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Oncolytic ImmunoViroTherapy: A long history of crosstalk between viruses and immune system for cancer treatment

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ABSTRACT

Cancer Immunotherapy relies on harnessing a patient's immune system to fine-tune specific anti-tumor responses and ultimately eradicate cancer. Among diverse therapeutic approaches, oncolytic viruses (OVs) have emerged as a novel form of cancer immunotherapy.

OVs are a naturally occurring or genetically modified class of viruses able to selectively kill cancer cells, leaving healthy cells unharmed; in the last two decades, the role of OVs has been redefined to act beyond their oncolytic activity. Indeed, the immunogenic cancer cell death mediated by OVs induces the release of tumor antigens that in turn induces anti-tumor immunity, allowing OVs to act as *in situ* therapeutic cancer vaccines. Additionally, OVs can be engineered for intratumoral delivery of immunostimulatory molecules such as tumor antigens or cytokines to further enhance anti-tumor response. Moreover, OVs can be used in combination with other cancer immunotherapeutic approaches such as Immune Checkpoint Inhibitors and CAR-T cells.

The current review first defines the three main mechanisms of action (MOA) of OVs currently used in cancer therapy that are: i) Oncolysis, ii) OV-induced cancer-specific immune activation, and iii) Exploiting pre-existing anti-viral immunity to enhance cancer therapy. Secondly, we focus on how OVs can induce and/or improve anti-cancer immunity in a specific or unspecific fashion, highlighting the importance of these approaches. Finally, the last part of the review analyses OVs combined with other cancer immunotherapies, revising present and future clinical applications.

Contents

1. Introduction

Oncolytic Viruses (OVs) are a class of viruses that infect and replicate preferentially in cancer cells, leaving the normal cellular component that surrounds the tumor unharmed. The use of viruses for the treatment of malignancies is the result of several observations originating

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in the mid-1880s leukemia patients could who would occasionally go into remission upon influenza infection (Pelner, Fowler, & Nauts, 1958). In the 1950s, virus-based therapies gained momentum and during that time, several pre-clinical and clinical trials were done in an attempt to capitalize on their full potential for cancer treatment. However, it was only with the introduction of recombinant DNA technology in the early 1990s, that enabled the enhancement of both the oncolytic properties and the safety profile of OVs that the virotherapy could provide engineered virus to exploit for cancer therapy. Currently, OVs are an emerging and important therapeutic agent for cancer treatment and several mechanisms of actions (MOA) make them an ideal therapy and here we summarize the main MOA involved in their therapeutic efficacy (Fig. 1).

1.1. Oncolysis: natural or engineered tropism for cancer cells

OVs specifically infect and lyse cancer cells and up to date two approaches, not mutually exclusive, have been exploited for specific targeting of human malignancies. The first approach relies on the natural tropism of viruses for tumors, exploiting either extracellular markers overexpressed in cancer cells or intracellular pathway or tumor-specific immune-avoidance mechanisms (Jayawardena, Poirier, Burga, & Bostina, 2020).

Indeed, viral infection is mediated through the interaction between viruses and cell surface receptors, and in this context, several OVs have been chosen based on the overexpression of their receptors in cancer cells compared to their normal counterparts. For instance, CD155 (also known as PVR) is upregulated in cancer cells as it protects the innate and adaptive immune systems through the interaction with the inhibitor receptor TIGIT on T and NK cells (Kucan Brlic et al., 2019). Tumors expressing CD155 are the ideal target for oncolytic poliovirus that owns a natural tropism for it. Moreover, the receptor CD46 protects malignant cells from complement damage, and the measles virus (Edmonston strain) has been reported to exert its oncolytic activity *via* interacting with this receptor, becoming a therapeutic option for tumors overexpressing CD46 (Dorig, Marcil, Chopra, & Richardson, 1993; Liszewski & Atkinson, 2015). Moreover, the overexpression of HVEM, nectin-1, and nectin-2 in cancer cells makes them more susceptible to oncolytic herpes virus infection such as T-VEC (Fu, Tao, Wang, Cripe, & Zhang, 2018). Likewise, the overexpression of ICAM-I (intracellular adhesion molecules I) and DAF (decay-accelerating factor) in breast cancer, multiple myeloma, and melanoma, have been successfully exploited to target and destroy cancer cells with coxsackievirus, such as CAV21 (Au, Lincz, Enno, & Shafren, 2007; Shafren et al., 2004). Interestingly, vesicular stomatitis virus (VSV) is highly sensitive to type I IFNs, showing attenuated lytic activity in interferon-responsive cells (Stojdl et al., 2003); however, the type I IFN pathway is defective in most types of cancers, unleashing VSV-mediated oncolysis in cancer cells. Additionally, the oncolytic activity of the Newcastle disease virus (NDV) has been reported to be tumor-specific due to defective activation of various antiviral signaling pathways, defects in type I IFN signaling pathway, defects in apoptotic pathways, and activation of RAS signaling (Zamarin & Palese, 2012).

Besides the natural cancer tropism displayed by some OVs, the possibility to genetically modify viruses has opened the opportunity to generate viruses that are both safer and more cancer cell-specific. In this sense, adenoviruses (Ad) have been extensively explored due to their large DNA genome to make them oncolytic. Upon infection, adenoviruses produce E1A proteins that modulate cell cycle progression from G0 or G1 into the S phase. Indeed, E1A binds Retinoblastoma (Rb), releasing E2F from the pre-existing complex E2F-Rb. The free E2F can in turn activate both E2 adenoviral promoter and several cell cycle regulatory genes. As cancer cells are characterized by a mutation in the Rb pathway, adenovirus bearing partially deleted E1A that is defective in binding Rb, lose the capability of replicating in normal cells, and can only replicate in malignant cells with mutations in the Rb pathway (Fueyo et al., 2000). Another interesting approach consists of placing the E1A gene under the control of a cancer-specific promoter. CV706, an OAd in which the E1A has been placed under the control of prostate-specific antigen (PSA) promoter, renders the adenovirus replication and consequently cell killing only in human prostate cancer cells (DeWeese et al., 2001). Another example is KH901 with E1A under the

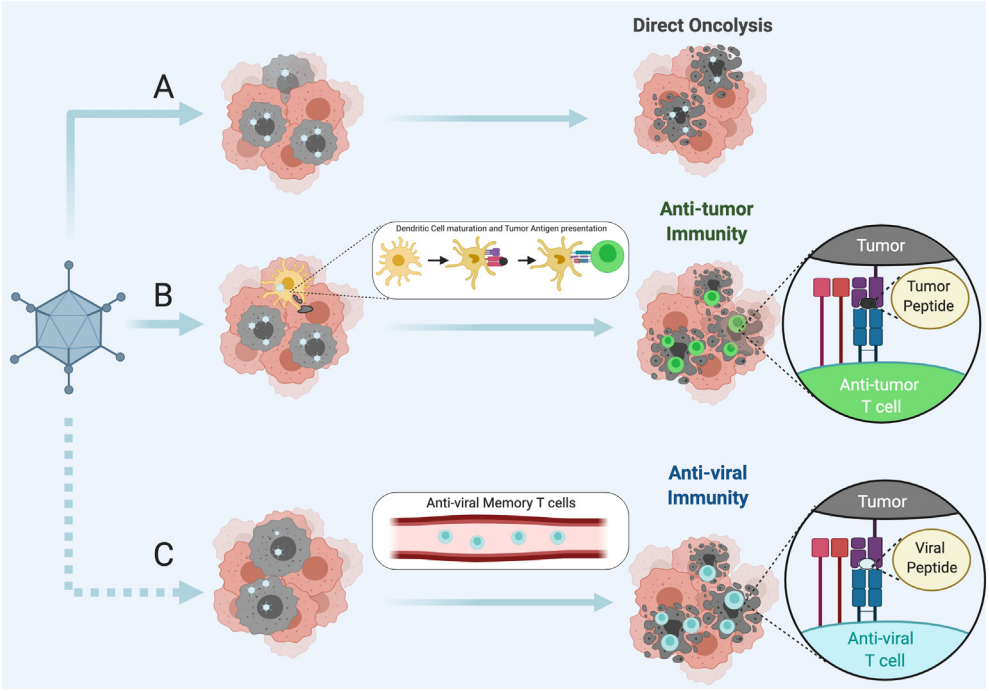

Fig. 1. Classification of Mechanism of Action (MoA) of OVs exploited in cancer therapy are depicted. Here the three main MoA A) Direct Oncolysis, B) OV-induced cancer-specific immune activation, and C) Viral pre-existing immunity are all shown as an emerging tool for cancer therapy.

control of the hTERT promoter, restricting the adenoviral replication to telomerase-positive tumor cells (Chang et al., 2009).

Also, the adenoviral protein E1B has been explored to restrict the viral replication in tumor cells; indeed, E1B usually binds and inactivates p53, preventing apoptosis in normal cells. Two OAd have been engineered to prevent the expression of E1B: ONYX-15 and H101 (Lei et al., 2015; Ries & Korn, 2002). These viruses replicate in and lyse only cancer cells bearing mutations or downregulation of p53, although retaining the capability of infecting normal cells. However, later research showed that ONYX-15 could still replicate in cells with a wild-type p53, showing that the replication was not controlled by p53 and p14 (ARF) (Edwards et al., 2002; Geoerger et al., 2002; Rothmann, Hengstermann, Whitaker, Scheffner, & zur Hausen H., 1998).

Moreover, viruses have been engineered to exploit defective antiviral mechanisms in cancer cells. In this context, HSV-1 has been made oncolytic through the introduction of deletions in ICP 34.5 and US11. Indeed, under physiological conditions, ICP34.5 and US11 prevent PKR activation, a signaling pathway that in presence of viral infection, block the protein synthesis (Poppers, Mulvey, Khoo, & Mohr, 2000). The attenuated virus (the backbone of T-VEC) is, therefore, able to infect and replicate preferentially in cancer cells.

1.2. Beyond oncolysis: exploiting OV-induced cancer-specific immune activation

Besides the direct viral-mediated oncolysis, the anticancer activity of OVs is strictly related to the modulation of the tumor microenvironment (TME), taking advantage of different mechanisms of viral actions.

First, OVs are an ideal and natural platform for the recruitment of CD8+ T cells and the priming of both innate and adaptive immune responses; indeed, following tumor lysis, the released tumor-associated antigens (TAA) and tumor neoantigens (TNA) are made available for the dendritic cells (DCs) to be eaten up and presented on the cellular surface to prime and boost a specific anti-tumor T cell response. Moreover, the massive release of viral epitopes induces the presentation of the antigenic viral epitopes on MHC-I complexes that induce signals in the TME and it can directly activate T cells bypassing the costimulatory signals (Li, Zhang, Gilbert, Conejo-Garcia, & Mule, 2021).

Additionally, OVs promote immunogenic cell death (ICD) (*e.g.*, necrosis, necroptosis, pyroptosis, autophagic cell death and immunogenic apoptosis) with the release of danger-associated molecular patterns (DAMPs) such as surface-exposed calreticulin (ecto-CRT), secreted adenosine triphosphate (ATP) and released high mobility group box 1 protein (HMGB1) (Ylosmaki & Cerullo, 2020). Moreover, pathogen-associated molecular patterns (*e.g.*, DNA, dsRNA, ssRNA, and 5′-triphosphate RNA) are freed following the tumor cells burst, and collectively with the DAMPs, they activate the innate immune system (DCs and NKs) through the interaction with pattern recognition receptors (PRRs). Interestingly, OVs such as vaccinia virus and HSV-1 can also infect and destroy endothelial cells, exerting an antiangiogenic effect, contributing to the overall efficacy of the viruses (Benencia et al., 2005; Breitbach et al., 2013).

Recent evidence has shown that viral infection can convert peripheral tissue into germinal center-containing tertiary lymphoid structures (TLSs) able to function as secondary lymphoid organs (Denton et al., 2019); the mechanism of this conversion is not yet well understood but it depends on type I IFN production upon viral infection with the consequent expression of CXCL13 that can convert non-lymphoid tissue into a functional TLS. From a cancer therapeutic point of view, this could implicate the recruitment of B cells, broadening the humoral response against tumor-specific antigens.

1.3. Exploiting pre-existing Immunity to enhance oncolytic cancer therapy

Patient's pre-existing or treatment-induced immunity to viral components is known to impair OVs systemic delivery, replication, and transgene expression and it is also responsible for premature OV clearance. Moreover, the presentation of viral epitopes on MHC-I molecules triggers the cytotoxic activity of pre-induced anti-viral T cells, converting the immune response towards the virus rather than the tumor. However, recent studies have shown the possibility of exploiting anti-viral/vector immunity to enhance cancer therapeutic approaches, shedding new light in the field. For example, Rica et al. (Ricca et al., 2018), showed a superior therapeutic effect of New Castle Disease virus (NDV) in mice bearing tumors if the mice were previously immunized with NDV compared to naïve mice. The authors highlighted that pre-existing immunity to NDV may have potentiated systemic cytotoxic anti-tumor immune response following intratumoral NDV treatment. Moreover, recent studies suggest that anti-viral T cells may be cross-reactive with tumor-associated antigens homologous to viral peptides, taking actively part in tumor clearance (Fluckiger et al., 2020; Rosato et al., 2019). The mechanism behind this "molecular mimicry" may be related to the intrinsic degeneracy of the T cell receptor (TCR), defined as the ability of a single TCR to recognize more than one antigen, allowing anti-viral memory CD8 + T cells generated by prior infections to recognize unrelated viruses (Welsh & Selin, 2002) (Fig. 2A). Interestingly, Chiaro et al. took advantage of this phenomenon, demonstrating that pre-induced anti-viral TCRs could recognize MHC-I restricted tumor peptides homologous to viral epitopes, controlling the tumor growth in two murine models of melanoma (B16.OVA and B16F10) upon oncolytic cancer vaccine treatment (Chiaro et al., 2021). In the same study, the presence of CD8 + T cells reactive to both Cytomegalovirus (CMV) peptides and CMV-homologous melanoma peptides positively correlated with the prolonged survival of CMV seropositive melanoma patients under immune checkpoint inhibitor (ICI) treatment, confirming a potential role of cross-reactive T cells in anti-cancer immune response (Chiaro et al., 2021). Additionally, Tähtinen et al. showed that pathogen-related CD4 + T cell memory populations can be re-engaged to support cytotoxic CD8 + T cells, converting a weak primary anti-tumor immune response into a stronger secondary-like one; indeed, the authors used tetanus toxoid-preimmunized mice bearing tumors and treated them with an oncolytic virus-based cancer vaccine (PeptiCRAd) coated with peptides that were specific for tetanus and the tumor. The tetanus toxoid-preimmunized mice showed better tumor control compared to the naïve mice. The same data were then validated using polio-boostrix-preimmunized mice, showing that the proposed mechanism of action was not restricted to tetanus, but the principle could be applied to other vaccine formulations as well (Tahtinen et al., 2020). From a cancer therapeutic point of view, as many factors are involved in inducing a spontaneously relevant T cell activation (Cusick, Libbey, & Fujinami, 2012), the stimulation of the molecular mimicry through *ad-hoc* peptides designed in accordance to patient's vaccination history and/or previous pathogen infections is an exciting approach for future therapeutic cancer vaccine development.

Even though several attempts have been made to prevent antibody-mediated neutralization of viruses such as viral re-targeting or chemical modification (Bah, Nace, Peng, Munoz-Alia, & Russell, 2020; Kuhn et al., 2008; Verheije & Rottier, 2012), nowadays the anti-viral humoral response, mainly neutralizing antibodies, can be exploited to defeat malignant cells. In this context, an interesting study focused on viral neutralizing antibodies that could promote MHC-mediated immunogenic antigen presentation *via* the interaction with the intracellular factor TRIM21 (Ng et al., 2019). Indeed, anti-viral antibodies would increase virus uptake both DCs and macrophages mediated, hampering, however, the oncolysis effect; this latter could be far from being a limitation as the role of viral replication in generating a systemic anti-tumor immunity as *in situ* vaccination is still open to discussion (Galivo et al., 2010; Prestwich et al., 2009; Roy et al., 2021). On the other hand, prioritizing APC infection may be an advantage for future therapeutic cancer vaccines (Gromeier & Nair, 2018) (Fig. 2B).

Another approach has taken full advantage of the anti-viral humoral response to redirect the OV-neutralizing antibodies to the tumor site.

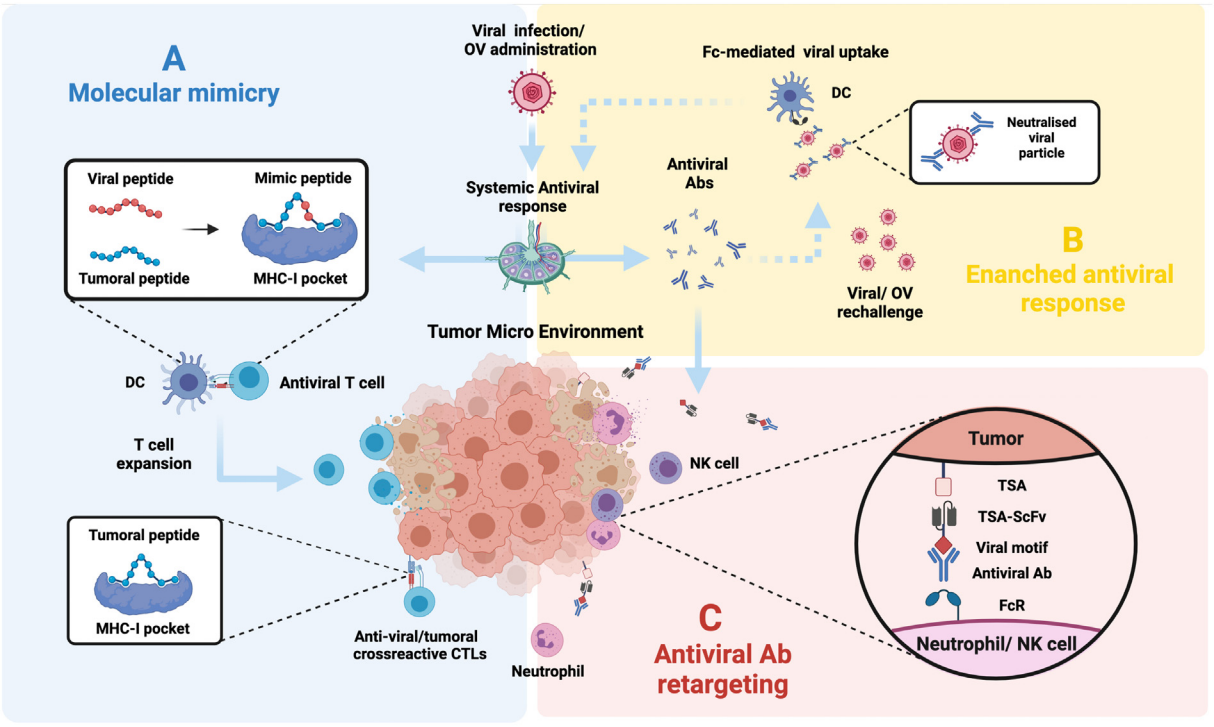

Fig. 2. Pre-existing Immunity to enhance cancer immunotherapy could take advantage of the following mechanisms of actions such as A) Molecular Mimicry B) Enhanced viral uptake in APCs to explore for cancer therapeutic vaccines C) Retargeting of antiviral Ab at the tumor site.

This has been shown by Niemann et al. who developed a bispecific molecule to re-target anti-Ad5 antibodies to polysialic acid (pSia) on the tumor surface; mice pre-immunized with Ad5 or Ad5-based oncolytic virus showed an improved tumor growth control and improved survival compared to naïve mice when treated with the bispecific adapter molecule after the establishment of a syngeneic adenocarcinoma or an aggressive melanoma (Niemann et al., 2019). Thus, Ab-retargeting represents a valid alternative to the TCR-MHC axis to kill cancer cells, as antibodies can mediate cancer cell killing through several mechanisms such as *via* antibody-dependent-cell mediated toxicity (ADCC) or complement-dependent cytotoxicity (CDC) (Fig. 2C). Most importantly, the possibility of exploiting pre-existing anti-viral immunity opens up a new era of OV-based applications in patients that have been either naturally or clinically challenged with multiple viruses during their life.

Despite the initial excitement for the use of OVs as cancer therapy, the impact of OVs as a single agent has unmet the expectations in clinical settings. For instance, the oncolysis can prolong the overall animal survival, but may not be able to initiate an antitumor immunity in advanced tumors (Du et al., 2014). Additionally, the release of a wide repertoire of proteins from cancer cells, as well as the viral immunodominance over the tumor antigens, fail at promoting an anti-tumor effector response of clinical significance, prioritizing instead, to anti-viral immunity (Kaufman, Kohlhapp, & Zloza, 2016). To further enhance the activation of specific anti-tumor immune responses and to overcome the immunosuppressive environment, OVs have been genetically modified to encode tumor antigens or diverse immune modulators. Additionally, several technologies based on decorating OVs with tumor antigens are emerging as well as alternative therapeutic approaches.

2. Oncolytic viruses as cancer vaccines: how to generate a specific anti-tumor response

2.1. OVs encoding tumor antigens

The rising interest in cancer immunology has contributed to making OVs as one of the most promising and versatile platforms for active immunotherapy. Indeed, due to their oncolytic activity, OVs induce the release of tumor-associated antigens (TAAs) and tumor-specific antigens (TSA) from cancer cells; in addition to that, OVs promote the maturation of antigen-presenting cells (APCs) and reverse the immune tolerance to MHC-I/-II-restricted tumor peptides (Delaunay et al., 2018; Gujar & Lee, 2014; Ma et al., 2020). Because of that, several clinical studies are currently evaluating OVs as adjuvants or as single agents for cancer immunotherapy. One strategy is employing OVs expressing tumor antigens (OVs-TA) to further amplify systemic anti-tumor immunity specific for the provided antigen upon intralesional administration, that is, OVs acting as *in situ* vaccination (Fig. 3 (1)).

In a work by Hodge et al., published in 1995, the authors demonstrated that the coadministration of two recombinant Vaccinia viruses (rVVs), expressing the carcinoembryonic antigen (CEA) and the murine CD28, into tumor-bearing mice, induced a detectable CEA-specific T-cell response; additionally, an impaired tumor establishment following the rechallenge suggested an efficient generation of immunological memory. In contrast, the administration of single viruses showed reduced or no effect on tumor growth (Hodge et al., 1995). Moreover, OVs can be armed with multiple antigenic epitopes to counterattack loss or heterogeneous antigenic expression (Olson & McNeel, 2012). For instance, the intranodal (IN) administration of an rVV expressing $gp100_{280–288}$, Melan-A/$MART\text{-}_{127–35}$ and $tyrosinase_{1–9}$ HLA-A0201 restricted epitopes, and CD80 and CD86 costimulatory molecules have successfully promoted a specific cytotoxic T leukocyte (CTL) response to at least one of the epitopes in 7 out of 10 patients with a stage III and IV melanoma (Adamina et al., 2010), showing that OVs-TA are a safe and an ideal tool for anti-cancer vaccination. As most common vaccines against pathogens, also peptide-based vaccines generate a relevant anti-tumor immune memory when included in prime-and-boost regimens. To avoid an unbalanced expansion of anti-viral CTL clones, heterologous prime-and-boost approaches exploit diverse OVs expressing the same TA in a multiple administration schedule (Atherton et al., 2017; Bridle et al., 2010). The effectiveness of this approach has been shown by Pol et al. who developed a vaccination regimen using a genetically modified Maraba virus (MG1) expressing dopachrome tautomerase (DCT or

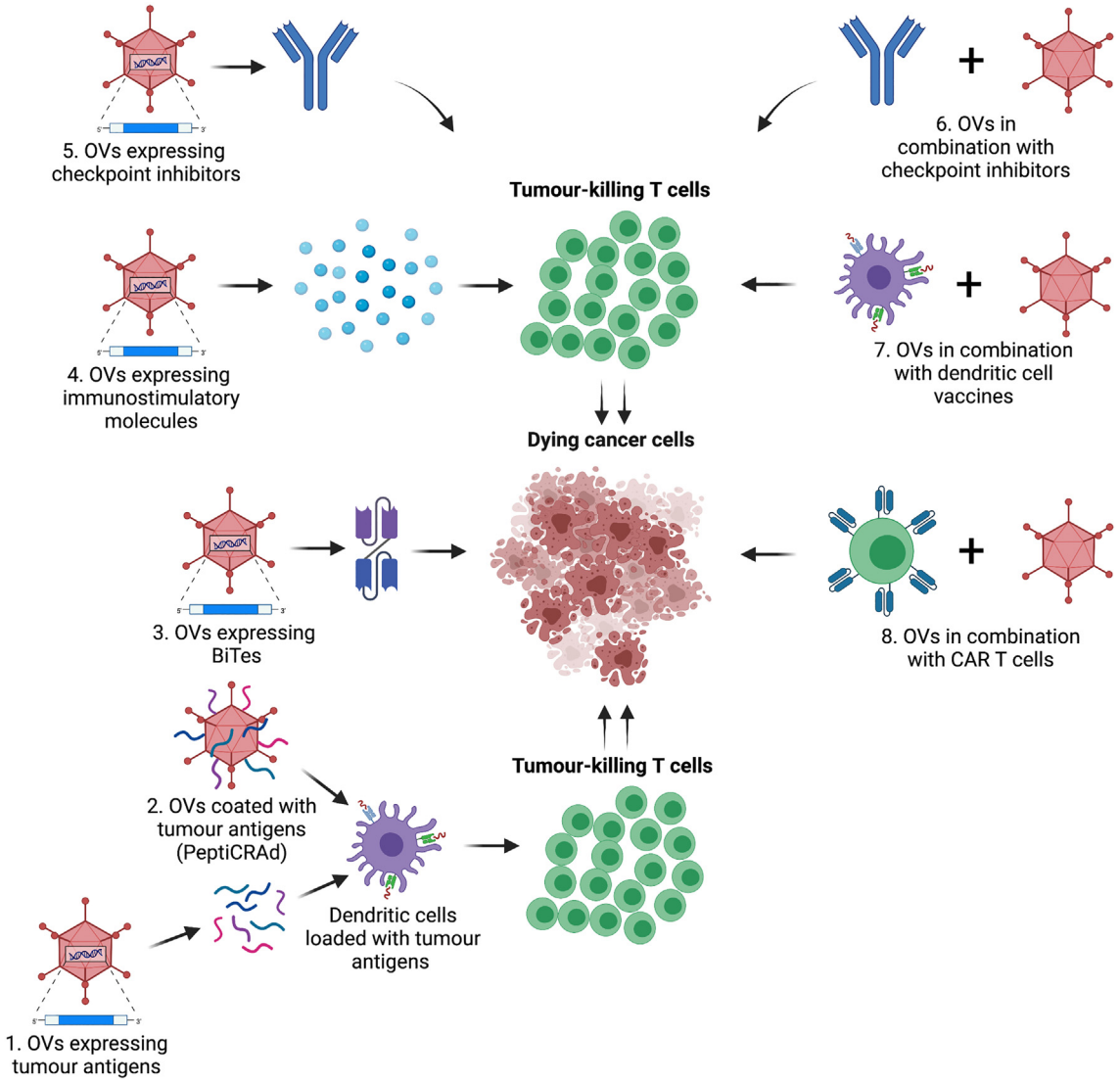

Fig. 3. Various genetically modified OVs as a monotherapy or in combination with cancer immunotherapy to generate and/or support anti-tumor immune responses. Several strategies have been exploited to capitalize on the potential anti-tumor role of OVs. The principal strategies are summarized in the figure. The approaches on the left involve the modification of OVs consisting of 1) OVs expressing tumor antigens; 2) OVs coated with tumor antigens; 3) OVs expressing BiTes; 4) OVs expressing immunostimulatory molecules; 5) OVs expressing checkpoint inhibitors. The right panel involves the combination of OVs with existing cancer therapies such as 6) Checkpoint inhibitors; 7) Dendritic cells vaccines; 8) CAR-T cells.

TRP2) in a B16F10 murine model. While ineffective alone as a priming agent, MG1-DCT was shown to rapidly induce a potent as well as specific T cell response when used as a booster following the administration of a replication-deficient Adenovirus (Ad) expressing the same antigen (Pol et al., 2014), showing that MG1-DCT owned a remarkable ability to boost anti-tumor response.

However, the generation of novel oncolytic vectors encoding one or more transgenes requires deep validation protocols, increasing the time and costs that are not always compatible with patient-tailored medicine approaches; additionally, a robust production and spreading of tumor antigens relies on OVs replication. To overcome this limitation, the use OVs in combination with tumor antigens have been proposed.

2.2. OVs in combination with tumor antigens

Nowadays, fast and high-quality GMP methods for synthetic peptide production are a breakthrough in the vaccine field as they have created the opportunity to use those peptides for cancer therapeutic approaches. Furthermore, as aforementioned, the use of OVs-TA is facing two main limitations: the time and cost of generated OVs-TA according to patient's requirements and the antigenic spreading.

In this context, Cerullo et al. has developed novel oncolytic cancer vaccine platforms based on coating OVs with tumor peptides for personalized cancer treatment approaches (Fig. 3 (2)). Different viruses, such as Ad, VV and HSV-1, have been tested in aggressive murine models of melanoma and triple-negative breast cancer (Capasso et al., 2016; Ylosmaki et al., 2018); the intratumoral administration of peptide-coated OVs was shown to increase anti-tumor specific T cell responses and enhance the control of tumor growth both in injected and non-injected lesions (Feola et al., 2021). Recently, the same group has expanded the concept to bacteria such as Bacillus Calmette–Guérin (BCG), used also as a booster for PeptiCRAd in a heterologous prime- and boost regimen (Ylosmaki et al., 2021).

In a recently published work, Roy et al. have shown that different OVs such as Adenovirus (Ad), Maraba virus (MRB), Vesicular Stomatitis Virus (VSV) and Vaccinia virus (VV), co-administered with synthetic tumor peptides (OVA, DCT, and neoantigen derived peptides) are as efficient as antigen-engineered OVs (OVs-TA) at controlling the tumor

growth in B16F10 and CT26 murine models. Interestingly, in the same study, the authors showed that the magnitude of immune response is independent of viral replication. Indeed, they obtained robust induction of anti-OVA CD8 + T cells by priming with replication-deficient E1/E3-deleted human type 5 Ad (Ad-OVA) and boosting with UV-inactivated MRB expressing OVA (MRB-OVA), suggesting that the viral replication was not required for adjuvanticity (Roy et al., 2021).

Overall, these results imply that using different and readily available MHC-I (and eventually MHC-II) -restricted tumor peptides co-administered with OVs may be a rapid alternative to generate a relevant anti-tumor specific immune response.

These observations further support the use of OVs in peptide-based cancer vaccine treatment. The clear benefit of using OVs and anti-tumor immunity inducing peptides together is the rapid adaptability of the technology by coating the OVs with a new set of tumor antigens, crucially important aspect for personalized cancer vaccines in clinical setting.

Additionally, the combination with multiple synthetic peptides, allowing the production of easily adaptable vaccines to individual patients, elicit a broader response against different tumor structures; furthermore, viral genomes can be engineered to express immunostimulatory molecules to enhance the vector immune-adjuvanticity or the antigenic signal (Ylosmaki et al., 2021). Currently, no clinical trials are evaluating OVs in combination with tumor peptides; however,

2.3. OVs encoding BiTes

Bispecific T cell Engagers (BiTEs) are bispecific antibodies that can cross-link on one side tumor surface antigens while engaging T cells *via* CD3-binding on the other side. In 2015, Blinatumomab (a CD3-CD19 BiTE) achieved the FDA approval for targeting malignant B lymphocytes in Philadelphia chromosome-negative acute lymphoblastic leukemia (pH.R/R B-ALL). However, despite the remarkable success in liquid malignancies, BiTEs have shown reduced efficacy in solid tumors; indeed, solid tumors represent a more challenging scenario for macromolecules to access the TME *via* intravenous delivery. Moreover, the expression of BiTE-recognized antigens in healthy tissues may cause severe 'on-target, off-tumor' T cell-mediated systemic toxicity (Pishvaian et al., 2016). To overcome these limitations, intratumorally-delivered OVs-expressing BiTEs (OV-BiTEs) based on VV, Adv (EnAdenotucirev (EnAd) and ICOVIR-15 K) and Measles Virus (MV) have been tested in multiple murine tumor models, showing local transgene secretion while avoiding systemic toxicity (Fig. 3 (3)). Moreover, OVs are a natural platform to increase CTL infiltration in TME that in turn it allows the BiTEs to work more efficiently.

Importantly, OVs encoding BiTEs or Tri-specific T cell Engagers (TriTEs) provide superior antitumor activity over OVs alone or locally delivered BiTEs (Fajardo et al., 2017; Speck et al., 2018; Yu et al., 2014). Interestingly, while OV replication may be responsible for enhanced transgene activity, Speck et al. observed similar therapeutic benefits when comparing a replication competent MV-mCD3-CD20 with the UV-inactivated counterpart in fully immunocompetent $CD20^{+/+}$ tumor-bearing mice, suggesting a non-significant role for direct oncolysis when using MV (Speck et al., 2018).

The second-generation BiTE constructs aim to direct T cell-mediated cytotoxicity to other leading actors in the TME (de Sostoa et al., 2019; Goebeler & Bargou, 2020; Khanali, Azangou-Khyavy, Boroomand-Saboor, Ghasemi, & Niknejad, 2021). Tumor-associated macrophages (TAMs) or cancer-associated fibroblasts (CAFs) constitute the tumor-supporting stroma, responsible for immunosuppressive cytokine release and checkpoint molecule, such as PD-L1 expression. In this context, due to their TME remodeling ability, OV-BiTEs represents a valuable solution to compensate for on-tumor approaches shortcomings. For example, Scott et al. designed and tested a EnAd-encoding TriTE (CD3-CD3-CD206/ folate receptor β (FRβ)) to target TAMs in malignant ascites; in this work, upon activation through the encoded TriTE ascites, T cells killed preferentially M2 over M1 macrophages in 4 out of 5 cancer patient samples (Scott et al., 2019). Furthermore, the same group engineered the same viral vector to express a fibroblast activation protein (FAP)-targeted BiTE to target CAFs in primary malignant ascites samples. Surprisingly, besides affecting the targeted population (engaged T cells- BiTE effect) and cancer cells (OV effect), the treatment induced macrophage repolarization towards M1-like phenotype. Notably, the increased expression of genes involved in cytotoxicity, lymphocyte functions, and pathogen defense within ascites immune cells suggests a broader immune-stimulating effect than provided by the BiTE-engaged T cells and oncolytic activity (Freedman et al., 2018). As aforementioned, OVs create feasible conditions for endogenous T cell activity within the TME; similarly, OV-BiTEs synergize with adoptive cell therapies (Fajardo et al., 2017; Goebeler & Bargou, 2020; Yu et al., 2014). Indeed, OV-BiTEs in combination with CAR T cells have been tested so far in xenograft models of colorectal and pancreatic cancers, showing enhanced intratumoral CAR T cell accumulation and activity (Fajardo et al., 2017; Wing et al., 2018). Interestingly, Porter et al. have recently evaluated the versatility of the platform by integrating a CD44v6-targeted BiTE within a scFv-antiPDL-1 and IL-12 -encoding high-capacity Adv (alternatively, helper-dependent Adenovirus, HDAdv) for enhanced HER.2.CAR-T activity against HER2+/+ and HER2−/− solid tumors *in vivo* (Porter et al., 2020). These promising results represent a crucial step for the clinical translation of adoptive T cell therapies in solid cancer settings.

In conclusion, one scenario to unravel the real potential of OVs-BiTEs maybe be the engagement of engineered specific antitumor cells (*e.g.*, CAR T cells); however, despite the excitement, further studies are needed for making this approach a clinical reality. Another resolution could be combining OV-BiTEs with therapeutic cancer vaccines to generate a pool of endogenous ready-to-kill antitumor T cells, exploiting in this way the full BiTEs' activity in future applications.

3. Arming OVs

As aforementioned, the generation of OVs encoding immunostimulatory molecules have been exploited to both support antitumor immune responses and to overcome the immunosuppressive TME. In this context, the most successful example is an oncolytic herpes simplex virus 1 (HSV-1) called T-VEC, the first OV approved by EMA in Europe and by FDA in the United States for the treatment of advanced melanoma (Rehman, Silk, Kane, & Kaufman, 2016). Indeed, T-VEC is a HSV-1 encoding human granulocyte-macrophage colony-stimulating factor (GM-CSF), which helps to promote the priming of T cell responses. Following the successful example of T-VEC, the generation and application of OVs encoding immune modulators have gained momentum in recent years. Indeed, the literature reports several examples regarding to the introduction of immunomodulators into viral vectors that increases responsiveness to anti-cancer treatment. In particular, cytokines, costimulatory molecules and immune checkpoint inhibitors have been exploited to engineer OVs.

Here, we review the current genetically modified OVs that are engineered to encode immune modulators and how these OVs have been used in the field of cancer immunotherapy.

3.1. Arming OVs with cytokines and Immunostimulatory molecules

Cytokines are a group of small polypeptides or glycoproteins (molecular weight 5–20 KDa) involved in growth, differentiation, inflammatory or/and anti-inflammatory signals in different cell types (Berraondo et al., 2019). Moreover, cytokines stimulate and regulate the immune system, and because of that, they have been exploited to potentiate anti-tumor responses, becoming among the first immunotherapeutic approaches for cancer treatment. For instance, IL-2 have been approved by the FDA for the treatment of renal cell carcinoma (RCC) and metastatic melanoma and interferon alpha (INF-α) was approved for the

treatment of hairy cell leukemia, follicular non-Hodgkin lymphoma, melanoma, and AIDS-related Kaposi's sarcoma (Berraondo et al., 2019; Fyfe et al., 1995). However, the systemic administration of cytokines has been related to severe toxicity, giving rise to major concerns and limitations for their use (Donnelly, Young, & Rosenberg, 2009). In this context, arming OVs with cytokines overcome this disadvantage as the OVs mediate *in-situ* delivery of the cytokine, producing it at high concentration within the tumor bed (Fig. 3 (4)).

3.2. GM-CSF

GM-CSF is one of the most exploited transgenes for arming oncolytic viruses because of its pleiotropic functions; indeed, GM-CSF induces myeloid precursor cells to proliferate and differentiate into neutrophils, monocytes, macrophages, and eosinophils; moreover, GM-CSF recruits and stimulates DCs and NK cells with the induction of tumor specific CD8+ T cells. The combination of GM-CSF with OV therapy has been proven being particularly effective. Indeed, following the immunogenic cell death induced by the viral replication, the release of tumor antigens provides *in situ* patient-specific anti-tumor response enhanced in presence of GM-CSF (Malhotra et al., 2007).

In this context, HSV-1 was the first oncolytic virus armed with GM-CSF and it was successfully used to lyse several human tumor cell lines, including melanoma (Rehman et al., 2016). Interestingly, in the murine model A20 both virus encoding GM-CSF and unarmed control virus inhibited the tumor growth of injected lesion; however, only HSV-1 encoding GM-CSF was able to control also the not injected contralateral tumor growth, showing the potential of arming an OVs with immunostimulatory molecules to induce systemic anti-tumor response beyond the oncolysis (Rehman et al., 2016). Afterwards, this virus was named Talimogene laherparepvec and evaluated for clinical applications eventually also in combination with immune checkpoint inhibitors (Ribas et al., 2018). In addition to T-VEC, several other oncolytic viruses have been armed with GM-CSF such as JX-594/PexaVEC (vaccinia virus) (Cripe et al., 2015), CG0070 (oncolytic adenoviruses) (Ramesh et al., 2006) and ONCOS-102 (oncolytic adenoviruses) (Kuryk et al., 2018).

3.3. IL-12

Under physiological conditions, IL-12 is secreted by antigen presenting cells (APCs) mostly DCs and macrophages) in response to bacteria, bacterial products, or intracellular parasites. IL-12 is a pleiotropic cytokine and among its functions, there are (i) Th1 differentiation, (ii) increased activation and cytotoxicity activity of T and NK cells, (iii) inhibition and/or reprogramming of immunosuppressive cells, (*i.e.*, tumor associated macrophages (TAM) and myeloid-derived suppressor cells (MDSCs)). Additionally, IL-12 stimulates the production of IFNγ that in turn has cytostatic/cytotoxic/ anti-angiogenic effects and upregulates MHC I and MHC II level for enhanced recognition and lysis of cancer cells (Nguyen et al., 2020; Toda, Martuza, Kojima, & Rabkin, 1998).

All together these features have made IL-12 as an attractive molecule to be used in cancer immunotherapy; however, the clinical use of IL-12 has been limited by severe toxicity when administered systemically. Therefore, taking advantage of the local delivery of transgenes by OVs, several OVs have been armed with IL-12. For instance, an oncolytic HSV-1 armed with IL-12 showed robust anti-tumor activity in a murine squamous cell carcinoma model (Wong et al., 2001).

Additionally, an HSV-1 encoding IL-12 exhibited tumor growth control and increased immune cell infiltration in murine models of prostate cancer, compared to both unarmed HSV-1 and HSV-1 encoding GM-CSF, demonstrating the advantage of combining immunoregulation and antiangiogenic effect mediated by IL-12 (Varghese et al., 2006). Likewise, an oncolytic adenovirus armed with IL-12 has been shown to improve local tumor growth control in a preclinical model of prostate cancer (Freytag, Barton, & Zhang, 2013); however, the data reported from the same group upon conclusion of phase I study indicated that treatment was well tolerated in patients, but not clinical benefits were reported (Barton et al., 2021). Overall, these data showed that OVs armed with IL-12 are potentially a valid tool to achieve better tumor growth control.

3.4. IL-2 and TNF-α

IL-2 is a 15.5 KDa cytokine involved in stimulation, proliferation and survival of T and NK cells. IL-2 is among the first example of immunotherapeutic drugs approved for the treatment of metastatic melanoma and renal cancer, however alongside with various other cytokines, the systemic administration of IL-2 was associated with severe side effects in humans (Rosenberg, 2014; Schwartz, Stover, & Dutcher, 2002). Therefore, local administration has been preferred and several OVs have been engineered to express IL-2. For instance, NDV (Newcastle Disease virus) encoding IL-2 was shown to be effective in generating tumor-specific cytotoxic T cells in a pre-clinical model of melanoma and hepatocellular carcinoma (HCC); interestingly, the use of NDV-IL-2 in those tumor models was also able to generate immune memory T cells, as demonstrated by long lasting anti-cancer immune protection upon tumor rechallenge (Bai et al., 2014). These conclusions were confirmed by Zamarin et al. in a malignant model of melanoma (Zamarin, Vigil, Kelly, Garcia-Sastre, & Fong, 2009) or using an adenovirus backbone for the IL-2 expression (Slos, De Meyer, Leroy, Rousseau, & Acres, 2001) for the treatment of a mastocytoma murine model, paving the way to future clinical applications. Like IL-2, Tumor Necrosis Factor alpha (TNF-α) was already in use in the ´80s for the treatment of melanoma; usually secreted by macrophages/monocytes during acute inflammation, TNF-α mediates several signaling pathways that lead to necrosis or apoptosis (Idriss & Naismith, 2000). Interestingly, the use of a soluble TNF-α is associated with promotion of M1 anti-tumor polarization in TAM population and attraction and stimulation of neutrophils and monocytes in the site of anti-tumor response (Josephs et al., 2018). Exploiting TNF-α mediated anti-tumor effects, several OVs have been modified to express TNF-α in order to enhance cell death and host immune system activation. Hirvinen et al. armed an oncolytic adenovirus Ad5/3-D24 with TNF-α and upon administration, the TNF-α expressing Ad showed increase in OVA-specific cytotoxic T cells in the poorly immunogenic model of melanoma B16.OVA (Hirvinen et al., 2015). Moreover, vesicular stomatitis virus (VSV) armed with TNF-α was able to significantly enhance tumor growth control in a murine model of mammary cancer EMT6 and most importantly in combination with SMCs (inhibitors of inhibitor of apoptosis proteins) was able to abrogate tumor blood perfusion through collapse of the neovascularate within the tumor bed (Beug et al., 2018). Interestingly, a TILT-123 (onocolytic adenovirus) armed with both IL-2 and TNF-α completely eradicate the tumor in a hamster model of pancreatic cancer (HapT1) when combined with TIL transfer, paving the way to combination therapy with adoptive cell therapy (Havunen et al., 2017).

3.5. OX40L and CD40L

Virus-mediated lysis of tumor cells releases tumor associated antigens and neoantigens, acting as *in-situ* cancer vaccine. However, the generation of specific anti-tumor T cells remain modest and can be further enhanced by the local expression of costimulatory molecules such as CD40L and OX40L (Zamarin & Pesonen, 2015). The former is a protein usually expressed on activated CD4 + T cells, B and NK cells able to license APCs (macrophages and DCs) to prime CD8 + T cells response (Croft, So, Duan, & Soroosh, 2009), activating the innate arm of the immune system; the latter, instead, act in the adaptive immune response, regulating several T cells functions through the engagement of OX40 receptor, such as cytokine production, expansion, survival, and generation of memory CD8+ T cells. OVs armed with CD40L and/or OX40L has been extensively explored (Ishii, Takahashi, Soroosh, & Sugamura,

2010). For instance, a Vaccinia Virus (VV) armed with CD40L showed tumor control in a murine model of melanoma and cancer cell apoptosis. Additionally, the use of VV encoding CD40L induced infiltration of NK cells and DCs to the tumor site (Parviainen et al., 2014). Another important feature of CD40L is the induction of Th1 immunity as showed by Diaconu et al. The authors reported increased expression of IFN-γ, TNF-α and RANTES, characteristic of a skewed Th1 response, in a murine model of urothelial carcinoma upon treatment with OAd encoding CD40L (Diaconu et al., 2012). Moreover, an OAd encoding OX40L (namely Delta-24-RGDOX) was successfully used for the treatment of mouse glioma tumor, enhancing the generation of tumor specific CD8+ T cells and causing specific immune memory (Jiang et al., 2017). Interestingly, the same virus was effective also against disseminated melanoma, showing the ability of generating effector CD8+ T cells both in injected tumor site and peripheral blood. Combination of both molecules has also been explored in order to take full advantage of licensing APCs through CD40/CD40L signaling pathway to drive CD8 + T cell responses that in turn is increased and sustained by OX40/OX40L interaction. This approach has been tested in an oncolytic cancer vaccine, in which the OAd encoding OX40L and CD40L was decorated with a model antigen SIINFEKL. In this work, the authors showed that the specific anti-tumor T cell response generated through their platform was enhanced and sustained in presence of immunostimulatory molecules (Ylosmaki, Ylosmaki, et al., 2021). Another interesting approach was exploited by Eriksson et al. In this work the authors engineered an OAd (LOAd703) to express CD40L and 4-1BBL. 4-1BBL is another tumor necrosis factor receptor family ligand, involved in stimulation of T cell expansion, acquisition of effector function, survival and development of T cell memory. LOAd703 was shown to be effective in *in vitro* assays to act as a potent immune activator that modulate the stroma facilitating the lymphocyte attachment and transmigration (Eriksson et al., 2017).

3.6. Arming OVs with immune checkpoint inhibitors

Cancer immunotherapy has made a tremendous breakthrough with the introduction of antibodies targeting immune checkpoints (ICI) such as CTLA-4, PD-1 and PDL-1 that unleash the breaks of the immune system, reviving and boosting the effector function of specific anti-tumor T cells (Pardoll, 2012). However, the clinical response is not yet satisfactory, reaching a response rate of 15–30% in most solid tumors and 45–60% in melanoma and in MSI-H (Microsatellites Instability High) tumors (Das & Johnson, 2019). The concomitant blockade of CTLA-4 and PD-1/PDL-1 has been shown to enhance the overall therapeutic response, but it also substantially increases the toxicity to patients from 16.3% (anti-PD-1 alone), 27.3% (CTLA-4 alone) to 55% (Seidel, Otsuka, & Kabashima, 2018). Additionally, ICIs therapy modulates a pre-existing anti-tumor response and therefore they are ineffective against immunologically "cold" tumors, characterized by low tumor infiltrating lymphocytes (TILs) (Hwang, Hong, & Yun, 2020). In this context, OVs has been proposed as the ideal candidates to synergize with ICIs. Indeed, OVs modulate the TME by turning "cold" tumors to "hot", increasing the cancer susceptibility to ICIs; moreover, the use of OVs encoding ICIs leads to localized antibody release, representing a safer option in comparison to systemic administration that has been associated with adverse events, mainly autoimmune related symptoms. Therefore, several OVs have been engineered to encode ICIs (Fig. 3 (5)). For instance, an OAd (SKL002) armed with anti-CTLA-4 antibody showed tumor growth control in a murine melanoma model (B16F10); most importantly no lung metastases were detected in mice treated with SKL002 compared to the untreated groups, demonstrating that the SKL002 induced abscopal effects (Du et al., 2014). In addition to these results, measles virus (MV) engineered to express antibody against PD-L1 (MV-aPD-L1) or CTLA-4 (MV-aCTLA-4) controlled tumor growth in a murine model of melanoma through modulation of the immune components within TME (Engeland et al., 2014); in particular, the authors in this work demonstrated that the localized release of ICIs induced increase infiltration of CD3+ T cells and decrease of FoxP3+ T cells at the tumor site; also, MV-aPD-L1 treatment increased cytotoxic CD8 + T cells and activated IFN-γ + expressing CD8 + T cells at the tumor site. These results were confirmed by Kleinpeter et al. (Kleinpeter et al., 2016). In this study, a modified a vaccinia virus was engineered to express an anti-PD-1 antibody and in addition to the immunological analysis, they demonstrated that the use of OVs encoding ICI is as efficient as the combination of unarmed virus with anti-PD-1 antibody, in terms of effect on tumor growth and survival in a preclinical model of melanoma. The clear advantage consists in local expression of the ICI at the tumor site, avoiding systemic administration with increased risk of adverse events. These were further investigated with an oncolytic myxoma (MYXV) virus encoding anti-PD-1 antibody; in both murine models of melanoma (B16F10) and lung cancer (LLC), either the use of MYXV encoding anti-PD-1 or the combination of an unarmed MYXV together with PD-1 blockade, controlled the tumor growth; however, the combination therapy was associated to alopecia that in contrast was severely reduced in the mice treated with MYXV armed with anti-PD1 (Bartee, Dunlap, & Bartee, 2017). Finally, Hamdan et al. (Hamdan et al., 2021) developed an interesting approach to reduce the ICI toxicity meanwhile taking full advantage of the effector mechanisms of an antibody against PD-L1. In this work, an OAd (Ad-Cab) was armed with a fusion peptide consisting of a chimeric Fc region of an IgA1 and IgG1 which is connected to a PD-1 ectodomain *via* a GGGS linker. The authors showed that the hybrid antibody could capitalize on the effector mechanisms of an IgG1 (activation of complement and NK) and of an IgA1 (activation of monocytes, macrophages and neutrophils) enhancing cancer cytotoxicity in various murine models and patients derived tumor organoids (PDOs).

Overall, the use of OVs armed with ICIs have shown great potential and the strategy could soon revolutionize the way we treat cancer.

4. OVs in combination with other cancer therapies

4.1. OVs in combination with immune checkpoint inhibitors

Immune checkpoint inhibitors (ICIs) have revolutionized the treatment of cancer, however, the number of patients responding to ICIs have remained modest (Schoenfeld & Hellmann, 2020). Patients responding to ICIs seem to have pre-existing anti-cancer immunity that ICIs can render effective (Fourcade et al., 2010) (Yuan et al., 2011). As OVs can effectively modulate and induce immune cell infiltration into the TME (Breitbach, Lichty, & Bell, 2016), there is a strong rationale for combining OVs with ICIs (Fig. 3 (6)). We and others have recently shown that OVs can increase the number of responders to ICI therapy in preclinical setting (Cervera-Carrascon et al., 2020; Cervera-Carrascon et al., 2021; Havunen et al., 2021; Kim et al., 2021; Lee et al., 2020; Liu et al., 2020; Lou et al., 2021; Martikainen et al., 2021; Masemann et al., 2021; Nakatake, Kuwano, Kaitsurumaru, Kurosaki, & Nakamura, 2021; Panagioti et al., 2021; Puigdelloses et al., 2021; Ylosmaki, Ylosmaki, et al., 2021). Initial data on early phase clinical trials combining OVs and ICIs have already been reported. The first reported phase 1b trial combined T-VEC and ipilimumab in previously untreated, unresectable stage IIIB-IV patients with melanoma (Puzanov et al., 2016). The authors of the study reported that the combination therapy group (T-VEC + ipilimumab) appeared to have greater efficacy than either T-VEC or ipilimumab monotherapies, with objective response rate of 50%. These results were later confirmed in a phase II study evaluating the efficacy and safety of T-VEC plus ipilimumab against ipilimumab alone in patients with advanced, unresectable melanoma (Chesney et al., 2018). In this study, 39% of patients in the combination arm and 18% of patients in the ipilimumab monotherapy arm had an objective response. Importantly, responses were not limited only to injected lesions since visceral lesion decreases were observed in 52% of patients

in the combination arm and 23% of patients in the ipilimumab monotherapy arm (Chesney et al., 2018). T-VEC has also been clinically tested in combination with pembrolizumab. In the first reported phase 1b trial testing T-VEC in combination with pembrolizumab in 21 patients with advanced melanoma, the confirmed objective response rate was 62% with a complete response rate of 33% (Ribas et al., 2017). Long-term analysis of this phase 1b trial showed that at nearly 5 years of follow-up, median progression free survival and overall survival were not reached for the patients in the combination arm. In addition, 92% of the responders remained in response at this follow-up time point (Fluckiger et al., 2020). An ongoing phase 3 clinical trial is currently comparing T-VEC plus pembrolizumab against placebo plus pembrolizumab in patients with stage IIIB-IV melanoma (NCT02263508). In addition to T-VEC, multiple OVs are being tested in combination with various different ICIs (see Table 1 for more information).

4.2. OVs in combination with other emerging immunotherapies

Recently, OVs have been combined with dendritic cell-based cancer vaccines (Komorowski, Tisonczyk, Kolakowska, Drozdz, & Kozbor, 2018; Koske et al., 2019; Zafar et al., 2018) (Fig. 3 (7)). Zafar et al. armed an oncolytic adenovirus with a CD40 ligand that can engage with CD40 receptor expressed on the surface of dendritic cells. CD40L engagement on the surface of dendritic cells licenses dendritic cells to mature and to trigger immune responses. By combining virally expressed CD40L-induced dendritic cell activation with tumor cell lysate-pulsed dendritic cell therapy, the authors showed significantly increased tumor growth control and survival over the single agent therapies (Zafar et al., 2018). The strategy of combining OVs and DC cancer vaccines has now entered into early clinical trials; intratumoral injection of autologous CD1c (BDCA-1)$^+$ myeloid DCs together with T-VEC is being tested in patients with non-visceral metastases of melanoma (NCT03747744).

OVs have also been shown to have synergistic effects with adoptive cell therapies (ACT) and preclinical studies suggest that the safety of ACT might be increased by using OVs to replace lymphodepleting preconditioning with high-dose chemotherapy (Cervera-Carrascon et al., 2018; Havunen et al., 2017). Havunen et al. used an oncolytic adenovirus armed with human interleukin 2 (IL-2) and tumor necrosis factor alpha (TNF-α) combined with tumor-infiltrating lymphocyte (TIL)

Table 1
Selected ongoing clinical trials with OVs.

OV	Transgene	In combination with	Indication	Clinical Phase	Identifier
T-VEC (HSV-1)	GM-CSF	Atezolizumab	Early breast cancer	Exploratory study	NCT03802604
T-VEC	GM-CSF	Pembrolizumab	Metastatic and/or locally advanced sarcoma	Phase II	NCT03069378
TVec	GM-CSF	Pembrolizumab	Melanoma	Phase II	NCT04068181
T-VEC	GM-CSF	Atezolizumab	Triple negative breast cancer and colorectal cancer with liver metastases	Phase Ib	NCT03256344
T-VEC	GM-CSF	Pembrolizumab	Melanoma	Phase II	NCT02965716
T-VEC	GM-CSF	Nivolumab	Sarcoma	Phase II	NCT03886311
T-VEC	GM-CSF	Pembrolizumab	Melanoma	Phase II	NTC03842943
T-VEC	GM-CSF	Pembrolizumab	Liver tumours	Phase Ib/II	NCT02509507
T-VEC	GM-CSF	Myeloid dendritic cells	Melanoma	Phase I	NCT03747744
Pexa-Vec; JX-594 (VV)	GM-CSF	Nivolumab	Hepatocellular carcinoma	Phase I/IIa	NCT03071094
Pexa-Vec; JX-594	GM-CSF	Ipilimumab	Metastatic/advanced solid tumours	Phase I	NCT02977156
Pexa-Vec; JX-594	GM-CSF	Durvalumab and/or Tremelilumab	Colorectal cancer	Phase I/II	NCT03206073
DNX-2401 (Ad)	None	Pembrolizumab	Brain cancers	Phase II	NCT02798406
ONCOS-102	GM-CSF	Durvalumab	Advanced peritoneal malignancies	Phase I/II	NCT02963831
Ad-MAGEA3 (Ad) MG1-MAGEA3 (MRB)	Melanoma-associated antigen 3	Pembrolizumab	Melanoma or cutaneous squamous cell carcinoma	Phase Ib	NCT03773744
Ad-MAGEA3 MG1-MAGEA3	Melanoma-associated antigen 3	Pembrolizumab	Non-small cell lung cancer	Phase I/II	NCT02879760
TBio-6517 (VV)	Flt3 ligand, anti-CTLA-4 antibody and IL-12	Pembrolizumab	Triple negative breast cancer or microsatellite stable colorectal cancer	Phase I/IIa	NCT04301011
ASP9801 (VV)	IL-7 and IL-12	—	Metastatic/advanced solid tumours	Phase I	NCT03954067
LOAd703 (Ad)	CD40L and 4-1BBL	Gemcitabine and nab-paclitaxel +/- Atezolizumab	Pancreatic cancer	Phase I/IIa	NCT02705196
LOAd703	CD40L and 4-1BBL	Atezolizumab	Malignant melanoma	Phase I/II	NCT04123470
LOAd703	CD40L and 4-1BBL	—	Pancreatic adenocarcinoma, ovarian cancer, biliary carcinoma and colorectal cancer	Phase I/II	NCT03225989
LOAd703	CD40L and 4-1BBL	Multiple immunotherapy-based treatment combinations	Metastatic colorectal cancer	Phase Ib/II	NCT03555149
OH2 (HSV-2)	GM-CSF	—	Pancreatic cancer	Phase Ib/II	NCT04637698
TILT-123 (Ad)	TNFα and IL-2	—	Advanced solid tumors	Phase I	NCT04695327
TILT-123	TNFα and IL-2	Adoptive T cell therapy	Metastatic melanoma	Phase I	NCT04217473
CAdVEC (OAd and non-replicating Ad vector)	IL-12p70, anti-PD-1 mini-antibody and HSVtk	HER2.CART cells	HER2-expressing solid tumours	Phase I	NCT03740256
MV-NIS (MV)	Thyroidal sodium iodide symporter	—	Recurrent medulloblastoma or recurrent atypical teratoid rhabdoid tumours	Phase I	NCT02962167
MV-NIS	Thyroidal sodium iodide symporter	—	Recurrent or Metastatic Squamous Cell Carcinoma of the Head and Neck Cancer or Metastatic Breast Cancer	Phase I	NCT01846091
MV-NIS	Thyroidal sodium iodide symporter	—	Bladder cancer	Phase I	NCT03171493
MV-s-NAP (MV)	Helicobacter pylori Neutrophil-activating Protein	—	Invasive metastatic breast cancer	Phase I	NCT04521764

therapy and showed that the combination therapy was able to cure 100% of treated tumor-bearing animals. In addition, cured animals were protected against tumor rechallenge, an indication of a systemic anti-tumor memory response (Havunen et al., 2017). This combination therapy is currently being tested in a phase I clinical trial in metastatic melanoma patients (NCT04217473).

4.3. OVs in combination with CAR T cells

Chimeric antigen receptor (CAR) T cell therapies use genetically engineered autologous T cells that can identify tumor cells in a non-MHC-restricted manner (Wu, Wei, Brzostek, & Gascoigne, 2020). CAR T cell therapies have shown significant clinical impact in patients with leukemia or lymphoma, but the clinical impact against solid tumors have been limited, in part, for the highly immunosuppressive TME (Guedan & Alemany, 2018). Since OVs can revert the immunosuppression in the TME, OVs have the potential to synergize with CAR T cell therapies (Fig. 3 (8)). Nishio et al. developed an oncolytic adenovirus encoding a chemokine RANTES and a cytokine IL-15 (Ad5Δ24.RANTES.IL15) to enhance trafficking and survival of T cells, respectively, and combined it with CAR T cells targeting the GD2 antigen expressed by neuroblastoma cells (GD2.CAR-T cells) (Nishio et al., 2014). They demonstrated that intratumoral treatment with Ad5Δ24.RANTES.IL15 increased the number of tumor-infiltrating T cells and when combined with GD2.CAR-T cells, enhanced tumor growth control and prolonged survival was observed. Moon et al. used an oncolytic vaccinia virus encoding CXCL11, a chemokine that attracts T cells into tumor site, in combination with mesothelin-targeted CAR T cells (Moon et al., 2018). Interestingly, they showed that intravenous administration of both therapies resulted in increased intratumoral CAR T cell infiltration and significant tumor growth control in mice bearing mesothelin-transduced TC1 tumors. Recently, Shaw et al. combined an oncolytic adeno-immunotherapy (comprised of an OAd and a helper-dependent adenoviral vector (HDAd) encoding human interleukin 12p70 (hIL-12p70), PD-L1 blocking mini-antibody, and herpes simplex virus thymidine kinase (HSVtk) safety switch expression cassettes) with HER2. CART cells for the treatment of pancreatic cancer (Rosewell Shaw et al., 2021). They showed that the oncolytic adeno-immunotherapy combined with CAR T cell therapy was highly effective in eradicating established solid tumors, and most importantly, the combination therapy was also efficient in controlling the growth of distant, untreated tumor sites. This combination therapy is currently being tested in a first in human Phase I clinical study in patients with HER2-positive tumors (NCT03740256).

In addition to arming OVs with cytokines or chemokines to attract T cells into tumor site, OVs can also be used to tag cancer cells for CAR T cell-targeted destruction. Park et al. engineered an oncolytic vaccinia virus to express a nonsignaling, truncated variant of CD19 (CD19t) protein that enables the use of CD19-specific CAR T cells (originally approved by the US FDA for the treatment of B-cell-derived hematologic malignancies) with multiple tumor types (Park et al., 2020). Similarly, Tang et al. engineered an oncolytic adenovirus to express CD19t to be used in combination with CD19-specific CAR T cells (Tang et al., 2020). Both studies elegantly established that OVs can be used to deliver CAR T cell targets to solid tumors broadening the applicability of CAR T cell therapies to solid tumors lacking tumor-restricted and/or homogenously expressed tumor antigens.

5. Conclusion and future perspectives

The use of OVs has radically changed the face of cancer treatments, emerging as important immunotherapeutic agents. Indeed, OVs provide the unique advantage of targeting and lysing solely cancer cells as part of their replication cycle, meanwhile stimulating the immune attack. Additionally, the development of DNA recombinant technologies and diverse oncolytic cancer platforms have capitalized on the immune response to shift the paradigm from an “antiviral” to “anti-tumor-specific” response. Moreover, the combination of OVs with other immunotherapeutic approaches such as ICI has the potential to improve the outcome in patients; indeed, T-VEC, as well as other OVs, is being tested in combination with ICIs or other immunotherapeutic treatments in several clinical trials and the results will open the new possibility on OVs as players in the cancer treatments.

We believe that OVs as cancer vaccines will help the filed to shift towards personalized cancer treatment; in particular, the use of “plug-and-play” technology based on decorating OVs (armed with immunostimulatory molecules) with the selected tumor-specific peptides will pay the way to fast generation of tailored therapeutic cancer vaccines in a future clinical application where personalized therapies represent one of the main goals for a successful treatment.

Author contributions

S.F., S.R., E.Y., and V.C. conceived the work, decided the topic, and drafted the outline. S.F., S.R., and E.Y wrote the bulk of the text, generated the figures, and found the references. V.C. supervised the work.

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Declaration of Competing Interest

Vincenzo Cerullo is a co-founder and shareholder at VALO Therapeutics. The other authors have no conflicts of interest.

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