reduced tumor burden by antibody-encoding OV, superior to parental virus, but no significance (antibody is specific for human VEGF)	[111]
Vaccinia virus (Western Reserve strain)	Expression of heavy and light chains fused via 2A peptide and fused to luciferase from separate transcription unit with viral early/late promoter	IgG	Murine TIGIT	• Syngeneic subcutaneous or intraperitoneal mouse tumor models/intratumoral or intraperitoneal OV injection: ○ Antibody-encoding OV shows stronger tumor growth inhibition and increased survival compared with parental virus ○ $CD8^+$-T cell infiltration induced by OV application, but not further increased by antibody expression; reduced fraction of $TIGIT^+$ T cells and increased IFN-γ by antibody expression ○ therapeutic effect of antibody-encoding OV abrogated by CD8 + T cell depletion, but not by NK cell depletion ○ Mice cured by antibody-encoding OV reject tumor rechallenge	[105]

[a]Expression of functional antibody *in vitro* shown; virus replicative and/or oncolytic features usually not or only slightly attenuated as analyzed *in vitro*.

intraperitoneally (i.p.) injected commercial mAb.[108] Durability of antibody expression by viro-MAb therapy in an immunocompetent organism was reported using an IgG-encoding oncolytic VV in a syngeneic mouse model showing, after i.t. virus injection, IgG expression for 11 and 5 d in tumors or serum, respectively, with a peak at day 5. These kinetics mirrored virus replication.[103] IgG concentrations in tumors were higher and longer lasting compared with mice that were injected i.t. with 10 µg of a matching commercial mAb, whereas serum antibody concentrations were 10-fold lower at day 1, but higher at later times.

Antibody-mediated therapeutic activity via different modes of action has been demonstrated for IgG-encoding OVs. The expression of trastuzumab by the oncolytic Ad resulted in enhanced tumor growth inhibition in a xenograft model when compared with monotherapies.[108] The activation of patient-derived T cells by cell culture supernatants of infected tumor cells *in vitro* has been reported for the oncolytic Ad encoding checkpoint inhibitor anti-CTLA-4.[106] Furthermore, OVs encoding an anti-murine PD-1, CTLA-4 or TIGIT antibody enabled tumor growth inhibition and survival superior to parental virus and for the PD-1 virus similar to combined parental virus with repeated high dose systemic mAb.[103,105,117] Similar results were obtained for an oncolytic HSV-2 encoding an anti-human PD-1 antibody in a mouse model with humanized PD-1.[92]

Expression of BiTEs (viro-BiTE therapy)

BiTEs are another type of genetically engineered antibodies besides mAbs that has been approved for cancer treatment. They are defined not only by the antibody format, two scFvs linked via a flexible peptide linker (tandem scFv, $scFv_2$) (Figure 3), but also by their specificities and mode of action: they bind T cells via CD3 and tumor targets via specific cell surface molecules. Thus, BiTEs activate T cells in the presence of target cells, irrespective of the TCR-specificity of T cells and irrespective of MHC expression by target cells. The latter makes BiTEs an especially interesting therapeutic approach for immunotherapy of cancers that lack MHC expression. Blinatumomab, a BiTE with specificity for the B cell marker CD19, is approved for treatment of relapsed or refractory B-ALL. However, BiTEs face the problems of (1) short half-lives in serum due to their small size, thus requiring continuous infusion; (2) immune-hostile TMEs in solid tumors lacking T cells required for retargeting by BiTEs or containing exhausted T cells; and/or (3) severe off-tumor toxicities. Viro-antibody therapy is a promising strategy to overcome some of the drawbacks of conventional BiTE therapy by intratumoral and extended BiTE expression (***viro-BiTE therapy***).[123,124] This mode of delivery facilitates enhanced accumulation and penetration of BiTEs in the tumor and minimizes systemic BiTE distribution to reduce off-tumor activities and adverse effects. Moreover, OVs induce inflammation, thereby triggering infiltration of T cells including potentially powerful antiviral T cells that can be redirected to tumor targets by the BiTEs. In light of these advantages, several viro-BiTE therapy studies have been reported investigating BiTE-encoding oncolytic VV, Ad, MeV, and HSV-1 (Table 2).

Efficacy of viro-BiTE therapy has been demonstrated for four different kinds of tumor targets: 1) direct targeting of cancer cells has been explored with OVs carrying a transgene encoding a BiTE directed against EphA2,[98] EGFR,[110,112,114] EpCAM,[74] CEA,[125] or CD20;[125] 2) targeting of cancer-associated fibroblasts with a FAP-specific BiTE;[75,104,113] 3) targeting of tumor-supportive M2 macrophages with a BiTE specific for the M2 marker folate receptor (FR)-β;[76] and recently 4) simultaneous targeting of cancer cells and immunosuppressive cells via a BiTE directed to PD-L1, that can be regarded as a pan-cancer marker.[93] The latter study explored a bispecific nanobody-scFv format as T cell engager, with the nanobody specific for PD-L1, in addition to the standard tandem scFv BiTE (see Figure 3), demonstrating comparable activity in co-culture models (see below).

The activation of T cells and resulting killing of target cells has been reported for OVs encoding BiTEs with specificity for the targets EphA2,[98] EGFR,[110,112] EpCAM,[74] or CEA[125] in co-cultures of tumor cells or recombinant cells expressing the corresponding BiTE-target with peripheral blood mononuclear cells (PBMCs) or PBMC-derived T cells, for OVs encoding BiTEs with specificity for FAP in co-cultures that in addition contain fibroblasts,[75,113] and for OVs encoding BiTEs with specificity for PD-L1 in co-cultures containing M2-like macrophages instead of or in addition to cancer cells.[93] Investigations of viro-BiTE therapy with oncolytic Ads in clinically relevant co-cultures derived from pleural effusion- and/or peritoneal ascites demonstrated T cell activation and depletion of either cancer cells using an EpCAM-BiTE, fibroblasts using an FAP-BiTE, M2 macrophages using an FR-β-BiTE, or cancer cells and M2 macrophages using an PD-L1-BiTE or PD-L1 nanobody T cell engager.[74–76,93] Here, the viro-BiTE treatments showed results similar to recombinant BiTE. Of note, these studies demonstrate that the viro-BiTE approach facilitates activation of patient-derived autologous T cells and that it is effective even in presence of the immunosuppressive tumor exudates. Notably, for the approach using the PD-L1-specific T cell engagers, superior T cell activation was reported in the presence of immunosuppressive ascites, likely resulting from increased expression of the BiTE target PD-L1. Thus, these BiTE- or nanobody T cell engager-encoding OVs converted an immunosuppressive tumor microenvironment into enhanced immunotherapeutic activity.[93] Furthermore, for the FAP- and FR-β-targeted viro-BiTE approach, TME repolarization toward a pro-inflammatory state was reported via analysis of T cell transcriptomes and/or repolarization of the remaining macrophages to the M1 phenotype, respectively.[75,76] The FAP study has prompted a clinical trial with oncolytic Ad, EnAd, encoding a FAP-specific BiTE and further immunostimulatory proteins (PsiOxus, virus NG-641, NCT 04053283).

In vivo therapeutic activity of viro-BiTE therapy was shown in tumor xenograft models with PBMCs or PBMC-derived T cells for oncolytic BiTE-encoding VV (injected i.v.), Ad (injected i.t. or i.p. in carrier cells), and MeV (injected i.t.).[98,110,113,114,125] These studies also revealed infiltration and activation of T cells and, for a BiTE specific for human and mouse FAP, reduced mouse FAP expression likely resulting from depletion of tumor-associated fibroblasts that are of mouse origin. In an effort to address tumor heterogeneity, one study investigated the combination of

Table 2. Overview of developed therapeutic antibody-encoding OVs: OVs expressing BiTEs (viro-BiTE therapy), sorted according to target and publication date.

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Vaccinia virus (vvDD, Western Reserve strain, TK^- and VGF^-)	Separate transcription unit, late viral promoter	BiTE	Human EphA2 × human CD3	• Co-cultures of infected tumor and unstimulated T cells or PBMCs: BiTE-encoding OV, not control virus, induces T cell activation, which depends on presence of EphA2-positive cells, and T cell-dependent bystander tumor cell killing • Subcutaneous xenograft mouse tumor model with mixed tumor cells and PBMCs/intraperitoneal virus application (immediately after cell injection): improved tumor growth inhibition (i.e., prevention of tumor growth), survival and PBMC activation compared with control virus • Lung metastasis xenograft mouse tumor model/intravenous OV and/or PBMC injection: BiTE-encoding OV shows significantly delayed tumor growth compared with controls	[98]
Ad (ICOVIR-15K: E1AΔ24, E2F binding site in E1A promoter, RGDK in fiber shaft)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter)	BiTE	Human EGFR (cetuximab-derived scFv) × human CD3	• Tumor cell-PBMC co-cultures: infection with BiTE-encoding OV but not parental virus, triggers T cell activation, proliferation and bystander target cell killing • Subcutaneous xenograft tumor mouse model/intravenous human PBMC or pre-activated T cells and intratumoral OV injection: ○ Increased accumulation and persistence of T cells observed for BiTE-encoding OV compared with parental virus ○ PBMC-dependent improvement of therapeutic outcome for BiTE-encoding OV compared with parental virus ○ injection of PBMC does not affect persistence of the virus	[110]
Ad (ICOVIR-15K: E1AΔ24, E2F binding site in E1A promoter, RGDK in fiber shaft)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter)	BiTE	Human EGFR (cetuximab-derived scFv) × human CD3	Combination therapy with folate receptor (FR)-targeted CAR-T cells • Co-cultures of infected tumor cells and CAR-T cells *in vitro*: ○ Efficacy and specificity of target cell killing by EGFR-BiTE-encoding OV + FR-targeted CAR-T superior to CAR-T alone or combination of EGFR-targeted CAR-T and FR-targeted CAR-T ○ BiTE-encoding OV triggers activation of CAR-T cells in absence of FR and activation of CAR^- T cell fraction in CAR-T cell preparations • Subcutaneous xenograft mouse tumor model/intratumoral OV and intravenous T cell injection: ○ BiTE-encoding OV + CAR-T combination therapy significantly decreased tumor growth and increased survival compared with monotherapies or combinations containing control T cells or OVs ○ Increase in CAR-T infiltration and activation for xenografts weakly expressing CAR-T target	[112]
Ad (ICOVIR-15K: E1AΔ24, E2F binding site in E1A promoter, RGDK in fiber shaft)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter)	BiTE	Human EGFR (cetuximab-derived scFv) × human CD3	• Subcutaneous xenograft mouse tumor model/intravenous injection of allogeneic PBMCs and intraperitoneal injection of OV or menstrual blood-derived mesenchymal stem cells (MenSC) infected with OV: ○ MenSC+BiTE-encoding OV treatment results in significant tumor growth inhibition compared with MenSC+parental virus or with BiTE-encoding OV ○ Reduced virus genome copies and viral protein immunostaining at end of experiment for MenSC+BiTE-encoding OV compared with MenSC+parental virus ○ Superior BiTE mRNA expression for MenSC+BiTE-encoding OV compared with BiTE-encoding OV	[114]

(*Continued*)

Table 2. (Continued).

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Ad (EnAd, chimeric type B Ad)	Inserted as separate transcription unit with CMV promoter (constitutive) or inserted in late viral transcription unit with splice acceptor sequence (expression from viral major late promoter, replication-dependent)	BiTE	Human EpCAM × human CD3	• Co-cultures of infected tumor cells and PBMC-derived T cells: BiTE-encoding OVs trigger superior activation of PBMC-derived T cells and tumor-specific cell killing compared with control viruses • *Ex vivo* tumor exudates (containing tumor and immune cells) from patients with EpCAM+ malignancies in 50% immunosuppressive exudate fluid: • BiTE-encoding OVs induce proliferation and activation of endogenous T cells and tumor cell depletion superior to control viruses and similar to BiTE protein • Splice acceptor sequence-driven BiTE expression, but not CMV promoter-driven BiTE expression restricted to tumor cells	[74]
MeV (Edmonston B vaccine strain)	Additional transcription unit downstream of H gene	BiTE	Human CEA × murine or human CD3, human CD20 × murine CD3	• Subcutaneous syngeneic mouse tumor models/intratumoral OV injection: BiTE-encoding OV results in (1) prolonged survival, in one of two models superior to control virus or direct BiTE injection; (ii) increased T cell infiltration and activation; and (3) protective immunity (to parental tumor cells not expressing the BiTE-target. Thus indicative of antigen spread, i.e., activation of endogenous T cells specific for tumor antigens) • Patient-derived subcutaneous xenograft mouse tumor model/intratumoral OV and PBMC injection: BiTE-encoding OV in combination with intratumoral PBMC injection shows superior survival compared with BiTE-encoding OV or PBMC alone	[125]
Vaccinia virus (Double-deleted VV vvDD, Western Reserve strain, TK^- and VGF^-)	Separate transcription unit, viral late promoter	BiTE	Murine FAP × murine CD3	• Co-cultures of infected FAP-encoding tumor cells and splenocytes: BiTE-encoding OV (but not control virus) results in T cell activation and enhanced cytotoxicity for (bystander) FAP-expressing cancer cells • Subcutaneous syngeneic mouse tumor model/intratumoral OV injection: BiTE-encoding OV (compared with control virus) results in reduction of FAP+ cells, an increase in virus titer per g tumor tissue, increased infiltration of CD4+ and CD8 + T cells in injected tumors, increased CD8- and CD4-response to tumor antigen (but not to FAP), and superior tumor growth inhibition of injected and contralateral tumors (in which infectious VVs were found) • Syngeneic lung metastases mouse model/intravenous OV injection: ○ BiTE-encoding OV reduces number of lung surface nodules compared with control virus ○ BiTE not detectable in blood, no systemic α-FAP activity after infection with BiTE-encoding OV	[104]
Ad (EnAd, chimeric type B Ad)	Inserted as separate transcription unit with CMV promoter (constitutive) or inserted in late viral transcription unit with splice acceptor sequence (expression from viral major late promoter, replication-dependent)	BiTE	human FAP × human CD3	• Co-cultures with PBMC-derived $CD3^+$ cells + cancer cell lines mixed with normal fibroblasts: ○ BiTE-encoding OVs but not control viruses mediate T cell activation and target cell killing ○ OV with BiTE transgene in the late transcription unit requires infection of cancer cells for replication-dependent (thus tumor-specific) antibody expression • *Ex vivo* ascites model with total ascites cells (including cancer cells, cancer-associated fibroblasts, immune cells): BiTE-encoding OVs, but not control viruses, trigger T cell activation and proliferation, depletion of FAP^+ fibroblasts, increase in inflammatory signature and repolarization of macrophages to M1 phenotype • *Ex vivo* culture of thin sections of prostate cancer biopsies: BiTE-encoding OV increases T cell activation and LDH release superior to control virus with infected cells surrounded by apoptotic cells	[75]

(*Continued*)

Table 2. (Continued).

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Ad (ICOVIR-15K: E1AΔ24, E2F binding site in E1A promoter, RGDK in fiber shaft)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter)	BiTE	Human/murine FAP × human CD3	• Co-cultures of infected tumor cells + stained FAP^+ cells + T cells: BiTE-encoding OV, not parental virus, induces (bystander) target cell killing • Subcutaneous xenograft mouse tumor model (inducing FAP^+ stroma)/ intratumoral OV injection and intravenous injection of T cells: BiTE-encoding OV compared with parental virus shows ○ Increased accumulation and persistence of intravenously injected pre-activated T cells in tumors ○ sSronger tumor growth inhibition and increased survival ○ Similar intratumoral Ad genome copy numbers, but reduced mFAP RNA copy numbers and protein staining	[113]
Ad (EnAd, chimeric type B Ad)	Inserted as separate transcription unit with CMV promoter	BiTE	Human folate receptor-β × human CD3	• *Ex vivo* ascites model with total ascites cells: BiTE-encoding OV induces T cell activation and expansion, depletion of macrophages, and increase of M1 markers on remaining macrophages (repolarization) superior to parental and control viruses • Order of scFvs in BiTE construct is critical for activity of BiTE-encoding OV	[76]
HSV-1 G207 ($ICP6^-$ and $ICP34.5^-$)	Inserted as separate transcription unit with CMV promoter	BiTE or nanobody-scFv fusion	Human PD-L1 (scFv or nanobody) × human CD3 (scFv)	• PD-L1-positivity of T cells does not prevent expansion or effector functions after activation by purified BiTE • Co-cultures of infected tumor cell line, PBMC-derived T cells and immunosuppressive ascites fluid: BiTE-encoding OVs, not control virus, induce depletion of tumor cells • Co-cultures of *in vitro*-differentiated and infected M2-like macrophages, PBMC-derived T cells and immunosuppressive ascites fluid: BiTE-encoding OVs, not control virus, induce expansion and activation of T cells and reduced expression of M2 marker in surviving cells • Co-cultures of tumor cell line, *in vitro*-differentiated M2-like macrophages, and PBMC-derived T cells were infected: BiTE-encoding OVs, not parental virus, induce increase in T cell number and killing of both tumor cells and macrophages • *Ex vivo* ascites model with total ascites cells: BiTE-encoding OVs, not parental or control viruses, induce T cell activation, which is stronger in presence of immunosuppressive ascites fluid, and reduced expression of PD-L1 and M2 markers in surviving cells	[93]

[a]Expression of functional antibody *in vitro* shown; virus replicative and/or oncolytic features usually not or only slightly attenuated as analyzed *in vitro*.

cell therapy using FR-α-directed CAR-T cells, which triggered tumor escape, with viro-BiTE therapy using an oncolytic Ad encoding an EGFR-specific BiTE.[112] For this combination therapy, redirection to EGFR-positive tumor cells and activation of CAR-T cells, notably including the CAR-negative fraction of CAR-T cell preparations, was demonstrated *in vitro* and decreased tumor growth with increased survival compared with monotherapies in a xenograft tumor model. CAR-T cell infiltration was increased for tumors not strongly expressing FR-α.

Viro-BiTE therapy was also explored in immunocompetent syngeneic animal models, demonstrating the induction of T cell infiltration, T cell activation and therapeutic responses, as observed after i.t. injection of a FAP-BiTE-encoding VV or a CD20-BiTE-encoding MeV.[104,125] FAP cells were depleted by the VV/FAP-BiTE treatment and the MeV/CD20-BiTE induced protective immunity resulting in the rejection of tumor cell rechallenge. Of note, the rechallenge was performed with matching tumor cells not expressing the BiTE target. Moreover, the MeV/CD20-BiTE treatment was superior to direct BiTE injection in the immunocompetent model. These observations point at an OV-triggered tumor vaccination effect responsible for the observed protective immunity and are supportive of the complementarity of oncolysis and BiTE treatment in the immunocompetent context. Another interesting observation was that this approach was active in a tumor model with low baseline T cell infiltration, but not in another model with high tumor-infiltrating lymphocytes content. Thus, analysis of the TME might inform about the responsiveness of tumors to viro-antibody therapy or rather suggest alternative treatments, such as OVs encoding different therapeutic proteins.

Expression of immune checkpoint inhibitors (viro-CHECKin therapy)

ICIs represent a major breakthrough in cancer treatment, as introduced above. With the success of ICIs and identification of their limitations, OVs have received considerable attention of cancer immunotherapy research toward sensitizing patients to ICIs that are unresponsive to ICI monotherapy. Specifically, several OVs have been reported in preclinical models to switch immune-"cold", thus ICI unresponsive tumors to "hot" by triggering intratumoral inflammation and activation and recruitment of tumor antigen-specific T cells, thereby facilitating enhanced therapeutic efficacy of combination treatment with OV and ICI.[5,6,19] Results from early clinical trials indicate improved therapeutic responses of OV and ICI combination treatment in patients.[22,23] In fact, the analysis of combination treatment with ICIs has emerged as a standard experiment widely used by labs analyzing the therapeutic activity of OVs. These studies, together with reports of effective intratumoral injection or expression of low dose ICIs,[126–129] established a clear rationale for viro-antibody therapy with ICI antibodies (***viro-CHECKin therapy***) implementing a single-agent OV-ICI combination therapy featuring local, genetic antibody delivery. This approach, in addition to enhancing therapeutic outcome compared with monotherapies, aims at reducing the severe systemic side effects of standard ICI therapy. Viro-CHECKin therapy has been explored with many OVs, i.e., Ad, VV, MeV, HSV, NDV, VSV, and influenza virus (Table 3). Furthermore, different antibody formats (IgGs [see also viro-MAb therapy above], scFvs, scFv-Fc fusion proteins, and scFv-cytokine fusion proteins [Figure 3]) and targets (CTLA-4, PD-1, PD-L1, and TIGIT), have been investigated. It should be mentioned that alternative ICI formats independent of antibody-derived variable domains have been explored for genetic delivery by OVs, using a soluble checkpoint molecule, exemplified by soluble PD-1 or soluble PD-1 fused to an Fc domain.[87,130]

Viro-CHECKin therapy studies demonstrated activation of T cells, including T cells from cancer patients, *in vitro*,[106,131] and therapeutic activity superior to parental viruses in syngeneic models *in vivo*,[91,92,103,105,117,132–134] indicating that effective levels of antibodies were expressed in the tumor. Some studies reported therapeutic outcomes similar to parental virus in combination with systemic or local ICI treatment.[91,92,103,134] Moreover, delayed growth of untreated contralateral tumors[91,117] and rejection of tumor cell rechallenge[89,91,92,105,134] revealed the establishment of systemic and long-term immunity. Mechanistic insights were provided by a study using an oncolytic HSV encoding an anti-PD-1 scFv that showed increased cross-presentation of a model tumor antigen by dendritic cells and enhanced T cell activation in a syngeneic tumor model.[91] Furthermore, an increased overall T cell infiltration, but reduced Treg infiltration, by viro-CHECKin therapy was reported using an oncolytic VV encoding an anti-PD-1 IgG and an oncolytic MeV encoding anti-CTLA-4 scFv-Fc or anti-PD-L1 scFv-Fc fusion proteins. Notably, viro-CHECKin therapy enhanced T cell infiltration compared with parental OV alone in the MeV study, but not in the VV study, as oncolytic VV alone, but not MeV alone, triggered massive T cell infiltration already.[103,133] In consequence, the choice of therapeutic genes for effective viro-antibody therapy should consider the OV vector and its specific oncolytic and immunological properties.

Interestingly, one viro-CHECKin therapy study compared different antibody formats of the same specificity, i.e., IgG and scFv. Using an oncolytic VV, this study showed stronger expression of the scFv, but similar tumor growth inhibition for both formats, which was superior to parental virus. A survival advantage compared with parental virus was shown to be significant only for the IgG format, but not for either the scFv format or the combination of parental virus with systemic anti-PD-1 treatment.[103]

The choice of the OV-encoded ICI determines the outcome of viro-CHECKin therapy, as revealed by a study that compared in a syngeneic mouse model oncolytic MeVs encoding anti-CTLA-4 versus anti-PD-L1, both in the scFv-Fc format.[133] Compared with control virus, the anti-CTLA-4 virus delayed tumor progression but did not significantly prolong survival, whereas the anti-PD-L1 virus prolonged survival without reducing early tumor growth. Tumor-specific splenocytes were activated early after treatment with the anti-CTLA-4 virus and late after anti-PD-L1 virus treatment. In contrast to other viro-CHECKin studies (see above), in the MeV study the combination of MeV with systemic mAb therapy was superior

Table 3. Overview of developed therapeutic antibody-encoding OVs: OVs expressing immune checkpoint inhibitors (viro-CHECKin therapy), sorted according to target and publication date.

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Ad (Ad5/3-E1AΔ24)	Replacement of early genes (E3) by Ig chains linked via IRES	IgG2	Human CTLA-4	See Table 1	[106]
Ad (Ad E2F promoter E1A)	Replacement of early genes (E3) by Ig chains linked via 2A	IgG2	Mouse CTLA-4	See Table 1	[107]
Influenza A virus (IAV)	Heavy chain in PB1 segment downstream of PB1 gene via 2A; light chain in PA segment downstream of PA gene via 2A; scFv cloned into both segments	IgG and scFv	Proof-of-concept (IgG) and murine CTLA4 (scFv)	See Table 1	[117]
HSV-1 (bioselected clinical strain, ICP34.5^{-} and ICP47^{-}, also encoding murine GM-CSF and a highly fusogenic glycoprotein)	Separate transcription unit, MMLV LTR promoter	scFv fused to mouse IgG1	Murine CTLA-4	• Bilateral subcutaneous syngeneic mouse tumor model/low dose intratumoral OV injection of right flank tumor: antibody-encoding OV increases tumor growth inhibition of injected and not injected tumors (although significance not reached)	[90]
NDV lentogenic strain	Additional transcription unit downstream of P gene	scFv	Murine CTLA-4	• Intradermal syngeneic mouse tumor model/irradiation/intratumoral OV injection: antibody-encoding OV + X-ray shows similarly increased survival and tumor growth inhibition than parental virus + X-ray + systemic α-CTLA-4 when compared with α-CTLA-4 alone	[135]
MeV (attenuated vaccine strain)	Separate transcription unit downstream of H gene	scFv-IgG1 Fc fusion	Murine CTLA-4, murine PD-L1	• Subcutaneous syngeneic mouse tumor model/intratumoral OV injection: ○ α-CTLA-4-encoding OV reduces tumor progression, whereas α-PD-L1-encoding OV prolongs survival both compared with control virus ○ Both antibody-encoding OVs increase T cell infiltration, decrease Treg infiltration and result in splenocyte activation (early after infection for the α-CTLA-4 virus or late for the α-PD-L1 virus) ○ For targeting CTLA-4, but not PD-L1 the combination treatment of systemically applied checkpoint inhibitor antibody with parental OV was significantly superior to the antibody-encoding OV • Subcutaneous xenograft mouse tumor model/intratumoral OV injection: antibody-encoding OVs are as effective as control virus	[133]
VSV (M51R mutant)	Additional transcription unit between G and L genes	scFv	Human PD-L1 (avelumab-derived)	• Subcutaneous syngeneic mouse tumor model with hPD-L1-expressing mouse tumor cells/intratumoral OV injection: ○ Antibody-encoding-OV or combination of parental OV + intraperitoneal scFv reduce tumor growth and improve survival in comparison to monotherapies, 5/6 mice cured with antibody-encoding-OV resist rechallenge with tumor cells ○ Increase of activated CD8^{+} T cells in spleen of mice cured after treatment with antibody-encoding-OV compared with normal mice	[134]
Vaccinia virus (CF33: chimera derived by recombination between 9 strains)	Additional transcription unit with viral H5 promoter	scFv	Human PD-L1	• Intraperitoneal xenograft mouse tumor model/intravenous or intraperitoneal injection of antibody-encoding OV: ○ Detection of scFv expression stronger after intraperitoneal application of antibody-encoding OV virus compared with intravenous application ○ scFv-encoding OV shows therapeutic activity which is superior after intraperitoneal application (no comparison to parental virus)	[131]
Vaccinia virus (CF33: chimera derived by recombination between 9 strains; also encoding hNIS)	Additional transcription unit with viral H5 promoter	scFv	Human PD-L1	• Co-cultures of PDAC cell lines and activated T cells were infected: parental OV results in translocation of PD-L1 to cell surface in cancer cells; antibody-encoding OV delivers sufficient α-PD-L1 scFv to block cell surface detection of PD-L1 on cancer cells; OV-encoded scFv increases granzyme B production and prevents OV-induced decrease in perforin release by T cells	[137]

(*Continued*)

Table 3. (Continued).

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
NDV (lentogenic strain)	Additional transcription unit downstream of P gene	scFvs and scFv-cytokine (scmIL-12) fusions (immunocytokines)	Murine PD-1, PD-L1, CD28	• Unilateral subcutaneous syngeneic mouse tumor model/intratumoral OV injection: ○ α-PD-1- and α-PD-L1-encoding OVs show superior therapeutic activity compared with parental virus, esp. when combined with systemic α-CTLA-4 ○ >50% complete remission for α-CD28-mIL12-encoding OV, α-PD-L1-encoding OV and α-PD-L1-mIL12-encoding OV, each combined with systemic α-CTLA-4 • Bilateral subcutaneous syngeneic mouse tumor model/intratumoral OV injection in one flank: ○ Tumor growth inhibition observed in contralateral tumors ○ Strongest survival benefit for α-PD-1-, α-PD-1-mIL-12- and α-PD-L1-mIL-12-encoding OV, each combined with systemic α-CTLA-4 ○ Strongest upregulation of immune markers in tumors for α-CD28-mIL12-encoding OV and α-PD-L1-mIL12-encoding OV, both combined with systemic α-CTLA-4	[132]
Vaccinia virus (Western Reserve strain, TK^- and RR^-)	Separate transcription unit with different viral promoters at TK locus	IgG, Fab, scFv	Murine PD-1	See Table 1	[103]
HSV-2 ($ICP34.5^-$ and $ICP47^-$)	Expression of HC and LC as separate transcription units from CMV and RSV promoters, respectively	IgG	Human PD-1	see Table 1	[92]
HSV-1 NG34 ($ICP6^-$ and $ICP34.5^-$, also expression of human GADD34 gene from nestin-hsp68 promoter)	Separate transcription unit with CMV promoter	scFv	Human/murine PD-1	• Orthotopic syngeneic mouse tumor model/intratumoral OV application: ○ Increased survival of antibody-encoding OV versus mock (significant) and parental OV (not significant), rechallenge of cured mice with tumor cells rejected (in 2 mouse models) ○ Detection of antibody and viral mRNA at 16 h post-infection, strongly reduced at 36 h, active virus not recoverable (but recoverable in orthotopic human xenograft) → inefficient OV replication in mouse tumors	[89]
HSV ($ICP34.5^-$, $ICP0^-$, ICP27 promoter replaced by hTERT promoter)	Separate transcription unit with CMV promoter	scFv	Murine PD-1	• *In vitro* phagocytosis assay with DCs and cancer cells: antibody-encoding OV increases phagocytosis compared with parental virus • Subcutaneous syngeneic mouse tumor model/intratumoral OV injection: antibody-encoding OV ○ Results in increasing scFv expression over 72 h ○ Increases cross-presentation of model antigen compared with control virus ○ Results in injected and distal tumor growth inhibition similar to combination of control OV with intratumoral scFv injection and superior to control OV alone, long-term regressors rejected tumor cell rechallenge ○ Triggers T cell infiltration in injected and non-injected tumors similar to control OV, but with higher activation status ○ Triggers MDSC infiltration in injected and non-injected tumors more than control OV ○ In combination with systemic α-TIGIT therapy results in superior tumor growth inhibition and infiltration of model tumor antigen-specific $CD8^+$-T cells compared with combination of control OV with α-TIGIT; tumor growth inhibition was dependent on $CD8^+$- and $CD4^+$-T cells	[91]
Vaccinia virus (Western Reserve strain)	Expression of HC and LC fused via 2A peptide and fused to luciferase from as separate transcription unit from viral early/late promoter	IgG	Mouse TIGIT	See Table 1	[105]

[a]Expression of functional antibody *in vitro* shown; virus replicative and/or oncolytic features usually not or only slightly attenuated as analyzed *in vitro*.

to genetic antibody delivery, with differences being significant for the anti-CTLA-4 virus, but not the anti-PD-L1 virus. The authors speculated that their results are due to different modes of action: the CTLA-4 checkpoint is active earlier in the immune response, especially during T cell activation in secondary lymphoid organs, while the PD-1 checkpoint is active during execution of T cell activity in the tumor. Therefore, anti-PD-L1 effects might appear later during treatment and the anti-CTLA-4 approach might benefit more from systemic antibody therapy, pointing at potential limitations of viro-antibody therapy, whenever systemic rather than intratumoral activity of antibodies is required.

Viro-CHECKin therapy can be combined with further therapeutics or treatment modalities to improve therapeutic outcome. One approach was inspired by the observation of increased tumor infiltration by CD155+ myeloid-derived suppressor cells after viro-CHECKin therapy with an oncolytic HSV encoding anti-PD-1 scFv in a syngeneic mouse model, in spite of improved therapeutic outcome (see ref.91). Correspondingly, anti-PD-1-encoding HSV was combined with an anti-TIGIT antibody that blocks the CD155-binding checkpoint molecule expressed on T cells. The combination resulted in an infiltration of tumor-specific T cells and therapeutic activity superior to both monotherapies and also to the combination of the parental virus with anti-TIGIT.[91] A different combination strategy was to combine viro-CHECKin therapy with radiation therapy. This was inspired by the observation that triple treatment of oncolytic NDV, irradiation and anti-CTLA-4 showed increased complete response rates compared with double treatments in a syngeneic mouse model. The combination of an anti-CTLA-4 scFv-encoding NDV combined with irradiation in the syngeneic model resulted in a similar survival compared with triple treatment with parental virus, irradiation and anti-CTLA-4, which was significantly improved compared with anti-CTLA-4 treatment alone.[135]

A distinct viro-CHECKin combination treatment was based on genetic fusion of an ICI scFv with a cytokine (immunocytokine), representing yet another recombinant antibody format and mode of action (Figures 3 and 4). Specifically, oncolytic NDVs encoding anti-PD1 or anti-PD-L1 scFvs fused to single-chain murine IL-12, scmIL-12, were explored.[132] In addition, this study investigated the combination of viro-CHECKin therapy targeting PD1/PD-L1 with systemic anti-CTLA-4 treatment. In a syngeneic mouse model, treatment with NDV-anti-PD1, NDV-anti-PD1-scmIL-12 and NDV-anti-PD-L1-scmIL-12 resulted in superior survival compared with parental virus, but only when combined with systemic anti-CTLA4. Moreover, NDV-anti-PD-L1-scmIL-12 was superior to NDV-anti-PD-L1 in the presence, not absence, of anti-CTLA-4 and triggered an especially strong increase in CD8 and TNF expression as determined by gene expression analysis. This study also reported an oncolytic NDV encoding an anti-CD28 scFv as T cell costimulatory superagonist, with or without fused IL-12, representing yet another mode of action of viro-antibody therapy, "viro-CoStim therapy" (see Figure 4). For the NDV-anti-CD28-scFv-scmIL-12 virus, superior survival outcome in comparison to parental virus was observed, but again only in the presence of anti-CTLA-4. The clearly different outcomes of viro-antibody therapies in the presence versus absence of anti-CTLA-4 in this study underscore the more global concept that the efficacy of OV-encoded checkpoint inhibitors or co-stimulatory molecules is determined by the overall immunological status, systemically or in the TME.

Expression of anti-angiogenic antibodies (viro-ANGin therapy)

Another viro-antibody therapy approach aims at inhibiting tumor angiogenesis (***viro-ANGin therapy***). To this end, oncolytic VV and Ad encoding recombinant antibodies binding and inhibiting the angiogenic growth factor VEGF have been engineered (Table 4) based on the clinically established conventional antibody therapy with Avastin® (bevacizumab).[136] An oncolytic Ad encoding a VEGF-binding IgG is discussed above. Several other studies with oncolytic VV explored human and mouse VEGF-specific scFvs, and demonstrated superior or more rapid tumor growth inhibition compared with parental virus after systemic[94,97,102] or intratumoral[99] injection in human xenograft models. In a lung cancer xenograft model, a reduction in malignant effusion was observed.[97] Further analyses revealed a reduced blood vessel density in infected, but not in noninfected, areas of tumors[94,99,102] and a noticeable reduced vascular flow in tumors,[99] with oncolytic VV alone triggering either an increase or a decrease of vascular density dependent on the tumor model. These results indicate that diffusion of OV-encoded scFv from infected tumor regions was insufficient to block VEGF in noninfected areas of the tumor. Of note, i.v. injection of VVs expressing the anti-VEGF scFv gene from strong promoters resulted in antibody concentrations 7 days post-infection that were 12–15 times higher in infected areas of tumors compared with sera,[94] underscoring the potential of viro-antibody therapy for targeted delivery of therapeutic antibodies to tumors.

A single OV can feature two distinct antibody-mediated modes of action (see Figure 4) by co-expression of different recombinant antibodies, i.e., as a "viro-double-antibody therapy." This has been explored for oncolytic VVs expressing an anti-VEGF scFv together with either an anti-EGFR nanobody (a single-domain antibody format, see Figure 3) or an anti-FAP scFv.[102] Viruses encoding anti-EGFR or anti-FAP alone showed more rapid tumor growth inhibition than the parental virus in xenograft models. Mechanistic studies revealed for the anti-EGFR virus an inhibition of proliferation similar to parental virus in infected tumor areas, but superior to parental virus in noninfected areas, indicating spread of the virus-encoded nanobody. The anti-FAP virus resulted in a reduction of FAP+ and CD31+ cells in both infected and noninfected tumor areas when compared with the parental virus, also indicating antibody spread. The anti-VEGF/anti-EGFR and anti-VEGF/anti-FAP double-antibody viruses showed superior tumor growth inhibition to parental VVs, however, reaching significance only in comparison to the VV not encoding an Ab. Importantly, the anti-VEGF/anti-EGFR virus combined suppression of both proliferation and angiogenesis, demonstrating "double-antibody" activity.

Table 4. Overview of developed therapeutic antibody-encoding OVs: OVs expressing anti-angiogenic antibodies (viro-ANGin therapy), sorted according to target and publication date.

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Vaccinia virus (GLV-1h68: Lister vaccine strain, triple mutant)	Separate transcription unit, one of 3 viral promoters (synthetic early [SE], synthetic early/late [SEL], or synthetic late [SL])	scFv	Mouse and human VEGF	• Promoter determines strength of antibody expression (SEL, SL > SE) • Subcutaneous xenograft mouse tumor model/intravenous OV injection: ○ Antibody detected in serum at 7, 21 and 37 d post-infection (SEL, SL viruses) ○ Antibody concentration in infected areas of tumors >10-fold higher than in sera at 7 d p.i. (SEL, SL viruses) ○ Antibody-encoding OVs result in stronger tumor growth inhibition compared with control virus (significant for SEL, SL viruses) ○ Negative correlation of late tumor size and early antibody concentration in blood ○ Reduced blood vessel density in infected areas of tumors for antibody-encoding OV	[94]
Vaccinia virus (GLV-1h68: Lister vaccine strain, triple mutant)	Separate transcription unit, synthetic late promoter	scFv	Mouse and human VEGF (binds canine VEGF)	• OVs replicate productively in canine cancer cells • Subcutaneous canine cancer xenograft mouse models/intravenous OV injection: ○ Tumor-specific virus biodistribution and tumor growth inhibition in 1 of 2 models (not compared with parental virus) ○ Reduced blood vessel density in infected but not noninfected tumor areas for antibody-encoding OV compared with control virus	[95]
Vaccinia virus (GLV-1h100: Lister vaccine strain, triple mutant)	Separate transcription unit, synthetic late promoter	scFv	Mouse and human VEGF	• OV-encoded α-VEGF scFv reverses VEGF-induced radioresistance of endothelial cells, but not tumor cells • Subcutaneous xenograft mouse tumor model/systemic virus application + irradiation: ○ Enhanced tumor growth inhibition and reduced intratumoral VEGF concentration and vessel number for antibody-encoding OV + irradiation compared with monotherapies or combination with control virus, durable responses for combination, only ○ Irradiation enhances OV spread in xenograft as measured by reporter expression	[96]
Vaccinia virus (GLV-1h68: Lister vaccine strain, triple mutant)	Separate transcription unit, synthetic late promoter	scFv	Mouse and human VEGF	• Subcutaneous xenograft and malignant effusion mouse lung cancer model/intravenous OV application: ○ Parental OV results in infection of tumor cells in vessels and at tumor borders, tumor growth inhibition and less, delayed or disappearing malignant effusion, but also in an increase in vascular density, CD31 expression and accumulation of leukocytes ○ Antibody-encoding OV results in stronger tumor growth inhibition and stronger reduction of malignant effusion compared with control virus	[97]
Vaccinia virus (GLV-1h100: Lister vaccine strain, triple mutant)	Separate transcription unit, synthetic late promoter	scFv	Mouse and human VEGF	• Orthotopic xenograft mouse breast cancer model/intratumoral OV injection: antibody-encoding OV shows significantly superior therapeutic activity, noticeable decreased vascular flow by *in vivo* Doppler ultrasonography and decreased vasculature by immunostaining compared with control virus	[99]
Vaccinia virus (Lister vaccine strain 6.1.1)	Separate transcription unit, synthetic early/late promoter	scFv	Mouse and human VEGF, binds feline VEGF	• Subcutaneous feline cancer xenograft mouse model/intravenous OV injection: antibody-encoding OV results in tumor growth inhibition similar to parental virus, reduced intratumoral functional VEGF and reduced blood vessel density in infected areas compared with parental virus	[100]
Vaccinia virus (Lister vaccine strain 6.1.1)	Separate transcription unit, synthetic early/late promoter	scFv	Mouse and human VEGF, binds canine VEGF	• Subcutaneous canine cancer xenograft mouse model/intravenous OV injection: antibody-encoding OV regresses tumor growth and reduces blood vessel density more than parental virus	[101]

(*Continued*)

Table 4. (Continued).

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV[a]	Reference
Vaccinia virus (GLV-1h68: Lister vaccine strain, triple mutant)	Separate transcription unit, viral promoters (SEL, SL [VEGF] or SEL+SL)	scFv, nanobody	VEGF (scFv) + EGFR (nanobody); VEGF (scFv) + cross-species FAP (scFv)	• Subcutaneous xenograft mouse tumor model/intravenous OV injection: ○ OVs encoding single antibodies (targeting EGFR, VEGF, or FAP) inhibit tumor growth more rapidly (one xenograft model) or stronger (other xenograft model) than control virus ○ OVs encoding two antibodies result in strongest tumor growth inhibition, significantly superior to control virus, significance not reached in comparison to single antibody-encoding OVs ○ Therapeutic activity of OV encoding antibodies targeting EGFR and VEGF is similar to combination of control virus with systemic Avastin and Erbitux ○ OVs encoding the EGFR-targeted antibody show superior suppression of cell proliferation in noninfected tumor areas compared with control virus ○ reduced blood vessel density in infected areas of OVs encoding the VEGF-targeted antibody in comparison to control virus	[102]
Ad (EnAd, chimeric type B Ad)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter), Ig chains linked via IRES	IgG1	Human VEGF	See Table 1	[111]

[a]Expression of functional antibody *in vitro* shown; virus replicative and/or oncolytic features usually not or only slightly attenuated as analyzed *in vitro*.

Viro-ANGin therapy has also been combined with radiotherapy, considering that irradiation-induced VEGF expression by tumor cells triggers radioresistance of endothelial cells. An oncolytic VV-encoded anti-VEGF scFv was reported to reverse VEGF-mediated radioresistance of ECs, but not tumor cells, *in vitro*. Moreover, radio-viro-ANGin therapy with focal fractionated irradiation achieved superior tumor growth inhibition with durable responses when compared with parental virus alone, irradiation alone, and with parental virus and irradiation combined.[96] This was correlated with superior reduction in blood vessel density and reduced VEGF levels in tumors. Of note, this study also detected irradiation-enhanced VV infection *in vivo*, which might contribute to superior outcome of combination therapy.

Expression of cytotoxic antibody fusion proteins (viro-iTOX therapy)

Recombinant antibody fusion molecules can direct effector functions of fused proteins to tumor antigens or combine them with antibody-mediated effector activities. Such immunofusion molecules represent yet another antibody format that has been explored in the context of viro-antibody therapy (Figure 3 and Tables 3 and 5). One example is the expression of immunocytokines,[32] as discussed above for OVs encoding fusion proteins of scFvs that combine checkpoint inhibition or costimulation with IL-12 activity. Two other studies explored OVs expressing tumor antigen-specific scFvs fused to cytotoxic proteins for direct bystander killing of cancer cells (***viro-iTOX therapy***): One study engineered an oncolytic Ad encoding an immunoRNase consisting of a cetuximab-derived scFv fused to an RNase that triggers cell death after scFv-mediated cellular uptake.[109] This approach required an optimized transgene expression strategy by the Ad to avoid interference of the immunoRNase with virus replication and infectious particle production (as discussed above). However, with the optimized expression strategy, this viro-iTOX therapy enabled a potent and specific bystander killing of EGFR-positive target cells *in vitro* and tumor growth inhibition after intratumoral injection in a xenograft model *in vivo*. The second study explored an oncolytic HSV encoding a HER2-specific scFv fused to an extracellular fragment of the apoptosis-inducing FasL and a collagen-derived trimerization domain.[88] This immunotoxin features a trimeric killer molecule with limited killing activity on its own, but potent killing of HER2+ target cells mediated by multivalent antigen-binding. Expression of this "immuno-killer" gene by the oncolytic HSV triggered apoptosis, but also somewhat reduced virus replication *in vitro*, likely due to its pro-apoptotic activity. This viro-iTOX therapy inhibited tumor growth after intratumoral injection in a xenograft (low dose) and a syngeneic mouse model (high dose), the latter using a virus strain adapted to the mouse tumor cell line by *in vivo* passaging.

Of note, immunofusion approaches are relevant also for tumors resistant to the parental antibody due to defects in downstream signaling of the target molecule, e.g., Ras mutations mediating resistance to cetuximab even when EGFR is still expressed. This is because of the different mode of action, i.e., cell killing by the scFv-targeted cell killing moiety. This has been demonstrated for viro-iTOX therapy with the immunoRNase-encoding Ad facilitating the destruction of cetuximab-resistant cancer cells.[109]

Conclusion, challenges and outlook

Viro-antibody cancer therapy uses OVs for intratumoral genetic and amplified delivery of therapeutic antibodies or, in other words, arms OVs with a gene encoding a therapeutic antibody to complement viral oncolysis with a tumor-targeted therapeutic mode of action. The emerging field has reached three major preclinical research milestones since the first publication in 2008.[121] First, functional therapeutic antibodies of different formats can be expressed in and released from cancer cells infected with different OVs *in vitro* and *in vivo*. To this end, gene expression strategies have been established for the expression of polymeric and single-chain antibodies in a manner compatible with productive OV replication. Interestingly, one study even reported that antibody expression indirectly improves viral oncolysis by antibody-mediated ECM destruction.[104] Antibody expression by OVs was shown to be much stronger compared with replication-deficient vectors of the same virus platform.[106] Moreover, tumor–blood ratios of antibody concentrations after OV delivery were reported to be dramatically higher compared with conventional application.[103,106] Second, viro-antibody therapy can implement several antibody-mediated modes of action, including direct tumor cell killing (some viro-MAb therapies, viro-iTOX therapy), anti-angiogenesis (viro-ANGin therapy) or indirect and systemic killing of tumor cells, tumor stroma cells or inhibitory immune cells by activation of adaptive immune responses (viro-BiTE therapy, viro-CHECKin therapy). For the latter, several studies used sophisticated patient-derived *ex vivo* models derived from tumor ascites or pleural effusions containing tumor, stroma and immune cells, or mouse models with human tumor and immune cell xenografts (see Table 2). Third, after genetic delivery to tumors by OVs, therapeutic antibodies improved therapeutic outcome, i.e., showed therapeutic activity complementary to viral oncolysis, as detected by increased tumor destruction or growth inhibition, improved survival advantage, and/or higher cure rates. For viro-BiTE therapy or viro-CHECKin therapy, long-term antitumor immunity was demonstrated.[89,91,125,134] Therapeutic activity has been reported to be similar or superior to combined application of parental OV with standard antibody treatment.[91,92,102,134,135] Still, for specific settings, when

Table 5. Overview of the developed therapeutic antibody-encoding OVs: OVs expressing cytotoxic immunofusions (viro-iTOX therapy).

Virus	Antibody gene expression strategy	Antibody format	Target	Key results for antibody-encoding OV	Reference
Ad (Ad5/3 [serotype 3 cell-binding knob] E1AΔ24)	Insertion into late transcription unit with splice acceptor sequence (expression from major late promoter)	scFv-RNase fusion (immunoRNase)	Human EGFR (cetuximab-derived)	• Optimized gene expression control for strictly late expression of immunoRNase required to avoid adverse effects on OV infectivity/replication • Potent and specific bystander killing of target cells, including cetuximab-resistant tumor cells (distinct mode of action) • Antibody-encoding OV induces strong increase in cytotoxicity for target cells compared with control virus *in vitro* and *in vivo* (subcutaneous xenograft mouse tumor model/intratumoral OV injection)	[109]
HSV-2	Separate transcription unit with RSV-LTR promoter	scFv-trimerization domain-human sFasL fusion	Human Her2 + human Fas	• Reduced virus replication of antibody-encoding OV compared with parental virus • Subcutaneous xenograft mouse tumor model/low dose intratumoral OV injection: antibody-encoding OV shows stronger tumor growth inhibition than parental virus, transient halt of tumor growth • Subcutaneous syngeneic mouse tumor model/high dose intratumoral OV injection: antibody-encoding OV (pre-passaged *in vivo* for improved replication) shows prolonged tumor regression compared with parental virus	[88]

systemic antibody activity is required, as it is believed to be the case for anti-CTLA4-mediated immune checkpoint inhibition, conventional antibody application in addition to OV injection might be superior to OV-encoded delivery.[133] However, this approach requires the development of two biotherapeutics. In this light, it is not surprising that the therapeutic outcome of viro-antibody therapy was further improved by conventional application of anti-CTLA-4, or of another immune checkpoint inhibitor, anti-TIGIT, as reported for OVs encoding PD-1, PD-L1-, or CD28-specific scFvs or scFv-cytokine fusions.[91,132] Other studies demonstrated effective combination regimen of viro-CHECKin therapy with radiotherapy and of viro-BiTE therapy with CAR-T cell application.[112,135]

Research discussed here has also revealed several challenges that remain for viro-antibody therapy development: The above-mentioned achievements have been reported for selected combinations of OV platforms, antibody formats and modes of action. Moreover, direct comparisons of different virus platforms, antibody formats or modes of action in a given viro-antibody therapy approach or tumor model have, with one exception,[103] not been done. With respect to virus platforms, gene therapy provides a lesson, having shown clear differences between AAV and Ad vectors with respect to the kinetics and durability of antibody expression.[59,61,65,138] Certainly, differences between OVs relevant for specific viro-antibody therapy approaches exist and need further exploration, such as differences in OV-triggered immune cell infiltration determining the outcome of antibody-mediated immune cell activation or defining the required complementary mode of action, i.e., recruitment versus activation of T cells. Notably, analyses of tumor perfusion and biodistribution of OV-encoded antibodies and of their possible improvement by switching antibody formats have been reported rarely or not at all, respectively. There are some indirect indications from reported data: OV-mediated expression of anti-VEGF scFvs was reported in several studies to result in reduced blood vessel density in infected, but not noninfected, tumor areas (see Table 4), indicating limited perfusion of the scFv in the tumor. In contrast, one study showed that a FAP-specific scFv or an EGFR-binding nanobody encoded by the same OV triggered depletion of FAP+ cells and reduced tumor cell proliferation also in noninfected tumor regions.[102] These results point at the antibody target and/or the antibody format being important parameters for intratumoral spread of antibody activity. Overall, these reports provide a clear rationale for further mechanistic studies as starting point for improving tumor perfusion by OV-encoded antibodies. Furthermore, studies to explore how antibody engineering can be exploited to reduce or increase release into the bloodstream are warranted for therapeutic approaches for which systemic toxicities need to be avoided or systemic activity is of therapeutic benefit, respectively. Finally, activity of viro-antibody therapy in patients remains to be shown. Initial clinical studies by PsiOxus exploring oAds encoding an anti-CD40 antibody or an anti-FAP BiTE and further immunostimulatory factors, are presently recruiting (NCT 03852511, NCT 04053283), but further strengthening of translational activities is warranted.

Future research and development of viro-antibody therapy will certainly benefit from established technologies for engineering of both OVs and antibodies. Such engineering technologies represent tools to (1) address the above-mentioned challenges and overcome emerging roadblocks and (2) seize opportunities for design of novel viro-antibody therapies toward the development and translation of antibody-encoding OVs as one-agent multifunctional biotherapeutics featuring fine-tuned antibody expression, distribution, potency and tumor-specificity.

On the OV side of future viro-antibody therapy research, besides comparative studies to identify the best fit OV for individual therapeutic approaches and tumor entities, virus engineering provides opportunities to further improve antibody expression. While antibody delivery in viro-antibody therapy is inherently coupled to virus infection, replication and spread, modification of gene expression control may improve strength and/or modulate the kinetics of antibody expression. The latter is of critical interest to avoid premature destruction of OV-producing cancer cells by direct or indirect antibody-mediated cell killing. For example, it has been shown that the timing of immune checkpoint inhibition by antibodies relative to oncolysis is critical for therapeutic outcome, as it determines the strength of anti-viral versus antitumor immune activation.[139] Also, as OV replication efficiency in individual patients is difficult to predict, safety switches blocking either OV replication or antibody expression in case of toxicity will be a relevant research topic, especially for viro-CHECKin and viro-BITE therapy.[123] OV engineering might also provide an opportunity to increase antibody release from OV infected cells by modulation of how and with what kinetics infected tumor cells are lysed (see, e.g., ref118). Lastly, for therapeutic approaches that benefit from extended or repeated antibody delivery, strategies facilitating repeated application of antibody-encoding OVs are of interest, e.g., by switching of OV (sub-) type or platform.

Recent progress in the development of antibody therapies provides a plethora of opportunities for future viro-antibody therapy research, not only regarding therapeutic concepts but also approaches to increase efficacy and safety. This includes engineering of antibody molecules to meet pharmaceutical and clinical requirements by improving biochemical and biophysical properties of the antibodies, e.g., to increase stability, and to reduce immunogenicity. Furthermore, functional properties of the antibodies are now routinely adapted to the therapeutic needs, including modifications of the Fc region to tailor antibody effector functions. For example, IgG molecules intended to recruit immune effector cells through binding to Fcγ receptors can benefit from an increased and selective binding to certain Fcγ receptors, e.g., FcγRIII on NK cells to increase ADCC, while strategies where such immune effector cells are detrimental will benefit from silenced Fc regions.[140,141] Many of these modifications are accessible through genetic engineering. However, it should also be mentioned that some modifications, such as glyco-engineering or conjugation of therapeutic compounds, are excluded from viro-antibody therapy.

An increasing number of new targets is being evaluated for cancer therapy.[142] This pipeline is fueled by novel tools of antibody discovery, e.g., moving from target-led strategies to phenotypic screening approaches,[26,143] including targets associated with the various stages of the metastatic process and the tumor microenvironment.[144] Thereby, the "high-hanging fruits" regarding target biology, target exposure and antibody modes of action are being pursued. The development of intracellularly produced antibodies, so-called intrabodies, which have recently been shown to work *in vivo*,[145,146] provides opportunities for developing a different kind of viro-antibody therapy. As such, the expression of cytosolic, nuclear or vesicular intrabodies facilitates the targeting of intracellular host restriction factors and of cellular molecules involved in immune activation at the protein level. By this means, specific epitopes, conformations, or post-translational modifications may be targeted and protein–protein interactions might be blocked. A recent study points at possible applications in virotherapy: it demonstrated increased replication of a VSV mutant by knockdown of interferon-α secretion using an ER intrabody with specificity for several interferon-α isoforms.[147]

Antibodies against novel targets, but also antibodies against established and clinically used targets, can be further improved by a fine-tuning of antibody properties, e.g., considering affinity, valency, effector functions, and epitope specificity.[148,149] Here, bi- or multi-specific antibodies for combinatorial targeting of cell surface antigens and/or soluble ligands are currently under investigation, also allowing tumor heterogeneity to be addressed.[150] However, bispecific antibodies offer much more. Through their dual-binding mode, bispecific antibodies can exert many new modes of action beyond those of natural antibodies, including, for example, target-mediated uptake and inhibition of intracellular structures, forced internalization and degradation, ligand or co-factor mimicry resulting in cis activation of target molecules, and targeted clustering of surface receptors *in trans*, just to name a few of them.[38] Here, an entire zoo of bispecific antibody formats is available to adapt the molecular configuration to the therapeutic need.[38] Many of these bispecific antibody formats are evaluated in clinical studies for T-cell retargeting. Thus, it can be expected that, besides the BiTE format currently used in combination with OVs, alternative formats with favorable functional properties will be implemented in the future, for example, increasing valency and tuning affinity for target antigens to increase potency and tumor cell selectivity. Furthermore, strategies are available to endow bispecific T-cell engagers with mechanisms for conditional activation at the tumor site, e.g., by local liberation of the binding site through proteolytic cleavage or as a response to pH or hypoxia.[54] Finally, the exploitation of antibody fusion proteins for targeted delivery of effector moieties offers further opportunities. With a plethora of immune-regulating ligands available, new immunocytokines might be used to foster an antitumor immune response or to inhibit immuno-suppressive activities, e.g., of regulatory T-cells, in OV therapy.[32,151]

In summary, viro-antibody therapy research has established preclinical proof of principle for a panel of OVs, antibody formats and modes of action, and has identified challenges for further research and translation. Importantly, available comprehensive tools and strategies for engineering of both OVs and antibodies will allow researchers to address these challenges toward the development of improved and/or novel antibody-encoding OVs for effective viro-antibody therapy.

List of abbreviations

AAV, adeno-associated virus; Ad, adenovirus; ADC, antibody drug–conjugate; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; BiTE, bispecific T cell engager; DAMPs damage-associated molecular patterns; ECM, extracellular matrix; Fab, antigen-binding antibody fragment; FAP, fibroblast-activating protein; HSV, herpes simplex viruses; IAV, influenza A virus; ICIs, Immune checkpoint inhibitors; Ig, immunoglobulin; IRES, internal ribosome entry site; mAbs, monoclonal antibodies; MDSC, myeloid-derived suppressor cells; MenSC, menstrual blood-derived mesenchymal stem cells; MeV, measles virus; NDV, Newcastle disease virus; OVs, oncolytic viruses; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; scFv, single-chain antibody fragments; TME, tumor microenvironment; VV, vaccinia virus; VSV, vesicular stomatitis virus

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Disclosure statement

G.U. is founder and CMO/CSO of CanVirex AG, a company developing oncolytic measles viruses as (cancer) immunotherapeutics. R.E.K. is co-inventor on patent/-applications covering therapeutic antibodies and proteins and reports licensing agreements and research support from Baliopharm, BioNTech, and Oncomatryx and consultancy fees from Oncomatryx, Roche, and Immatics. D.M.N. declares no conflict of interest.

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