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Developing oncolytic viruses for clinical use: A consortium approach

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ABSTRACT

The use of oncolytic viruses forms an appealing approach for cancer treatment. On the one hand the viruses replicate in, and kill, tumor cells, leading to their intra-tumoral amplification. On the other hand the viral infection will activate virus-directed immune responses, and may trigger immune responses directed against tumor cells and tumor antigens. To date, a wide variety of oncolytic viruses is being developed for use in cancer treatment. While the development of oncolytic viruses has often been initiated by researchers in academia and other public institutions, a large majority of the final product development and the testing of these products in clinical trials is industry led. As a consequence relatively few pre-clinical and clinical studies evaluated different oncolytic viruses in competitive side-by-side preclinical or clinical studies. In this review we will summarize the steps and considerations essential in the development and characterization of oncolytic viruses, and describe our multidisciplinary academic consortium, which involves a dozen departments in three different Dutch universities, collaborating in the development of oncolytic viruses. This consortium has the ambition to develop a small series of oncolytic viruses and to evaluate these in various cancers.

1. Brief introduction on viro-immunotherapy

Oncolytic viruses are viruses that either have a natural preference to enter, replicate in, and/or kill cancer cells as opposed to normal cells, or are engineered to do so. Therefore, they are widely studied as anti-cancer agents in what is called oncolytic virotherapy. In the early 1900s, the first observations were recorded of potential anti-cancer effects by viruses, e.g. a leukemia patient that went briefly into spontaneous remission after contracting a presumed influenza infection [1]. In the 1950s, when techniques for virus growth, purification and characterization became available, this remarkable phenomenon was further investigated [2]. A variety of clinical trials were initiated, but due to somewhat disappointing results and safety concerns, oncolytic virotherapy lost interest as chemotherapy gained ground. The general idea at that time was that oncolytic viruses punched a hole in the tumor and were then cleared by anti-viral immunity. It was in the early 1990s when oncolytic viruses gained renewed interest, due to a change in the dogma: oncolytic viruses were no longer regarded as simple cancer cell killers, but instead as potent inducers of strong, long-lasting anti-cancer immunity. Thus, oncolytic virus therapy is now considered a novel form of immunotherapy, i.e. viro-immunotherapy. The rising numbers of publications on oncolytic virus therapy demonstrate the increased interest in the topic ever since [3]. The notion that oncolytic viruses are reputable anti-cancer agents is continuously growing, highlighted by the approval T-VEC in 2015 in the United States (U.S.), the European Union (E.U.) and Australia for treatment of a subset of patients suffering from advanced melanoma.

1.1. Some advanced oncolytic viruses

T-VEC is an oncolytic herpes simplex virus lacking its ribonucleotide reductase gene and expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) [4]. In light of this approval, herpes simplex virus is intensively studied and accounts for nearly a quarter of all ongoing clinical trials using oncolytic viruses. This enveloped virus harboring a dsDNA genome is currently tested for the treatment of various cancer types (including gastrointestinal cancer, breast cancer, melanoma, and brain cancers), both as a single agent and in combination with other anti-cancer therapies.

In addition to herpes simplex viruses, adenoviruses are ‘popular’ oncolytic viruses accounting for another quarter of all ongoing clinical trials investigating oncolytic virotherapy. Adenovirus is a non-

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enveloped virus with a dsDNA genome. The rise of recombinant DNA techniques in the 1980s, relative straightforward genetic modification, high-titer virus batches, and a good safety profile in clinical trials have made adenovirus one of the most frequently studied viruses in vaccine development, gene therapy, and oncolytic virotherapy. Because of this widespread application in several research areas, extensive knowledge is at hand about virus biology, virus-host interactions, immune modulation, and therapeutic application.

Roughly another quarter of clinical trials for oncolytic virotherapy is performed with vaccinia virus. This is an enveloped dsDNA poxvirus of which extensive data is available about safety in humans due to its prolonged use as a vaccine virus for the smallpox eradication programs. Together with a high transgene packaging capacity, vaccinia virus is an attractive platform for oncolytic virotherapy design.

Other regularly used oncolytic viruses include reovirus, measles virus, Newcastle disease virus, vesicular stomatitis virus, Maraba virus, Coxsackievirus, poliovirus, and retrovirus. Reovirus is a non-enveloped dsRNA virus that possesses natural oncolytic properties and is not associated with serious human disease. Clinical trials have demonstrated the safety of reovirus administration, and it is currently tested for efficacy in a variety of studies for several cancer types [5,6].

Measles virus is an enveloped negative-sense RNA virus that is the causative agent for measles disease. Oncolytic measles virus is based on vaccine strains of the virus, and preferentially lyses tumor cells through recruitment of CD46 as its receptor [7,8]. Its proven safety record, stability, and the feasibility to incorporate transgenes, make it a very attractive candidate for anti-cancer therapy. However anti-cancer efficacy may be hampered by anti-viral host immunity [8].

Another enveloped negative-sense RNA virus, Newcastle disease virus (NDV), is a virus with a natural avian host range that also displays tumor killing and the level of virulence in birds correlates with the degree of oncolysis [9]. Research efforts primarily focus on the generation of NDVs with improved efficacy and safety profiles. Several modified strains of NDV have been generated harboring therapeutic transgenes, and have demonstrated anti-cancer potency in a variety of clinical trials [10].

Vesicular stomatitis virus (VSV) is a negative-sense RNA rhabdovirus virus, has natural tumor specificity, a fast replication cycle, and potent immunomodulatory properties. Importantly, elevated doses seem to cause toxicities and, to date, only a pseudotyped VSV vaccine vector, lacking the G gene and instead containing the non-neurotropic glycoprotein of lymphocytic choriomeningitis virus (LCMV), and an attenuated oncolytic VSV expressing human interferon β have proceeded to clinical trials [11].

Maraba virus is also a rhabdovirus and shows some homology with VSV. No virus-related pathology has been reported in humans, and the prevalence of anti-viral antibodies is negligible, making it an interesting agent for virotherapeutic purposes. Safety and immunogenicity have been demonstrated in early-phase clinical trials and follow-up studies are currently performed in monotherapy as well as combination therapy approaches [12].

Coxsackievirus is a picornavirus harboring a positive-sense RNA genome and a prevalent human pathogen. This non-enveloped virus also has oncolytic potency, and Coxsackievirus A21 is currently evaluated in several clinical trials. Administration appears to be safe without serious adverse events. As the viruses can give severe disease in humans, care should be taken in evaluating this virus in clinical trials, and attenuation may be necessary [13].

Another positive-sense RNA virus, poliovirus, is a neurotropic virus that has been genetically modified to attenuate neurovirulence and retain cancer-specific cytotoxicity [14]. The resulting PVSRIPO virus has shown successful targeting of glioblastoma and has been granted “breakthrough therapy” and fast track designations by the U.S. Food and Drug Administration (FDA), expediting research into poliovirus therapy [15].

Toca 511 is a replication-competent retroviral vector based on a modified murine leukemia virus that specifically infects actively dividing cells. Its tumor selectivity is presumably enhanced by defects in anti-viral immunity. The combination of Toca 511 and the prodrug Toca FC has obtained the “breakthrough therapy” and fast track designations by FDA, as well as Priority Medicine designation from the European Medicines Agency (EMA), and is currently tested in a large phase III trial in patients with recurrent high grade glioma [16].

1.2. Human vs non-human viruses

Humans are the natural host of many of the studied oncolytic viruses. The efficacy of oncolytic virus therapy could be severely impeded by pre-existing immunity in the human population. Although conflicting data is reported on this, neutralizing antibodies may reduce the infectivity, penetration, and spread of the viruses. To circumvent this, viruses that are rare or absent in the human population have been studied as alternative oncolytic agents. Some of these have proceeded to clinical trials in humans, such as rare serotype adenoviruses and Newcastle disease virus [17,18]. While this approach may by-pass the barrier of pre-existing immunity, it could bring along new biological risks with regard to potential virus adaptation to the human hosts [19]. These genetic alterations should be closely examined to rule out possible changes to key features of the virus, e.g. affecting shedding, stability, and human-to-human transmission.

2. Development of viro-immunotherapy as a concerted action of disciplines

The therapeutic platform of oncolytic viruses encompasses many different aspects, and therefore the clinical development of this therapy is dependent on a multidisciplinary approach. Virology expertise is needed for the production of viruses and the characterization of the virus cycle in tumor cells *in vitro* and *in vivo*. The success of future clinical studies can be greatly affected by the identification and the control of the factors that are important for viral entry, replication, spread, oncolysis, and immune activation. These factors can differ between the various oncolytic viruses, between tumor types, and in fact, between individual cancer patients. Better stratification of patients, therefore, is warranted. Moreover, information on the immune composition of the various cancer types is essential, as well as insight in the immunological consequences of the viro-immunotherapy. On top of that, in-depth knowledge on the (molecular) biology of the different cancer types and their bidirectional heterotypic interactions with the supportive microenvironment is needed. What is known about the mutations that drive progression of the cancer cells, the proportion and properties of cellular and extracellular surrounding stroma? What are prognostic markers of tumor progression? How do the tumors escape from destruction by conventional therapeutic approaches? And lastly, a firm basis of medical data should be available on the effects and impact of conventional therapies, which could set the baseline for the standard of care, and which could form a basis of historical control data to be used as comparator in the initial experiments with viro-immunotherapy.

3. Viro-immunotherapy application

In the past decade(s), immunotherapy has become a hot topic in the field of cancer therapy. Many approaches are being explored, including cancer vaccines, CAR-T cells, checkpoint inhibitors, and viro-immunotherapy. The approval of T-VEC by the FDA and EMA may pave the way for other oncolytic viruses to reach the clinic. Most clinical trials using oncolytic viruses as a monotherapy have shown at least a single round of virus replication and transgene expression (if encoded by the virus), followed by moderate anti-cancer activity. Importantly, multiple oncolytic viruses show durable albeit moderate clinical activity, which is increased when combined with other chemotherapeutic

and immunotherapeutic agents [20]. Nevertheless, many viro-immunotherapies are outcompeted by alternative (non-viral) approaches that show similar effects. Therefore, a major focus of the viro-immunotherapy research field focuses on obtaining potency-enhanced viruses. Oncolytic viruses offer a promising therapeutic option in particular for the treatment of cancers that are nearly always fatal, difficult-to-treat, and with limited improvement by other treatment strategies. The development of effective treatments for such aggressive cancer types including pancreatic cancer, advanced prostate cancer and glioblastoma is lagging. Patients suffering from these tumors have a very poor prognosis, with 5-year overall survival rates between 3–31 % [21–25], warranting the urgent need for novel innovative treatment options.

4. Determinants of tumor sensitivity and therapeutic efficacy

Importantly, tumors are very heterogeneous and complex entities, that are often partially susceptible and partially resistant to oncolytic viro-immunotherapy [4]. This has led to partial therapeutic responses and relapses. There are many barriers to overcome before oncolytic viruses can reach and eradicate a tumor. First, a physical barrier of endothelial cells, abnormal lymphatic and vascular networks, tumor-associated fibroblasts, and dense layers of extracellular matrix can impair the efficient infiltration of the viruses and immune cells into the tumor. Moreover, tumors generally exist in an immunosuppressive tumor microenvironment (TME), escaping immune surveillance, dividing rapidly, and disseminating to distant locations. They secrete many factors that suppress the activity of immune cells and may recruit immunosuppressive cells. Therefore, upon reaching the tumor, oncolytic viruses need to retain their infectivity within an immunosuppressive environment. On the other hand, they can induce strong innate anti-viral immune responses upon intracellular viral sensing, or by interactions with antigen-presenting cells (APCs). Furthermore, the viruses can be bound by pre-existing antibodies or complement factors in the circulation, hampering effective tumor infection, although therapy-enhancing effects of pre-existing immunity have also been reported [26–28]. Therefore, it may be challenging to get sufficient amounts of the viruses at the tumor site before they are cleared by the host's immune system. Once the viruses reach the tumor, a productive virus infection must be established. Viruses commonly infect cells by interacting with cell surface receptors. Cancers can downregulate the expression of these receptors, thus impeding viral entry. After virus entry, intracellular anti-viral mechanisms should be evaded and the cellular genome replication as well as protein production machineries should be hijacked to allow for efficient virus multiplication. Lastly, the virus should exit the cells to allow for viral spread. Ideally, the virus escapes through the induction of immunogenic cell death rather than a 'clean' form of cell death, since the 'sterile' release of dead cell components as well as virus particles presumably leads to very little immune stimulation. Overall, immunological effects seem to be more crucial than oncolytic effects. Effects on immune (memory) cells generally are long-lasting, as opposed to direct oncolytic effects that will stop when the viruses are cleared. Moreover, immunological effects can be systemic, despite the absence of virus at distant locations.

5. Patient stratification

In most clinical studies only a single oncolytic virus was evaluated. This virus was often chosen on the basis of availability for the study. Also remarkably, few pre-clinical studies compared the anti-tumor efficacy of different oncolytic viruses. In order to rank oncolytic viruses for use in a particular patient group, the anti-tumor efficacy of different oncolytic viruses should be compared in a single model. The results of such comparative studies can be used to identify determinants of susceptibility to virus infection, oncolysis, and immune activation, and may allow the generation of predictive molecular tumor signatures that aid in selecting the best available virus for a patient cohort. In a next step such approaches may also allow to select an oncolytic virus that may be more likely than others to be efficacious in a particular patient, in other words matching a virus to an individual patient. The generation of predictor profiles can thus be exploited to stratify patients and design personalized therapeutic plans. Treatment personalization requires reliable prediction of an individual patient's response. Therefore, relevant models that represent the inter-patient heterogeneity are crucial.

5.1. Preclinical and 'near-patient' models

Many *in vitro* studies are performed in monolayer cultures of cancer cell lines, which likely do not optimally represent the patient's cancer and the complex multicellular interactions found *in vivo*. To maximize the therapeutic relevance of findings in the laboratory, models that faithfully represent the patient's tumor (i.e. 'near-patient' models) are needed. A key uncertainty in extrapolating the experimental results obtained in such models is to what extent these models represent the tumor in the patient, and how we can assure that the outcomes of experiments with these models have a predictive value for the clinical situation.

We and others have shown that serum-free cultures of glioblastoma patient-derived tumor cells are a useful model for anti-cancer drug screenings [29,30]. They are superior to serum-supplemented cultures with regard to the tumors' genetic makeup, gene expression profile, histological phenotype in xenografts, and recapitulation of the intra- and inter-tumoral heterogeneity [31]. Using this model system, we have established a large biobank of patient-derived gliomas that have been molecularly characterized. The predictive value of such a drug-screening platform has been highlighted by a significant correlation between the response of clinical and corresponding patient-derived cell culture upon temozolomide (TMZ) treatment, the standard of care agent for the glioma patients [32]. Importantly, the results of screenings can be correlated with known molecular features of the tumors, possibly revealing predictive markers of response to treatment as well as insight into underlying pathways. Currently, the potencies of several oncolytic viruses are evaluated on the glioma cells, and correlated to molecular tumor properties such as gene expression and secreted factors (unpublished data).

Actual tumors are three-dimensional structures, as opposed to two-dimensional cell cultures. To incorporate the three-dimensional character of tumors in a model system, spheroid culture models can be used. It seems easier for a virus to reach all tumor cells in a monolayer than in the three-dimensional structure of actual tumors. Interestingly, we have previously found that some tumor cells resist oncolytic reovirus infection in monolayer, whereas the cells are efficiently infected as spheroids [33]. Receptor expression seems not to be the only determinant of susceptibility to virus infection. We demonstrated that spheroids secrete high amounts of cathepsins (like many tumors). These enzymes can extracellularly convert the virus particles into a form that can infect cells independent of the canonical receptor. This process mimics the proteolytic activation of the reovirus particles in the endosomes. Spheroids can be grown from patient tumor cells as well. These spheroids may represent the tumor cells more genuinely and therefore the clinical relevance of cells in spheroid culture may be higher than the same cells grown in monolayer cultures. Moreover, tumor stroma cells can be incorporated in the spheroids, to better represent the composition of patient tumors [34–36]. Alternatively, spheroids can be derived directly from fresh tumor tissue by cutting the tissue into small pieces, which round up in culture. Such structures, known as organotypic multicellular spheroids, retain the characteristics of the primary tumor including the presence of stroma, immune cells, and endothelial cells and have proven to be a versatile model for studying viral infection, penetration and oncolysis in three-dimensional setting (Fig. 1, upper panel) [37].

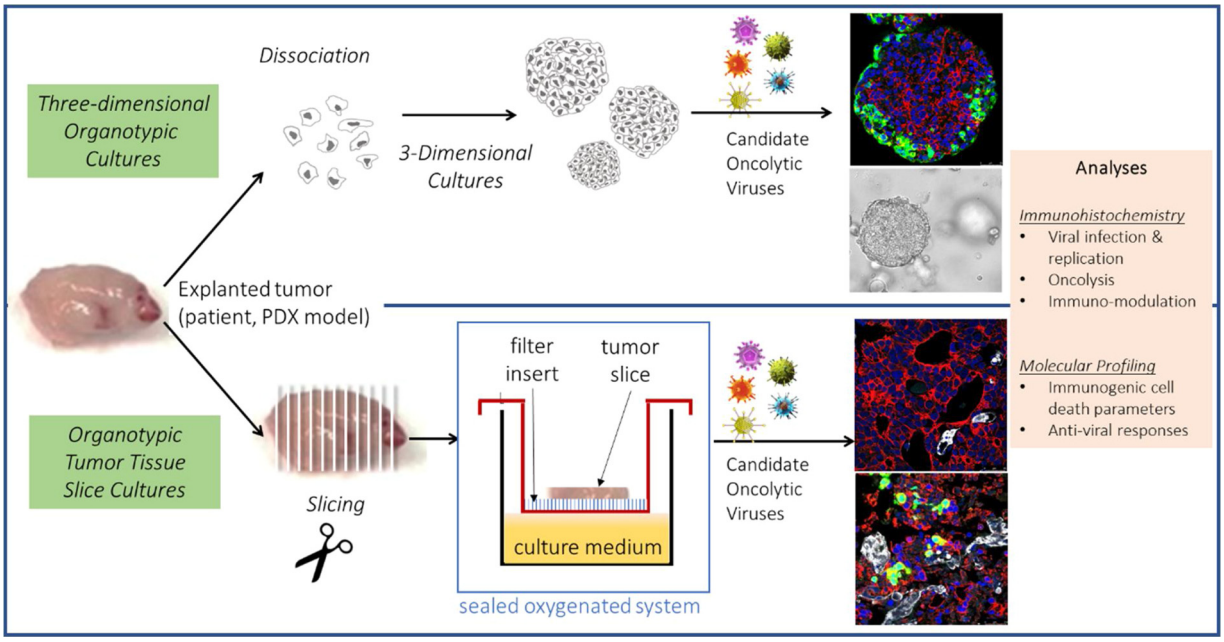

Fig. 1. "Near-patient" models that can be exploited to determine the direct and immuno-modulatory anti-tumor efficacy of candidate oncolytic viruses.
For the generation of patient-derived three-dimensional cultures (upper panel), the tissue is sliced and dissociated to obtain a single cell suspension. Subsequently, the cell suspension is cultured in the presence of specific growth factors and/or extracellular matrices that facilitate the assembly of three-dimensional cellular structures. Established three-dimensional cultures can be implemented for drug screening purposes.
Organotypic tumor tissue slices (lower panel) can be generated from neoplastic tissue types and from different locations in the human body (e.g. tissue of origin, lymph nodes, metastases). The impact of treatment with a panel of oncolytic viruses can be examined and compared on cancer cells, stromal cells (including CAFs and immune cells) and extracellular matrix molecules.

Similarly, thin slices of fresh patient-derived tumor material can be generated and maintained *in vitro*. This culture system was originally established for breast tumors [38], but has subsequently been extended to other cancer types including pancreatic adenocarcinoma [39], glioma [40], prostate [41,42], and bladder cancer [42]. This model incorporates the stromal cells and natural architecture of the tumor, allowing the monitoring of infection, penetration, and spread in a three-dimensional clinically relevant tumor model (Fig. 1, lower panel).

Another approach is to grow the tumors on the chorioallantoic membrane (CAM) of embryonated chicken eggs. In this model, tumors are implanted on the CAM, which is easily accessible by making a window in the egg shell. This system incorporates a functioning blood circulation, albeit consisting of non-human cells. It has proven useful to study pro- or anti-angiogenic agents, metastasis formation, and intra-tumoral spread of oncolytic viruses [43].

Animal models (e.g. syngeneic tumors in mice, other rodents, or spontaneous tumors in dogs) not only contain the tumor but also a blood circulation and a functional immune system. Typically, tumor cell lines are implanted into the rodents to grow tumors; these however are not representative for the tumor diversity and heterogeneity encountered in patients. Although the syngeneic model is useful to test general toxicity and immune responses to treatment, it may therefore have limited predictability for the patients, considering the large inter- and intra-tumoral variation. Another limitation is that various oncolytic viruses have a narrow host range, e.g. adenovirus does not replicate efficiently in murine cells, whereas most preclinical studies are performed in mice. Alternatively, patient-derived xenograft (PDX) models can be used, in which human material is engrafted in immunodeficient mice [44]. When using patient-derived tumor tissues, this system may retain the molecular characteristics of the original tumor, the tissue architecture, the stromal components, and the inter- and intra-tumoral heterogeneity. Therefore, it may reflect with some accuracy the complex interactions between tumor cells and tumor microenvironment in the patient. However, xenograft model experiments require substantial amounts of patient resection material, as well as time and money. Notably, xenograft models usually cannot be used to study the effect of immune modulation as this requires immune-competent models. Consequently, the downsides of available mouse models pose a challenge in selecting a suitable in vivo model for preclinical OV testing. It may therefore be useful to test virotherapy approaches in alternative 'near-patient' model systems to predict the potential antitumor efficacy of particular oncolytic viruses.

5.2. Data and sample collection from OV trials

Apart from the development of representative in vitro and in vivo models to study heterogeneity in response to various OV strains, efforts should be made to gain as much information as possible from clinical trials testing these agents. Comprehensive clinical data as well as clinical samples such as blood, post-treatment tissue biopsies or CSF can allow correlation of patient response to clinical parameters, molecular features or specific biomarker profiles of the patient or tumor and should therefore be collected and stored as much as possible, if not for prospective analysis then for future retrospective validation studies. Such an approach was taken for an oncolytic measles virus tested in glioblastoma patients. Analysis of sensitive versus resistant PDX xenografts identified constitutive interferon pathway activation as the key determinant for MV replication. Interestingly, analysis of patient tumors from a clinical trial testing this OV, revealed that viral replication in patient tumors was indeed inversely correlated with expression of this resistance gene signature, supporting the translational value of predictive profiles identified using preclinical model systems [45]. In two Finnish studies, retrospective analysis of clinical variables and laboratory tests of over 200 patients treated with various adenovirus-based OVs, revealed associations between treatment outcome and several variables such as pretreatment neutralizing antibodies, tumor burden, total leukocyte count, neutrophil counts, and HMGB1 baseline status [46,47]. Together, these results highlight the importance of collecting clinical data and samples. Future clinical studies are required to validate these potential biomarkers of response and explore their

relevance in also other types of OVs.

6. Choice of oncolytic virus candidates

With every individual researcher or research group typically studying his/her 'favorite' virus, the question remains how we can objectively determine which virus is optimal in a particular cancer or even a specific patient. Here, we discuss some considerations in virus selection, in addition to the obvious importance of tumor selectivity, virus pathogenicity, immunogenicity, and stability. There are no general rules for predicting how effective a virus can function as an oncolytic agent. It goes without saying that the candidate virus should not be pathogenic in humans, and not pose a risk to non-human hosts to which a virus could be transmitted. Candidate viruses have been found in all realms of virology, ranging from small non-enveloped and single-stranded RNA viruses (e.g. attenuated poliovirus, a picornavirus) to large enveloped double-stranded DNA viruses (e.g. attenuated herpes simplex virus). The viruses can be of human origin (e.g. attenuated human adenovirus type 5), or from animal origin (e.g. Newcastle disease virus, an avian paramyxovirus, or the rat H1 parvovirus [48]). For some of the viruses there is a known safety profile, for instance because the virus has been in use as a prophylactic vaccine. This is the case for the Copenhagen strain of the vaccinia virus, a pox virus [49]. Further modifications, such as deletion of the TK and the F1L gene, can increase the tumor selectivity, and further reduce the chance of reversion to a pathogenic phenotype. For other viruses such pre-existing safety profile is absent, nevertheless, they could be developed for clinical evaluation (e.g. Seneca Valley Virus, a picornavirus putatively of porcine origin [50]).

Conclusively, an efficacious oncolytic virus should not cause any disease, preferentially infect cancer cells over normal cells, efficiently enters cancer cells, should not be hampered by pre-existing anti-viral immune responses, and should kill all cancer cells in an immunogenic manner that induces strong, systemic, and lasting anti-cancer immunity.

The cancer cells on the other hand show plasticity and diversity, and this may allow cancer cell populations to escape from the viruses. Therefore, it seems unrealistic that a single oncolytic virus used as a monotherapy is able to cure cancers in all patients. As a result, they are increasingly used in combination therapies. At this moment clinical studies combining oncolytic viruses with immune checkpoint inhibition are exploring whether the viruses can be used to activate the immune system in those tumors exhibiting a paucity of tumor T cell infiltration, the so called "cold" tumors [51]. For such an approach the availability of an oncolytic virus that can infect the tumor cells and kill the cells in an immunogenic manner, is a necessity.

7. The consortium approach

The promising (pre)clinical results obtained with oncolytic viro-immunotherapy stimulates the further development of the approach, especially for the aggressive cancer types for which the current therapy options are grossly inadequate. The complexity and multi-faceted nature of the approach make the translation of oncolytic virus research into an effective therapeutic approach ambitious and challenging.

In 2012, the Dutch Oncolytic Viro-ImmunoTherapy (OVIT) consortium was established. This multi-disciplinary consortium consists of researchers and medical specialists of a dozen different departments in three Dutch universities (Rotterdam, Leiden, and Utrecht), as well as research centers such as Sanquin. The various participants include experts in oncology, virology, gene therapy, tumor biology, immunology, as well as several excellent medical oncology specialists. They combine their knowledge, expertise, research protocols, and preclinical models with the goal to jointly develop and implement innovative viro-immunotherapeutic approaches for different types of cancer in the Netherlands. Whereas traditionally, research groups only cover a relatively narrow research area, this consortium approach accelerates the execution of preclinical research and gears up for subsequent clinical trials. The participants collectively address the challenges that are faced in developing and implementing oncolytic viro-immunotherapy in the clinic, such as the generation of oncolytic viruses with improved potency and safety, the pre-clinical efficacy testing in near-patient models, the development of strategies for overcoming heterogeneous tumor responses, and the production of clinical-grade virus batches. The primary focus lies on difficult-to-treat, aggressive cancer types that urgently need new treatment strategies: pancreatic cancer, bladder cancer, prostate cancer, and glioblastoma. To this end, participating researchers of the OVIT consortium are currently performing comparative pre-clinical studies to define the therapeutic potential of adenovirus, Newcastle disease virus, and reovirus in near-patient models, to identify which virus is most potent in which tumor, and to elucidate underlying factors.

7.1. Viruses studied within the consortium

Adenoviruses are one of the most extensively studied oncolytic viruses due to their good safety profile in clinical trials, relative straightforward genetic modification, and feasibility to generate high-titer virus batches. We have extensively evaluated the therapeutic potential of the conditionally replicative adenovirus Ad5-Delta24RGD in preclinical and clinical phase I/II studies for glioblastoma. This virus has, in addition to the 24-bp deletion in E1A to improve cancer cell selectivity, an expanded tropism toward αvβ3/αvβ5 integrins by insertion of an Arg-Gly-Asp (RGD) motif into the fiber knob [52]. In our preclinical studies, we have shown that Ad5-Delta24RGD lyses patient-derived malignant glioma- and glioma stem cells, as well as both xenograft and syngeneic glioblastoma tumors in mice [53–55]. This occurs at a highly variable efficacy, with some being efficiently eradicated whereas others are largely resistant to the virus. Analysis of T cell reactivity and local cytokine levels revealed that the efficacy of this virus seems to be dependent on the virus-induced anti-tumor immune responses [55]. Analysis of CSF samples of GBM patients treated with Ad5-Delta24RGD, also revealed interpatient variability in cytokine induction, and concurrent variability in macrophage activation toward the proinflammatory phenotype [56]. Importantly, neutralizing antibodies for Ad5 are prevalent in the human population. To circumvent potential detrimental effects of pre-existing immunity on treatment efficacy, current efforts aim to generate and test oncolytic adenoviruses of non-human primate origin, in addition to Ad5-Delta24RGD.

Newcastle disease virus (NDV) has a natural avian host range but also displays anti-tumor activity, with the degree of virulence in birds correlating with the oncolytic potential [9]. Research efforts primarily focus on the generation of NDVs with improved efficacy and safety profile. We have performed preclinical tests with wild-type NDV, demonstrating anti-tumor activity in cancer models *in vitro* and *in vivo*, as well as safety in non-human primates [57–59]. Moreover, we have generated several modified NDVs harboring immunomodulatory transgenes, which showed improved oncolytic efficacy, without dramatically affecting the virulence [58]. The virulence of NDV strains poses an environmental risk, due to high susceptibility of poultry to infection. Therefore, the safety profile of the virus required continuous caution and care.

Reovirus has inherent oncolytic properties and is not associated with serious human disease. Clinical trials have demonstrated safety and moderate anti-tumor activity. Although presumably not the only determinant [33], scarcity of reovirus receptors on tumors could confer resistance to reovirus infection [60–62]. We previously described the isolation of reoviruses with an expanded tropism, beyond the canonical receptor JAM-A [63]. These mutants represent a promising virotherapeutic agent to target those tumors that display limited expression or inaccessibility of JAM-A.

7.2. Clinical translation

Next to comparative studies to define the therapeutic potential of the viruses, the production process of clinical-grade virus batches is being streamlined within the OVIT research groups. Furthermore, the consortium is involved in an extensive study on the risk assessment of the oncolytic viruses, investigating the risks involved in virus stability, shedding, and pathogenicity. This project is unique in that it convenes twice a year with the stakeholders involved, including the local and governmental authorities. In addition to the design of safe therapeutic approaches, the acquired data will be used to establish a modernized framework for regulations and reviewing of such approaches.

The consortium as well as its participating groups have benefitted from the concerted visions and goals. The consortium teamed up with the Dutch foundation “Overleven met Alvleesklierkanker” (Surviving with Pancreatic Cancer), which provides support to participating groups for distinct subprojects and invests in the infrastructure necessary to produce clinical grade vectors. Moreover, the individual groups members have successfully acquired grants for subprojects at several other grant organizations. The involvement in the consortium has allowed the participating groups to expand the ambition and will allow us to become internationally competitive, while maintaining a large degree of freedom to plan and perform clinical trials with one or more of our oncolytic agents. This should lead to efficacious and affordable treatment strategies in which oncolytic viruses are used to improve the therapy for those cancer patients who currently have dismal prospects. The help of the patients and the patient societies in this is key!

CRediT authorship contribution statement

Vera Kemp: Writing - original draft, Writing - review & editing. **Martine L.M. Lamfers:** Writing - review & editing. **Gabri van der Pluijm:** Visualization, Writing - review & editing. **Bernadette G. van den Hoogen:** Writing - review & editing. **Rob C. Hoeben:** Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

Vera Kemp and Rob C. Hoeben are inventors of patent applications on new adenoviruses for use in oncolytic virus therapy. These applications are assigned to LUMC. Rob C. Hoeben has received research funds from Janssen Vaccines & Prevention, Leiden, for studies on adenoviruses. Martine L.M. Lamfers has consulted for DNAtrix Therapeutics Inc. for which Erasmus MC has received compensation. Gabri van der Pluijm and Bernadette van den Hoogen declare no competing interests.

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Appendix A. Supplementary data

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Dr. Martine Lamfers received a MSc degree in Cell Biology from the University of Amsterdam and a PhD in Gene Therapy for vascular disease from Leiden University before joining the Dept of Neurosurgery at the VU Medical Center in Amsterdam for post-doctoral research focusing on gene and viral strategies to combat malignant brain tumors. Additional post-doctoral training was done in the Neuro-Oncology Laboratories at Massachusetts General Hospital in Boston. In 2007 she was appointed at assistant professor and in 2017 as associate professor, at the Dept of Neurosurgery, Erasmus MC Rotterdam, where she heads the Neurosurgery lab. Her research is focused on the development of novel treatments for malignant glioma using innovative patient-derived model systems. She has set up a drug development pipeline encompassing biobanking of patient tissue and derived cell cultures, in vitro 2D and 3D drug screening and in vivo validation. She was also involved in the preclinical development of the oncolytic virus Delta24-RGD and in a trial-associated study on the immune response to Delta24-RGD infusion in recurrent glioblastoma patients. Ongoing research is focused on strategies to improve efficacy of oncolytic viral therapy as well as implementing patient-derived models for predicting response to therapy and/or selecting the most optimal (viral) treatment for a specific patient. Martine is a co-founder of the consortium on 'Oncolytic-Immuno-Viro- Therapy (OVIT).

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Dr. Bernadette van den Hoogen heads the research groups on "Pneumoviruses and interactions with the host immune system'' and on "Oncolytic viruses" at the department of Viroscience, Erasmus MC. She obtained her PhD at this department on "the Discovery and Characterization of the Human Metapneumovirus (HMPV)". Since then, she has been investigating all aspects of the interaction between HMPV and the human immune system. For this research she was funded by the Netherlands Organisation for Scientific Research (NWO) with a VENI grant and by the EU's Horizon2020 research and innovation programme for her Innovative Training Network (ITN) 'INITIATE" (https://initiate-itn.eu/). The Oncolytic Virus Research group of Bernadette has been focusing on the use of viro-immuno therapy with NDV for treatment pancreatic adenocarcinoma's. Together with Prof. R. Fouchier and Prof. R. Hoeben (LUMC) she received a grant from the Netherlands Organisation for Scientific Research (NWO) to establish the safety of these oncolytic viruses for humans and poultry. Bernadette is co-founder of the consortium on 'Oncolytic-Immuno-Viro- Therapy (OVIT), in which Dutch researchers collaborate with the aim to bring oncolytic virotherapy to the clinic. To this end, Bernadette's research group also received funding from the foundation "Overleven met alvleesklier kanker' (OAK).

Dr. Rob Hoeben is a 1978 Biology graduate of Utrecht University. He performed his PhD research at Leiden University where he obtained his PhD in 1986. Dr Hoeben performed his post-doctoral studies at Leiden University with Dr. Alex van der Eb from 1991 to 2000. In 1996 he became a staff member of the Department of Medical Biochemistry at Leiden University, and in 2000 he was appointed as full professor at Leiden University and has been working at the Department of Cell and Chemical Biology of the Leiden University Medical Center. Here he heads the Virus and Stem Cell Biology lab. The current work of his group is focused on developing new viruses for use in oncolytic virus strategies. In these studies he employs primarily human reoviruses and adenoviruses. Dr. Hoeben has been one of the founding members of the Dutch Society of Gene and Cell Therapy and served on the board for 18 years. Rob is also co-founder of the OVIT consortium, in which Dutch researchers collaborate with the aim to bring oncolytic virotherapy to the clinic.