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2019-05-04
Taylor and Francis Ltd.
http://hdl.handle.net/10138/322184
Cervera-Carrascon, V, Havunen, R & Hemminki, A 2019, 'Oncolytic adenoviruses : a game changer approach in the battle between cancer and the immune system.', Expert Opinion on Biological Therapy, vol. 19, no. 5, pp. 443-455. https://doi.org/10.1080/14712598.2019.1595582

Oncolytic adenoviruses: a game changer approach in the battle between cancer and the immune system.

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Acknowledgements

Work on this paper was supported by Jane and Aatos Erkko Foundation, HUCH Research Funds (EVO), Sigrid Juselius Foundation, Finnish Cancer Organizations, University of Helsinki, TILT Biotherapeutics Ltd, European Commission Marie Curie Innovative Training Network (ITN) grant VIRION (H2020-MSCA-ITN-2014 project number 643130), and by University of Helsinki Doctoral Programme in Clinical Research.

ABSTRACT

Introduction: Oncolytic adenoviruses are among the most studied oncolytic viruses because of their tumor selectivity, safety, and transgene-delivery capability. With a growing number of different immunotherapies against cancer, the extraordinary immunogenicity of the adenovirus has emerged as a differentiating strength. Enabling T-cell related therapies with oncolytic adenoviruses appears a promising approach due to its inherent ability to elicit responses from the adaptive immune compartment. Areas covered: These viruses have successfully enhanced both adoptive T-cell therapies and immunecheckpoint therapies. Oncolytic viruses induce several effects at the tumor and on the systemic level that help to circumvent current limitations of T-cells and related therapies, such as T-cell trafficking, tumor immune suppressivity and antigen spreading. Expert opinion: Taking into account the multitude of possibilities of treating cancer with immunotherapies, learning to optimize the combinations and administration strategies of these drugs, could lead to durable responses in patients with currently incurable cancers.

Introduction

After the mid-19th century, a number of case reports observed tumor regression in patients undergoing viral infections. Those observations are probably the daybreak of modern oncolytic virotherapy. It is particularly relevant to emphasize the fact that, at that time, the existence of viruses and their role in causing diseases was unknown. It wasn't until the 20th century when the first viruses were discovered and characterized. In addition to their role in pathogenesis of infections, there was always an interest in their role in cancer. Mostly they were studied for a causative role but also as a potential treatment agent.

The primordial era of oncolytic virotherapy for cancer is a fascinating story including phases of excitement in the scientific community, and subsequent disappointment in patients (1). However, the same was true for all other cancer drugs as well. Contributing factors include the inability to measure response. Clinicians unrealistically thought that tumors would disappear and the patient become cured, and when this didn't happen they were unsatisfied. Conventional X-ray is not very useful for evaluating tumor size and clinical examination is practical in only few tumor types. Evaluation of the actual efficacy of cancer treatments only became reliable after randomization and computer tomography became standard in the 1980s.

The oncolytic virus field re-emerged in the late 1990s with rationally designed viruses, instead of the wild type viruses used earlier, although one such virus, Rigvir, had been approved in Latvia, Georgia and Armenia (2). Not long after, in 2005, , the first genetically modified oncolytic virus, Oncorine (H101), was approved by the Chinese Food and Drug administration (3). This adenovirus is engineered to replicate in p53-deficient cells and it is approved for head and neck squamous-cell carcinoma (4). The only oncolytic virus approved by US Food and Drug Administration and European Medicines Agency is talimogene laherparepvec for unresectable metastatic melanoma. This virus, a herpes simplex virus coding for granulocyte and macrophage colony stimulating factor (GMCSF), was approved in 2015 (5). Besides these three approvals, there are 84 clinical trials ongoing (source: Clinicaltrials.gov. Search criteria; Condition or disease: Cancer; Other terms: Oncolytic virus. Search date: December 26th 2018), and at least 95 viruses are being developed at the preclinical stage (6), proving the general interest of the oncology field in the approach.

The term "oncolytic virus" refers to viruses that are able to replicate selectively in cancer cells and leave normal cells unharmed (7). Some viruses have natural selectivity (parvoviruses, reovirus, Newcastle disease virus, myxoma viruses, Seneca Valley virus, and Coxsackie virus (7, 8)), often relating to interferon sensitivity. Other viruses can be rendered tumor selective through genetic modification (adenovirus, herpes simplex virus, measles virus, poliovirus, and vesicular stomatitis virus (7)). Besides the direct antitumor effect derived from the destruction of the tumor cell after virus replication, immunogenic cell death caused by oncolytic viruses develops immune reactions against the tumors (9, 10). Of note, this was first realized in patients, not in the laboratory (11). In this context, pathogen and danger associated molecular patterns (PAMPs and DAMPs) are released to the tumor microenvironment, which helps to attract the attention of the immune system towards the tumor (12). At the same time, tumor-associated antigens and tumor neoantigens are released, which, in the presence of viruses, enhances epitope spreading and further immune reactions against uninfected tumor cells (7, 13) (Figure 1).

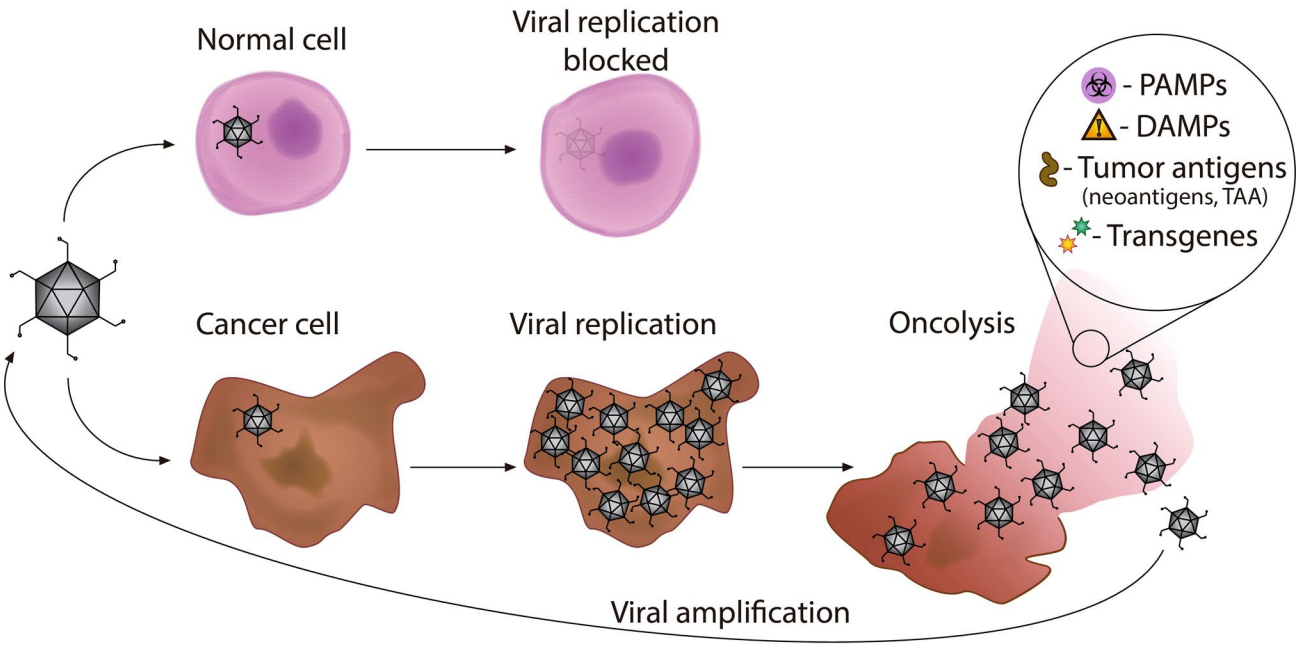

Figure 1. Oncolytic virus mechanism of action. Oncolytic viruses are able to selectively replicate in tumor cells where healthy cells remain unharmed. Besides the direct lytic effect of tumor cells, oncolysis releases pathogen and danger associated molecular patterns (PAMPs and DAMPs), tumor antigens (such as neoantigens and tumor associated antigens) and new viral particles to propagate to other cells. Furthermore, oncolytic viruses can be used to deliver transgenes in the tumor microenvironment.

OVs also face limitations, such as pre-existing and newly created antiviral immunity, physical and chemical barriers within tumors, or adverse events caused by off-site virus replication (7). Neutralizing antibodies and complement proteins can hinder the virus delivery to the target tumor cell (14, 15), but this dogma is starting to shift as antiviral responses are also known to trigger stronger anti-tumor immunity after the tumor environment is changed and epitope spreading is promoted (16-18). Inside the tumor, hypoxic conditions and a dense extracellular matrix might hinder viral replication and spreading (19, 20).

Regarding the entry of the virus into the cell, cancer cells sometimes downregulate proteins the viruses use as entry receptors (13). An illustrative example regards the coxsackie-adenovirus receptor, as this protein used by some coxsackie and adenovirus serotypes is strongly suppressed in many cancers (21). For this reason, the serotypes utilizing non-downregulated receptors should preferentially be used as therapeutic agents. For example, while adenovirus serotype 5 uses CAR, adenovirus serotype 3 uses desmoglein-2 as receptors, which are more commonly expressed on cancer cells (22, 23). One appealing embodiment is using just a part of a different serotype in an approach called serotype chimerism. This retains favorable features of Ad5 while allowing more effective entry (21).

In this review, we will focus on studying the development of oncolytic adenoviruses, one of the most studied oncolytic virus due to its convenient natural and artificially-designed properties, for their use as immunotherapy against cancer.

Oncolytic Adenovirus selectivity

Tumor selectivity in not a natural feature of adenoviruses, but it is a characteristic they can gain after certain modifications are implemented on their genome (8). In this regard, the E1A protein plays a paramount role, as it is the firstly expressed protein and it initiates the replicative cycle (24). One of the explored alternatives to restrict adenoviral replication to cancer cells is to use a promoter that is overexpressed in malignant cells. In 2001, DeWeese et al. (25) ran a phase I clinical trial on prostate cancer using an oncolytic adenovirus, whose E1A expression was confined to cells expressing the prostate specific antigen (PSA). Another relevant protein used as promoter is the human telomerase reverse transcriptase (hTERT), which is commonly overexpressed across different types of cancers (26). In 2009, Chang et al. (27) ran a phase I clinical trial, in which they treated head and neck cancer patients with a virus using hTERT as promoter for the expression of E1A.

Normally, E1A directs the cell towards S-phase when interacting with the retinoblastoma (pRb) protein (28) (Figure 2). The pRb function is to avoid excessive replication of the cell, which is accomplished by creating a complex with E2F family of DNA binding transcription factors (29). The E1A protein hijacks the pRb protein from that complex releasing the E2F, leading to host cell S-Phase (30, 31). Defects in the p16/pRb pathway are universal in cancer cells, resulting in an increased amount of free E2F (32). In that scenario, the activity of E1A is nonessential, as E2F is already free and the cell is in a replicative state, which constitutes one of the defining features of cancer (31, 32).

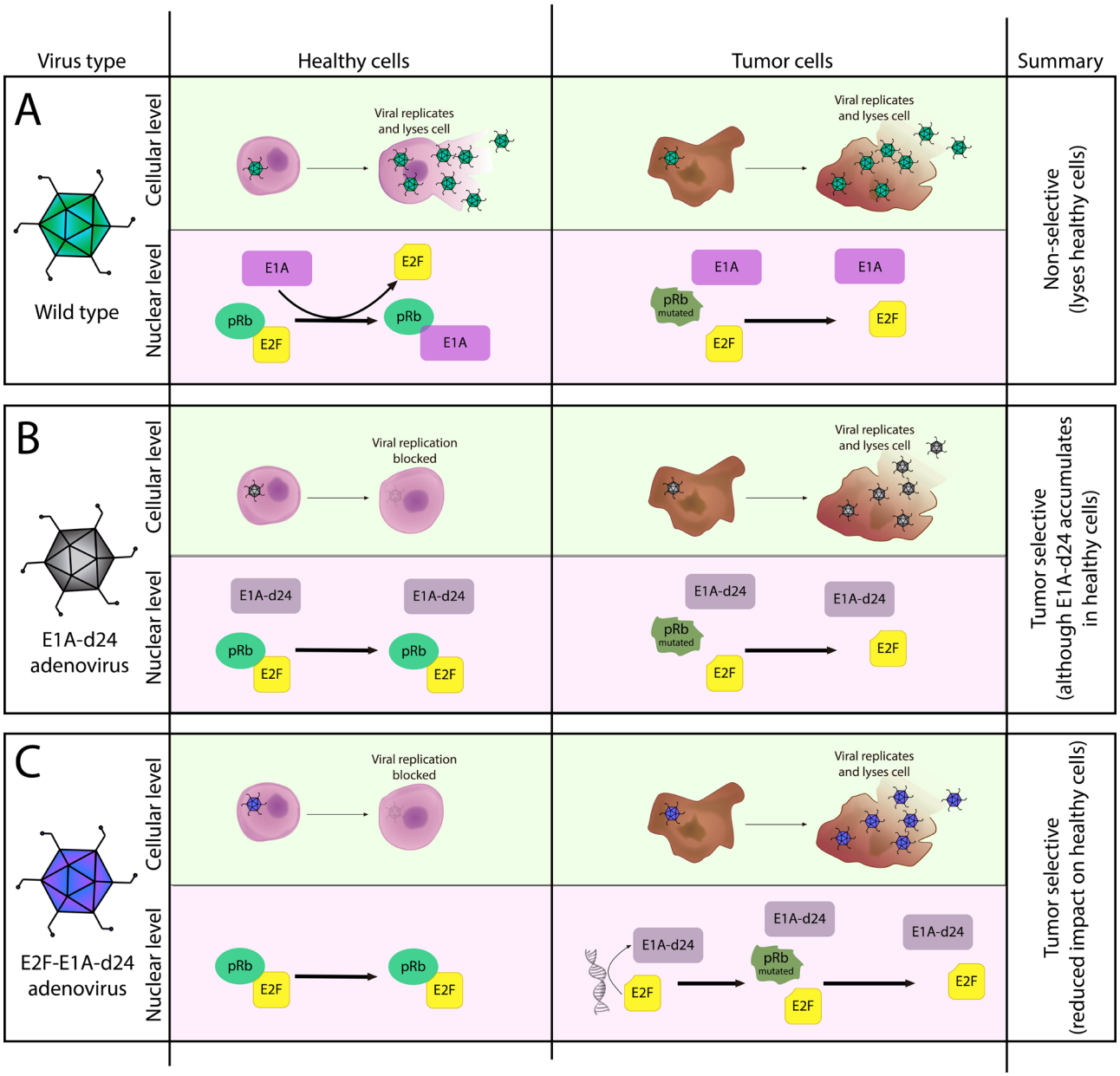

Figure 2. E1A-related mechanisms of tumor selectivity. A) Wild type adenovirus produces E1A, which in healthy cells binds pRb releasing E2F triggering a series of processes resulting in lysis of the healthy cell. In tumor cells, deficient pRb makes E2F available even without the action of the viral E1A protein. B) Upon infection the E1A-d24 (deficient E1A after a 24bp deletion) protein is unable to interact with the pRb/E2F complex in healthy cells, but the viral protein is still produced. In tumor cells the cell lysis occurs due to the presence of free E2F, making the function of E1A not-needed. C) The E2F-E1A-d24 virus starts its replication using E2F as a promoter, making replication or even production of E1A-d24 in healthy cells non-viable. On tumor cells, free E2F acts as a promoter of E1A-d24 and then drives viral replication and ultimately cell lysis.

These circumstances open the way for another selectivity mechanism, where *E1A* is made defective. A virus unable to express a functional E1A will not be able to replicate in normal cells, but can do it in cancer cells, as they are defective in the p16/pRb pathway (32). This safety mechanism has been embodied as a 24-bp deletion on the *E1A* gene (*E1A-d24*) and it has been used in a clinical set-up (18, 33-35). An additional layer of selectivity can be introduced with the addition of an E2F promoter before the *E1A-d24* gene (21, 36).

Similar trans-complementation between the virus and the tumor cell can be established around the p53 protein. In parallel to p16/Rb, another universally mutated pathway is p53/p14ARF (37, 38). The E1B protein expressed by wild type adenoviruses binds p53, rendering it inactive. When the *E1B* gene is deleted,

replication is crippled in normal cells (39). This selectivity approach is implemented in oncolytic adenoviruses used in humans, such as ONYX-015 and H101 (39, 40). In contrast to the D24 deletion, which does not reduce replicativity of the virus in tumor cells, an E1B or E1b55k deletion reduces replication in both target and non-target cells (41).

Oncolytic Adenoviruses as vectors

Besides the modifications in the adenoviral genome that render them tumor-selective, it is also possible to engineer them to express one or multiple transgenes. Adenoviruses can accommodate several thousand base pairs of extragenomic DNA depending on how much genome is previously deleted (13), that can be used for arming the virus with cytokines or other proteins, which enhance the antitumor efficacy (42). Delivering recombinant cytokines with viruses has two major differences when compared to regular systemic delivery methods: i) the expression is local, and ii) the expression is continuous. Taking the interleukin-2 (IL-2) treatment used in several therapeutic approaches (43-45) as an example, its administration has historically been systemic (intravenous or subcutaneous). This means that the cytokine is diluted into the whole body, reaching the tumor only passively, and at the same concentration as normal organs. In contrast, when the IL-2 is delivered locally by the virus, the ratio of intratumoral to systemic can be over a thousand times higher (46). The restriction of systemic IL-2 exposure could help to reduce the toxicities from recombinant cytokines. Also efficacy could be increased as higher IL2 concentrations are achieved locally where it matters (13, 46).

The second difference relies on the evolution of cytokine levels over time. As it has been shown for other active compounds with low half-life, sustained release is desirable way to avoid peaks in concentration (47, 48). Several cytokines, including IL-2, have a short half-life (5-7 minutes (49)). In that sense, the armed viruses turn the tumor into a prolonged release device for the transgenes. In addition to IL-2, preclinical and clinical studies have been conducted with adenoviruses expressing other cytokines (tumor necrosis factor alpha (TNFa)(50, 51), GMCSF (18, 33, 52, 53), IL-12 (54-56), IL-15 (57), IL-18 (55), CCL20 (57)), soluble immune ligands (CD40L (35, 58), CD80 (56), 4-1BBL (54), OX-40L (59), GITRL (60)), antibodies (anti-CTLA4 (52, 61, 62), anti-PD-L1 (63), anti-EpCAM/anti-CD3 bispecific T-cell engager (64)), stroma degrading proteins (hyaluronidase (65), relaxin (66)), antiangiogenic compounds (VEGI (67), anti-VEGFanti-VEGFR-1 (68)), RNA-interference tools (69), or combinations of these (51, 52, 54-57).

Besides the use of oncolytic viruses to deliver a protein cargo to tumors, it is possible to arm them to deliver genes to replace malfunctioning copies in target cell in a similar way as it has been classically done in gene therapy (70) or to insert suicide genes in tumor cells (71). Beyond these, other approaches have been developed, in which adenoviruses are used to deliver tumor-associated antigens (72) in some cases without the need for engineering the viral genome. In this last approach, tumor-associated antigens are attached to the surface of the virus by electrostatic bond (73). A key design aspect to keep in mind is that infected tumor cells are predicted to die. Therefore, the cargo would optimally have paracrine, not intracellular, activity.

Oncolytic Adenoviruses to enable other immune therapies

Perhaps the most valuable clinical lesson learned from trials performed in the last ten years with oncolytic viruses is that they interact with the immune system in a way that can be exploited to enhance the overall antitumor response (74-76). Before that realization, because these agents were mostly studied in the laboratory with systems lacking immune cells, this critical aspect was mostly missed or ignored (77, 78). The potential interest in the immune effects coming from oncolytic viruses emerged concurrently with the success of other immune therapies, especially checkpoint antibodies (1, 74).

Oncolytic viruses consistently showed that in their replication cycles they increase pathogen and damage associated molecular patterns (PAMPs and DAMPs) in the tumor niche, which increases immune trafficking towards the tumor (12, 79). In addition, the release of neoantigens or tumor-associated antigens, in the context of the broad immunostimulatory effects of these viruses, turns the combination of immune therapies with oncolytic viruses into a coherent way to improve antitumor responses (7, 79, 80).

Each of the different oncolytic viruses studied has distinct effects on the immune compartment. Those properties should be taken into account when choosing the best combinations of immunotherapies. For example, RNA viruses replicate in the cytoplasm and are able to kill the host cell faster than viruses replicating in the nucleus, such as the adenovirus. Furthermore, viruses that replicate in the cytoplasm depend on defects in the interferon signaling pathways (81). These facts make oncolytic RNA viruses elicit less selective anti-tumor responses. Also, because of the requirement for interferon deficiency, less pronounced interferon production is seen in the tumor microenvironment, resulting in less prominent T-cell responses (82). Moreover, interferon produced by T-cells or the tumor microenvironment could attenuate RNA viruses.

In contrast, adenovirus selectivity is due to non-interferon related mechanisms and produces anti-interferon proteins VA1 and VA2 allowing it to replicate despite interferon production. Thus, the virus remains active even in the presence of a prominent interferon response, which is a feature of a potent anti-tumor immune response. With regard to wild type adenovirus (DNA virus) infection in humans, recovery is strongly dependent on T-cell immunity (83-85). This fact highlights the strong relationship between adenoviruses and the T-cell compartment. That is why it is coherent to exploit such interaction by using oncolytic adenoviruses to potentiate antitumor T-cell related therapies, such as adoptive-cell therapies or immune-checkpoint therapies.

Adoptive-cell therapies

Currently, adoptive-cell therapy delivers promising results against a selected few cancer types (44). This approach comprises treatments in which cells are collected from a patient, grown artificially in a process that might include modifications of the cells, and then infused back to the patient as a graft. Current embodiments of adoptive-cell therapy typically involves T cells as the main cell type transferred (86). There are three main flavors: tumor-infiltrating lymphocytes (TIL), chimeric antigen receptor T cells (CAR T) and T-cell receptor modified T-cells (TCR).

➔ **Adoptive TIL therapy**

Adoptive TIL therapy is an approach in which patient's autologous tumor resident T cells are extracted and isolated from a tumor biopsy, activated, and expanded before infusing them back to the patient to exert an antitumor effect (87). The successful application of TIL therapy in patients began in 1986, after Rosenberg, S.A. and colleagues implemented a preconditioning regimen of lymphodepleting cyclophosphamide and a postconditioning with high-dose IL-2 administration together with the TILs (88, 89). A closely similar approach is used today, rendering about a 50% response rate in melanoma (87). There are examples of efficacy also in many other tumor types, but far fewer trials (90). There is one randomized trial (91) showing the efficacy of the approach when used as adjuvant therapy for high risk nodally recurrent melanoma. No randomized trials have been completed in the metastatic setting.

Besides the intrinsic ability of adenoviruses to interact with the T-cell compartment, this relationship can be enhanced by selecting an arming device oriented towards such purpose. In a data driven approach, several cytokines potentially attractive in this regard were studied (92). The combination of IL2 and TNFa was found to have non-overlapping synergistic effects on T-cells (93). The most prominent effects of TNFa relate to danger signaling and T-cell trafficking, while IL2 is important in the context of T-cell activation, propagation and prevention of exhaustion (93). These findings resulted in construction of Ad5/3-E2F-D24-TNFa-IL2, also known as TILT-123 (51).

IL-2 was previously known as T-cell growth factor and as a recombinant protein has the potential to induce tumor regression (94, 95), presumably by its effects on lymphocytes, and not the tumor itself (96). In addition to the previously commented benefits of local delivery of cytokines, the use of oncolytic adenoviruses coding for IL-2 can also improve the current standards of TIL therapy. Santos JM et al. (97) showed how the local IL-2 production from an IL-2 armed adenovirus made the addition of systemic postconditioning with high-dose IL-2 unnecessary, which also reduced off-target toxicities (46).

Furthermore, Santos et al demonstrated that TILT-123 removed the need for lymphodepleting preconditioning. Virus injections improved antitumor efficacy while avoiding the classic chemotherapy toxicities observed in control groups. Selection of the arming device has been shown to be critical not only with regard to antitumor efficacy, but also in generating stronger antitumor memory and abscopal effect (92, 98).

Clinical proof-of-concept data was generated with a non-replicative adenovirus coding for interferon gamma (TG1042). This virus was developed with the aim of countering the tumor immune suppression by producing the cytokine locally facilitate antitumor efficacy by TILs (99). In a phase I/II clinical trial in metastatic melanoma, this combination achieved objective responses in 5 out of 13 patients (38.5%), including 3 complete responses (23.1%). In this study safety of the virus was also achieved (99). Further gains could be achieved with an oncolytic platform, and with a different choice of transgene (92).

➔ **Adoptive TCR therapy**

Generation of adoptive TCR treatments includes the use of viral vectors (*e.g.* retrovirus or lentivirus) to transduce recombinant TCRs into T cells (previously harvested by apheresis from the patients), which are expanded and selected by target affinity before reinfusing them to the patient (100). This approach initiated in the 1990s after TIL studies on melanoma showed that some of those TILs were antigen-specific against MART-1 and gp100 (101). The idea was to transduce the TCRs against these antigens into T-cells to get "off-

the-shelf" antitumor-specific T cells from patient's own cells (100). The major obstacles for adoptive TCR therapies are the same as for TIL therapy: attracting the T-cell graft towards the tumor and changing the immune microenvironment to support lasting antitumor responses.

One of the most studied animal models in cancer immunotherapy, the B16.OVA cell line with OVA-specific OT-I T cell transfer, is a common model of adoptive TCR therapy (102). Using this model, it was shown that adenoviruses can improve the antitumor efficacy of adoptive TCR therapy regardless of pre-existing immunity against adenovirus (103). Furthermore, the virus treatment creates *de novo* antitumor responses against other tumor antigens besides OVA (103). Adenoviruses can additionally increase the trafficking of labeled transgenic-TCR T cells towards the tumor and, again, different arming devices improved the trafficking to different extent (93).

➔ **Adoptive CAR T therapy**

The only difference between adoptive TCR therapy and adoptive CAR T therapy is that instead of using a regular T-cell receptor, the cells in CAR T therapy are transduced with a chimeric receptor composed of antibody-binding domains fused to T-cell signaling domains (104). The first generation of CAR T cells was designed before 1989 by Gros G et al. The second and third generations included one or two intracellular costimulatory domains, respectively, to increase the proliferation and the survival of the CAR T cells (105).

CAR T therapy shows impressive results in hematological malignancies and was recently approved by FDA for such indications. However, few beneficial effects are seen in patients with solid tumors (82). Responses are also sometimes seen in some solid tumor types, such as GD2-positive tumors, virally-induced tumors, glioma, melanoma, sarcoma patients (106). One important mechanism of relevance is that CAR T mediated cell lysis triggers the release of all the tumor antigens, which are then cross-presented, promoting a phenomenon called epitope spreading (107, 108). Besides the direct benefit of oncolytic viruses enhancing CAR T therapy activity, an added value might rise from the increased epitope spreading as both therapies are causing tumor cell killing in parallel, perhaps of different tumor cell populations.

Similarly to other ACT approaches, the limitation of CAR T include cell trafficking, proliferation, and persistence at the tumor (109), tumor-associated immune suppression (110), and reduced presence of target antigens due to tumor heterogeneity (111) or antigen loss (112). However, oncolytic viruses are able to potentially reduce every single limitation presented for CAR T cell therapy: they increase tumor trafficking, revert immune suppression, and use a different antitumor mechanism to cope with antigen loss, for example. Appropriately, the combination of oncolytic viruses and CAR T is a topic recently covered in original papers (113)and reviews (82, 105).

Oncolytic adenoviruses with double transgenes, IL-15 and RANTES (114) as well as TNFa and IL-2 (113), have demonstrated an ability to enhance CAR T cell therapy, increasing the amount of CAR T cells at the tumor. In the second study (113), Watanabe K et al. proved the efficacy of dual treatment (CAR T cells plus OAd armed with TNFa and IL-2) in controlling both the virus injected primary lesion and the uninjected metastases, using both immune defective and immune competent models. Monotherapy controls failed in achieving such results, making the case for the synergistic combination of these therapies.

Other approaches with CAR T therapy include the use of oncolytic adenoviruses coding for checkpoint inhibitors (CPI) to reduce T-cell anergy (63, 115) or oncolytic adenoviruses coding for a bi-specific T cell

engager targeting a second antigen to reduce the chances of tumor escape due to antigen loss or tumor heterogeneity (116). Among the possibilities when combining oncolytic adenoviruses and CAR T cells, a novel approach is to use the engineered CAR T cells as carriers to deliver the oncolytic adenoviruses at the tumor niche, protecting them from neutralization, while they are in the systemic compartment (117).

Immune-checkpoint therapies

Immune-checkpoints include inhibitory and stimulatory pathways that regulate self-tolerance and immune responses. The immune checkpoint pathways are classified into inhibitory pathways (reduce immune activity) and stimulatory ones (increase immune activity). In cancer, inhibitory checkpoint pathways are typically active, because most tumors feature abnormal proteins, which would be targeted by the immune system unless suppressed (118).

The idea for utilizing immune-checkpoints as cancer therapeutic targets came about in the 1990s with the parallel work on the programmed cell death protein 1 (PD-1)(119) and the cytotoxic T-lymphocyte antigen 4 (CTLA-4)(120). In subsequent years, therapeutic antibodies targeting CTLA-4, PD-1, and PD-L1 were studied in the clinical set-up, where they showed promising results in a subgroup of patients. These data nevertheless have revolutionized the field of immunotherapy (121), because many of the responses have been durable, which hadn't been seen with other oncology drugs. Unfortunately, responses are seen in a minority of patients, only 10-40% with single agent therapy (122). Similarly to ACT, immune-checkpoint therapies trigger (or release) antitumor responses via T cells, but antibodies are technically much simpler and easier to use than cell-based therapies, which has facilitated their breakthrough.

In the field of cancer immunotherapy, a major effort is being invested on understanding how to increase the percentage of patients benefitting from immune-checkpoint therapies. So far, it has been noted that total mutational burden, the presence of TILs (123, 124) and upregulated inflammatory cytokine signatures (125-127) are among the strongest predictive factors for response to the therapy (10). Oncolytic viruses could be rationally used to increase the response rate to immune-checkpoint therapies, as they have consistently shown to induce T-cell trafficking (93) and a proinflammatory shift in the tumor, either by their natural properties (12) or by the delivery of the engineered cytokine cargo (51). Concerning PD-L1 expression, it has been used as an indication-defining marker for the use of certain CPIs (128), for example in lung cancer. As viruses can also influence immune-checkpoint expression, it is paramount to understand the interaction in between therapies, as it determines the optimal sequence of administration (129, 130).

➔ **Immune-checkpoint inhibitors**

CPIs are drugs that block certain pathways in immune cells, aiming to release certain braking mechanisms and, thus, resulting in antitumor activities. CPIs are usually antibodies that block checkpoints such as CTLA-4, PD-1, PD-L1, PD-L2, TIM-3, LAG-3, and BTLA (118). When anti-PD-1 antibodies were tested together with adenoviruses expressing TNFa and IL-2 in a murine melanoma model, a synergistic effect between the therapies was seen. Complete responses occurred in all the treated animals, when the regimens were administered in a specific sequence which started with virus alone and the continued with the combination (129). The observed synergy can be hypothetically attributed to a positive impact of each therapy on the other: the virus increases the amount of T cells in the tumor and the antibodies protect them from anergy.

Similar results have been obtained also with other adenoviruses, confirming that combined use increases tumor growth control and the development and maintaining of antitumor specific responses (129, 131, 132).

In another preclinical study, Capuccini, F. et al. showed that when combining oncolytic viruses and CPIs, the selection of the specific targets is not a trivial choice as in their study the combination of a double oncolytic virus platform (adenovirus and a modified vaccinia Ankara virus) with anti-PD-1 was able to improve tumor control and overall survival. However, when they tried to reproduce the results using anti-PD-L1 instead, there was just a minimal effect in controlling the tumors, suggesting that PD-1/PD-L2 interactions could be relevant in that model (133).

More evidence legitimating the use of oncolytic adenoviruses with CPIs was shown after a phase I clinical study with ONCOS-102 (an OAd expressing GMCSF)(33) and a preclinical study with ADV/HSV-tk (an OAd coding for a suicide gene)(134), as they both showed that the use of these oncolytic adenoviruses efficiently increased the amount of CD8+ T cells and increased the expression of PD-L1 at the tumor niche. These findings motivated the start of new clinical trials, in which those viruses were tested together with anti-PD-1 antibodies (NCT03003676, NCT03004183). In a different clinical trial, the use of an unarmed OAd (DNX-2401) in malignant glioma patients resulted in a downregulation of TIM-3 but not of PD-1/PD-L1 (135). This finding led to a subsequent clinical trial (NCT02798406), as the investigators speculated that the use of PD-1 inhibitors in combination with the virus might reduce the tumor immune suppression even more.

Exploiting the possibilities given by the adenovirus engineering, some oncolytic adenoviruses have been armed with CPIs, such as anti-CTLA-4 (52, 61, 62) or anti-PD-L1 (63). The preclinical models using those viruses show high levels of antibodies produced, successful induction of T-cell activity, and antitumor efficacy both *in vitro* and *in vivo*. The strongest point of the "cis-approach" is that by local delivery of the antibodies, adverse events associated with the CPIs could be diminished. On the other hand, the current use of CPIs at the clinics sometimes triggers high-grade adverse events that require discontinuation of the antibodies, which could be more complex to do if the CPIs are being produced by the oncolytic adenoviruses .

➔ **Immune-checkpoint stimulators**

Similarly to CPIs, there are checkpoint stimulators (CPS). CPSs are drugs that activate certain pathways in immune cells, with the aim of triggering immune activation and thus granting antitumor activities. CPSs are usually antibodies that activate immune-checkpoints, such as OX-40, OX-40L, GITR, 41BB, and ICOS (118).

The therapeutic activation of those pathways is less advanced clinically, perhaps in part because the safety record of this approach is not unblemished (136). The only OAd currently under clinical development is LOAd703 (NCT02705196, NCT03225989), an adenovirus expressing trimerized membrane-bound CD40L and 4-1BBL. This virus carries two immune-stimulating transgenes, one for the myeloid compartment (CD40L) and the other to induce proliferation and survival of the lymphoid compartment (4-1BBL). In a preclinical pancreatic murine model, the virus treatment induced tumor growth control and a deep shift towards an immunostimulatory status (137).

Other viruses armed with CPSs, such as OX40L (138) and 4-1BBL (139), have showed limited tumor growth control as single agents in animal models, coupled to unwanted PD-L1 and PD-L2 upregulation. To control those tumors, anti-PD-1 therapy was combined with the virotherapy, improving the efficacy. In that sense, it seems that the inherent immune-stimulatory activity of the oncolytic adenoviruses coupled with the immunostimulatory effect of those CPSs triggered a strong immune suppression seen in the PD-1 pathway, which counters part of the therapeutic effect.

Some other molecules characterized before the immune-checkpoint era are starting to be included under this category as well: CD40, CD40L, CD28, CD80, CD86, CD27 and CD70 (118). In that sense, the use of oncolytic adenoviruses in combination with these CPSs has been explored both preclinically (56, 58, 140) and clinically (35). Those patients treated with the mentioned OAd coding for CD40L, and effective shift towards Th1 immunity was seen supporting the theoretical basis for this approach (35).

Immunotherapy beyond T cells

Currently, the antitumor immune therapies which work, are mostly oriented towards the T-cell compartment, but other cell types are being studied for their potential as well. Natural killer (NK) cells might be an interesting option due their ability to recognize cells with low expression of major histocompatibility complex-I (MHC-I), a phenomenon shared between virus-infected and a high proportion of cancer cells (141, 142). The effect of NK cells on virally infected tumor cells might reduce the amplification of the OV, but some preclinical studies concluded that the overall effect of NK cells in the context of oncolytic virus therapies is positive (141-144). Furthermore, NK cells might contribute to dendritic cell (DC) maturation and differentiation of cancer stem cells and other poorly differentiated cancer cells through the secretion of immunostimulatory cytokines, such as TNFa and IFNg (145, 146).

Also DC-based immunotherapy coupled to specifically designed oncolytic viruses is showing preclinical promise (54, 58, 140, 147, 148).

Oncolytic Adenoviruses: clinical progress

In 1956, Huebner, R.J. et al. carried out the first clinical trial with adenovirus (adenoidal-pharyngeal-conjunctival virus) in patients with cervical carcinoma (149). Today (December 26th 2018), oncolytic adenoviruses represent above 25% (21/82) of all the clinical trials regarding oncolytic viruses for cancer (source: Clinicaltrials.gov. Search criteria; Condition or disease: Cancer; Other terms: Oncolytic virus), making it the most studied type of oncolytic virus under clinical studies (Table 1).

Table 1: Oncolytic adenovirus trials. IL-12; Interleukin-12. HSV-tk; herpes simplex virus-1 thymidine kinase. GMCSF; granulocyte and macrophage colony stimulating factor. TMZ-CD40L; trimerized membrane-bound CD40 ligand. HPV; human papilloma virus. NSCLC; non-small-cell lung cancer. TNBC; triple negative breast cancer. RCC; renal-cell carcinoma. MG1-E6E7; maraba oncolytic virus expressing E6E7. SBRT; stereotactic body radiotherapy. Nab; neutralizing antibody. Of note, not all clinical trials using oncolytic viruses

*included the specific term 'oncolytic virus' under the description of the trial and thus, those studies will not appear in the previously described systematic search*Estimated enrollment. **First posted date.*

Virus name	Serotype	Transgene(s)	Indication	Phase	Nº of participants	Start date	Other treatments	Status	Clinical trial identifier
Ad5-yCD/mutTKSR39rep-hIL12	5	IL-12	Prostate cancer	I	15*	Sep-15	-	Recruiting	NCT02555397
ADV/HSV-tk	5	HSV-tk	NSCLC, TNBC	II	57*	Dec-16	Valacyclovir, SBRT, anti-PD1	Recruiting	NCT03004183
CAdVEC	5/3	anti-PDL1	Solid tumors	I	39*	Nov-18	-	Not yet recruiting	NCT03740256
CG0070	5	GMCSF	Bladder Cancer	II/III	22	Sep-11	-	Terminated	NCT01438112
CG0070	5	GMCSF	Bladder Cancer	II	-	May-14**	-	No longer available	NCT02143804
CG0070	5	GMCSF	Bladder Cancer	II	66	Feb-15	-	Active, not recruiting	NCT02365818
CGTG-102	5/3	GMCSF	Solid tumors	I	-	Sep-11	-	Withdrawn	NCT01437280
CGTG-102	5/3	GMCSF	Solid tumors	I	12	Oct-14	-	Completed	NCT01598129
ColoAd1	11/3	-	Colon cancer, NSCLC, Bladder cancer, RCC	I	17	Feb-14	-	Completed	NCT02053220
CRAd-Survivin-pk7	5	-	Glioma	I	36*	Mar-17	Neural stem cells	Recruiting	NCT03072134
DNX-2401	5	-	Glioblastoma, Gliosarcoma	I	37	Jul-14	Interferon-gamma	Completed	NCT02197169
DNX-2401	5	-	Brain tumors	II	48*	Jun-16	Anti-PD1	Recruiting	NCT02798406
DNX-2401	5	-	Glioblastoma	I	24	Oct-18	-	Recruiting	NCT03714334
DNX-2401	5	-	Glioblastoma	I	31*	Oct-13	Temozolomide	Completed	NCT01956734
DNX-2401	5	-	Brainstem glioma	I	12*	Jun-17	-	Recruiting	NCT03178032
ICOVIR-5	5	-	Melanoma	I	14	May-13	-	Completed	NCT01864759
LOAd703	5/35	TMZ-CD40L, 41BBL	Pancreatic cancer	I/II	26*	Nov-16	Gemcitabine, NAb paclitaxel	Recruiting	NCT02705196
LOAd703	5/35	TMZ-CD40L, 41BBL	Pancreatic adenocarcinoma, Ovarian Cancer, Biliary carcinoma, Colorrectal cancer	I/II	50*	Jul-17	-	Recruiting	NCT03225989
OBP-301	5	-	Melanoma	II	50*	Jun-17	-	Recruiting	NCT03190824
VCN-01	5	PH20 hyaluronidase	Solid tumors	I	36*	Jan-14	Gemcitabine, NAb paclitaxel	Recruiting	NCT02045602
VCN-01	5	PH20 hyaluronidase	Pancreatic adenocarcinoma	I	8	Jan-14	Gemcitabine, NAb paclitaxel	Completed	NCT02045589

To summarize 63 years of OAd clinical history, we focus on some key studies. The trial performed in 1956 (149) on patients with advanced epidermoid carcinoma of the cervix, was carried out using a wild type adenovirus (known as adenoidal-pharyngeal-conjunctival virus at the time). Despite lack of engineered selectivity, treatment was mostly safe and appeared effective. Some tumor necrosis was seen in two thirds (26/40) of the patients upon clinical observation (1). Similar results were obtained with other non-attenuated viruses, creating a need for safer and more effective viruses (150). The technical means to achieve tumor selectivity required genetic engineering tools, which arrived only in 1981 (151).

The first engineered oncolytic virus considered safe and efficacious to be approved by a regulatory agency (2005, Chinese Food and Drug Agency) is "Oncorine", an adenovirus also known as H-101. This virus heavily relies on its predecessor, Onyx-015, which achieved tumor selectivity through phenotype trans-complementation, restricting its replication to p53/p14ARF-deficient cells (81). However, while Onyx-015 (originally dl1520) was a wild type strain (152), H-101 was engineered. After promising results in phase I and II clinical trials (153), combination of H101 with chemotherapy in phase III resulted in a 79% response rate, almost the double over chemotherapy alone (40%)(3). Oncorine is still on the market since its launching in 2006 for nasopharyngeal carcinoma in combination with chemotherapy (4).

An individualized therapy program (rather than a clinical trial) called the Advanced Therapy Access Program, utilized ten different oncolytic adenoviruses in a personalized manner between 2007 and 2012 (11, 154). In this program 290 solid tumor patients refractory to standard treatments received one or more oncolytic adenoviruses from ten alternatives (Ad5-d24-GMCSF, Ad5/3-d24-GMCSF, Ad5-RGD-d24- GMCSF, Ad5/3-E2F-d24-GMCSF, Ad3-hTERT-E1, Ad5-d24- RGD, ICOVIR-7, Ad5/3-Cox2L-d24, Ad5/3-hTERT-CD40L, Ad5/3-d24-hNIS). Besides the treatments being safe and well tolerated, practical patient experience unveiled several aspects of the approach. The key observation was that T-cell predominated immune response triggered by adenoviral oncolysis might be the most important clinical mechanism of action. (53, 155-158). Of note, it was seen in patients that oncolytic adenoviruses seem to work best in immunologically "cold" tumors, lacking T-cells at baseline (159). This is in stark contrast to checkpoint inhibitors, which work only in "hot" tumors,

characterized by PDL-1, TILs and a high mutational burden (160). Therefore, the combination of T-cell stimulating adenoviruses with checkpoint inhibitors appears particularly appealing (129).

Currently, a dozen clinical trials featuring oncolytic adenoviruses are ongoing, and it is likely that more will be seen in the future. The accumulating experience with their use seems to point to the immune system as the real antitumor weapon making these oncolytic viruses, good candidates to unlock its power. Although many different virus types are being studied for their utility in cancer immunotherapy, their immunogenicity varies. Adenovirus appears as an extraordinary candidate due to its ability for stimulation of the T-cell arm of the immune system (82, 83, 131).

Expert opinion

Phenotypic features of cancer cells include many similarities to those features adopted by normal cells during virus infection. These circumstances allow the phenotypic complementation exploited by many oncolytic viruses to achieve tumor selectivity (81). Besides the safety (resulting from tumor selectivity) of oncolytic viruses, two other capabilities make them pluripotent tools: the possibility of transgene delivery and the immunostimulatory effects of oncolysis. Using viruses for transgene delivery, compared to traditional delivery routes, is an interesting approach, as they enable spontaneously amplifying but local transgene expression at the tumor niche. This reduces systemic adverse events of the transgene product, which can be a cytokine, for example (46). At the same time, the transgenes engineered into the virus can trigger immunological reactions, in addition to the ones the virus itself induces. These effect can be key to modulating the tumor microenvironment towards effective anti-tumor immunity. Among the most important immune consequences of using oncolytic viruses, are the increase of immune cell trafficking towards the tumor, and the release of tumor antigens in a context of immunogenic cell death and immunological danger signaling. Under these circumstances, there are higher chances to develop tumor specific responses and epitope spreading (131).

On the other hand, the immune system is also one of the classic proposed limitations to the therapeutic use of oncolytic viruses. Antibody-mediated neutralization (NAb) of non-enveloped viruses and complement activity for enveloped ones can reduce the efficacy of systemic administration, but even this dogma is starting to be questioned. Based on clinical results, there is no correlation between the anti-virus NAb titer and antitumor effects (155, 161, 162). The underlying mechanisms are being unveiled in preclinical models (16, 163). A deeper understanding of these mechanisms could end up improving the way oncolytic viruses are used in patients. There are ways for both increasing (*e.g.* checkpoint inhibitors (118) or low-dose cyclophosphamide (164)) and decreasing (*e.g.* high dose cyclophosphamide or anti-CD20 antibody (165)) immune activity. It might depend on patient and tumor which is more appealing.

Currently, most oncolytic viruses are delivered intratumorally due to efficacy and safety concerns. Ideally, the same antitumor and immunological effects, without further toxicities, could be achieved by systemic administration of the virus. This is probably not the most critical aspect to improve in the oncolytic virus field as systemic antitumor effect can be achieved after intratumoral administration (98), but pragmatically, intravenous administration is easier to perform. However, the technology needed for intratumoral injection is also available in all hospitals, even smaller ones. One of the most common radiological procedures is thin needle biopsy. Intratumoral injection uses the exact same technique, except that instead of aspirating a biopsy, the radiologist plunges the syringe to inject the virus.

Besides the antiviral immunity concern, there are some other crucial limitations to large tumor specific drugs such as oncolytic viruses. For example, dense extracellular matrix, stromal barriers, or hypoxic conditions can limit their replication and spreading (19, 20). These intratumoral complexities are an important reason for repeated intratumoral delivery, which ensures effective transduction of sufficient regions of the heterogeneous tumor. Another possible solution for overcoming these obstacles is to use oncolytic viruses together with other immunotherapies, chemotherapy, targeted therapies or radiation.

The adenovirus ranks among the most relevant viruses used as OV, especially when used together with other immune therapies. The main features making oncolytic adenoviruses good oncolytic virus include the clinically tested tumor selectivity, high cytopathic effects on infected cells, and the triggered immunogenic cell death able to broaden tumor specific T-cell responses (33, 34, 82, 131). Other important characteristics from the practical point of view, related to the use of oncolytic adenoviruses , is that there is abundant knowledge to engineer them, manufacture them at clinically relevant titers, and to use them safely in hospitals. These properties are what make oncolytic adenoviruses an attractive tool in cancer therapy.

While many oncolytic adenoviruses are now being tested together with other immunotherapies, especially checkpoint inhibitors, there are few viruses designed with T-cells in mind. In this regard, it is noteworthy that such viruses, *e.g.* TILT-123, achieve 100% complete responses in different models of T-cell related therapies. These include adoptive CAR T therapy (113), adoptive TIL transfer (51) and immune-checkpoint inhibitors (129). The ultimate goal of such preclinical work is obviously to translate the approach into clinical trials. The first trial with TILT-123 is set to start in 2019, and will feature virus injections in melanoma patients receiving an adoptive TIL therapy.

In summary, oncolytic adenoviruses are promising tools for combination immunotherapy. Specifically, their ability to stimulate the adaptive arm of the immune system is useful. There appears to be a therapeutic niche for oncolytic viruses in the way cancer will be treated in this century. No other technology achieves as potent immunomodulatory effects at the tumor and its microenvironment. Oncolytic viruses are also among the safest oncological approaches, which facilitates combination use.

Article highlight box

- After two decades of clinical experience and technical development, adenoviruses appear safe and effective therapies against cancer, but only one product has been approved heretofore.

- Oncolytic viruses can act as transgene delivery tools, achieving higher efficacy and lower toxicity than conventional delivery approaches.

- Oncolytic viruses trigger a series of immune-related processes that help to develop anti-tumor specific responses, while reducing immunosuppression.

- Due to adenovirus biology, oncolytic adenoviruses are interesting candidates to enable T-cell related therapies such as adoptive cell therapies or checkpoint inhibitors.

- Rational pairing and administration of immune therapies is needed to optimize treatment outcomes.

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