Changing Faces in Virology: The Dutch Shift from Oncogenic to Oncolytic Viruses

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Abstract

Viruses have two opposing faces. On the one hand, they can cause harm and disease. A virus may manifest directly as a contagious disease with a clinical pathology of varying significance. A viral infection can also have delayed consequences, and in rare cases may cause cellular transformation and cancer. On the other hand, viruses may provide hope: hope for an efficacious treatment of serious disease. Examples of the latter are the use of viruses as a vaccine, as transfer vector for therapeutic genes in a gene therapy setting, or, more directly, as therapeutic anticancer agent in an oncolytic-virus therapy setting. Already there is evidence for antitumor activity of oncolytic viruses. The antitumor efficacy seems linked to their capacity to induce a tumor-directed immune response. Here, we will provide an overview on the development of oncolytic viruses and their clinical evaluation from the Dutch perspective.

A Historic Perspective

FROM THE 1960s ONWARD, the relation between viruses and their host cells has been much studied in the Netherlands. Initially, the focus was on the biology of bacteriophages as well as on viruses from higher eukaryotes. Subsequently, viruses were studied as model systems for cellular processes. In Utrecht, Sussenbach and Van der Vliet studied the fate of adenovirus DNA in the host cells and the mechanisms that adenoviruses use to replicate their DNA genome. This shed light on the viral and cellular proteins that are involved in adenovirus replication. These studies identified the role of the human transcription factors NF-I and NF-III/OCT1 in orchestrating the initiation complex involved in adenovirus type 5 DNA replication, illustrating the interplay between cellular and viral processes involved in adenovirus replication [reviewed in (Hoeben and Uil, 2013)].

The observations that some viruses could transform cells were intriguing and stimulated studies to obtain insights in the processes involved in cellular transformation. Van den Noordaa in Amsterdam and Van der Eb in Leiden used adenovirus and SV40 as tumor-inducing agents in rodent models. Graham and Van der Eb demonstrated that human cells too could be transformed by naked adenovirus DNA, using their calcium phosphate co-precipitation technique for the efficient transfer of naked DNA into target cells (Graham and van der

Eb, 1973). Subsequently, these techniques were used to study the differences in oncogenic potential of the adenovirus types 5 and 12. The difference was found to correlate with the differential effects of the adenovirus E1 proteins on the expression of cellular MHC-class I molecules (Bernards *et al.*, 1983; Schrier *et al.*, 1983). This linked antigen presentation with oncogenicity. This and subsequent research also yielded the 293, 911, and PER.C6 cell lines that we now use so often as tools for research- and clinical-grade production of adenovirus vectors (Fallaux *et al.*, 1999).

At the Radiobiological Institute in Rijswijk, Bentvelzen studied transmission of the mouse mammary tumor virus (MMTV) and the way MMTV induced breast cancer in rodents. In the 1980s a similar approach was taken by Berns at the Netherlands Cancer Institute in Amsterdam, who analyzed murine leukemia viruses' integration sites to identify cellular genes and pathways involved in leukemogenesis in mice. Hence, oncogenic viruses and their associated cancers were actively being investigated by several Dutch groups.

It was in this environment that the Dutch entered the gene therapy arena. In the Van der Eb lab, Valerio was a pioneer in this field and has been among the first to clone the adenosine-deaminase gene and its cDNA (Valerio *et al.*, 1985). He demonstrated that retroviral vectors could be constructed for functional expression of the cDNA. Valerio continued his research in the Radiobiological Institute in Rijswijk, where he

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benefitted from the expertise in hematopoietic stem cell research of Van Bekkum and of the facilities for bone marrow transplantation research in monkeys. Following the pioneering work of the team led by W. French Anderson, R. Michael Blaese, and Ken Culver at the NIH, who were the first to treat children suffering from severe combined immune deficiency due to adenosine deaminase deficiency (ADA-SCID) with gene-modified autologous T cells (Culver *et al.*, 1991), Valerio and his team focused on the modification of bone marrow stem cells. Their preclinical studies (van Beusechem *et al.*, 1992) finally led to one of the first clinical studies in which retroviral vectors were used for transfer of the ADA gene into hematopoietic stem cells (Hoogerbrugge *et al.*, 1996). These studies offered hope for a cure for ADA-SCID patients. Indeed, this application of gene therapy has been a trail blazer ever since and to date gene therapy can be successfully applied in patients with ADA-SCID.

Cancer Gene Therapy

In the 1990s cancer became a more prominent target for gene therapy research in the Netherlands. Osanto and collaborators initiated a clinical study involving vaccination with allogeneic tumor cells that were modified to express interleukin 2 (IL-2), in patients with metastatic melanoma (Osanto *et al.*, 1993). Subsequently, several groups developed tumor-targeted replicating and nonreplicating vectors for cancer therapy. The scientists were very keen in developing not only new vectors but also new systems for testing the safety and efficacy of their approaches. The preclinical testing involved a range of different assays involving tumor cells grown in monolayer and in spheroid cultures. In addition, new testing systems have been developed that mimic tumors more faithfully. These models include tumor cell explants, tissue slices, and human tumors grown on embryonated chicken eggs (Grill *et al.*, 2002; Rots *et al.*, 2006; Durupt *et al.*, 2012). Recently, the merits of the various preclinical tumor models have been excellently reviewed (de Jong *et al.*, 2014).

Several of replication-defective adenovirus and retrovirus vectors have been evaluated in early-phase clinical trials in the Netherlands. At least four clinical centers participated in cancer gene therapy clinical trials (namely, Erasmus Medical Center, Rotterdam; Leiden University Medical Center; the VU University Medical Center, Amsterdam; and the University Medical Center Groningen). In these studies, vectors encoding prodrug-activating enzymes were used. In addition, in several other studies the focus has been on immunogene therapy approaches for cancer, employing adenovirus, canary pox, or plasmid vectors (Schenk-Braat *et al.*, 2007).

Viruses as Replication-Competent Anticancer Agents

Also, the use of replication-competent viruses that preferentially infect and kill cancer cells has been explored for cancer treatment. Such viruses are designated as oncolytic viruses. So far, adenovirus has been the prime oncolytic virus in Dutch preclinical research on viral anticancer agents. A main theme in Dutch research on adenovirus vector development was directed at tumor targeting. Transcriptional targeting of gliomas could be achieved by inclusion of promoters specifically expressed in glial and glioma cells (de Leeuw *et al.*, 2006). In Amsterdam, Bosma's group targeted gas-

trointestinal tumors (Wesseling *et al.*, 2001). Several approaches for capsid modification and use of bispecific antibody targeting adapter molecules that were first evaluated on replication-defective gene transfer vectors (van Beusechem *et al.*, 2000, 2002a; Vellinga *et al.*, 2004; Brouwer *et al.*, 2007; Schagen *et al.*, 2008; Kuhlmann *et al.*, 2009; de Vrij *et al.*, 2012) were later implemented in oncolytic adenoviruses (van Beusechem *et al.*, 2003; Carette *et al.*, 2007; Sebestyen *et al.*, 2007). A transcriptionally targeted oncolytic adenovirus has recently undergone an extensive preclinical safety assessment and is currently being evaluated in an investigator-driven clinical trial in Rotterdam (Schenk *et al.*, 2014).

In addition, successful tropism modifications developed abroad were adopted, such as the incorporation of an integrin-binding RGD motif in the adenovirus fiber, the use of fiber-switch mutants, or the use of affibody molecules as targeting ligands (Lamfers *et al.*, 2002; Witlox *et al.*, 2004; Magnusson *et al.*, 2012). A second focus of Dutch oncolytic adenovirus research was on augmenting lytic replication in cancer cells and spread in solid tumors. Examples include the p53 tumor suppressor protein that promoted adenovirus-induced cell death resulting in accelerated progeny virus release and more effective tumor eradication (van Beusechem *et al.*, 2002b; Georger *et al.*, 2004; Heideman *et al.*, 2005), a carboxylesterase prodrug-converting enzyme for more effective combined oncolytic adenovirus plus enzyme prodrug therapy (Oosterhoff *et al.*, 2005), and proapoptotic and anti-angiogenic tissue inhibitor of metalloproteinase-3 (Lamfers *et al.*, 2005). Vectors were designed for targeting a variety of cancer types involving all major cancer types and evaluated in relevant preclinical models.

More recently, the Dutch biotechnology company ORCA Therapeutics constructed an oncolytic adenovirus that incorporates a gain-of-function mutation that was identified by Alemany and colleagues in Spain (Gros *et al.*, 2008). The mutation relocates the E3/19K protein to the plasma membrane, inducing membrane permeabilization and enhanced virus release. The virus is currently in development for clinical testing in prostate cancer. In addition, Dutch researchers developed technology to exploit RNA interference in the context of a replicating oncolytic adenovirus (Carette *et al.*, 2004). A short hairpin expressed from the genome of an oncolytic adenovirus that was replicating in human cancer cells was properly processed by the cellular RNAi machinery and selectively silenced its target gene. Hence, it now became possible not only to express exogenous transgenes to strengthen oncolytic virus replication, but also to silence endogenous host gene expression that might inhibit effective oncolytic adenovirus replication. This opened new possibilities for improved oncolytic virus design. The oncolytic adenovirus RNAi can also be used to modulate immune responses in the tumor microenvironment (see below).

There is a high medical need to treat advanced cancers. Therefore, virus delivery methods are being sought that allow systemic administration to reach tumor metastases. Intravascular delivery of adenovirus 5 vectors is hampered by the effective sequestration in the liver, interaction with blood cells and plasma proteins, and effective recognition by the host innate immune system [reviewed in (Hendrickx *et al.*, 2014)]. These effects have been widely recognized as confounding effective therapy and have directed research into

adenovirus vector engineering. Contributions of Dutch researchers in this area include exploiting the natural diversity in adenovirus serotypes to construct adenovirus vectors that bypass preexisting immunity (Vogels *et al.*, 2003; Holterman *et al.*, 2004) and evaluating Ad5 capsid modifications that abolish interactions with natural receptors (van Beusechem *et al.*, 2000; Schagen *et al.*, 2006). Schagen *et al.* presented an adenovirus vector lacking binding sites for native receptors, blood-clotting factors, and complement components in its capsid. Consequently, the vector exhibited diminished capacity to bind human erythrocytes, strongly reduced hepatic tropism, and improved bioavailability upon systemic delivery to mice (Schagen *et al.*, 2008). Also, physical coating of adenovirus particles has been shown to contribute to systemic anticancer efficacy with acceptable toxicity, at least in animal models (Morrison *et al.*, 2008; Doronin *et al.*, 2009).

Interestingly, mutant adenoviruses that were obtained by bioselection displayed improved systemic antitumor efficacy (Kuhn *et al.*, 2008) or increased blood persistence and enhanced antitumor efficacy (Gros *et al.*, 2008). Such bioselections will further be facilitated by the use of error-prone adenovirus DNA polymerase mutants for generating the genetic diversity (Uil *et al.*, 2011). The use of such mutants enhances the spontaneous mutation rate during adenovirus replication by more than two orders of magnitude. The evolution and bioselection of replicating adenoviruses on SKOV-3 human ovary carcinoma cells yielded viruses with a mutation in the splice acceptor site preceding the open reading frame encoding the adenovirus death protein (ADP). The mutation results in premature expression of ADP and accelerates tumor cell lysis (Uil *et al.*, 2011).

An interesting development is the use of cellular carriers to transport oncolytic viruses through the bloodstream toward tumors (Nakashima *et al.*, 2010). This way, the virus may be protected from clearance by the immune system and from sequestration by nontarget tissues. A promising cell type for this purpose is mesenchymal stem cells (MSC), which have the ability to home toward tumors. Oncolytic adenoviruses were successfully delivered at orthotopic breast and lung tumors following intravenous administration (Hakkarainen *et al.*, 2007) and to orthotopic brain tumors following carotid artery injection (Yong *et al.*, 2009). Intriguingly, adenovirus infection was found to compromise the homing capacity of adipose tissue-derived stem cells in mice bearing orthotopic human glioma xenografts (Lamfers *et al.*, 2009). The mechanism responsible for the observed inhibition remains to be elucidated. Furthermore, it is not known whether the cargo virus should be replication competent in the MSC or that the MSC merely hand off the virus particles to which they were exposed. An interesting approach is the use of pharmaceutical induction of oncolytic virus release after MSC infiltration into the tumor (Hsiao *et al.*, 2012). Another carrier system was studied by Balvers *et al.* (2014), who demonstrated the potential of employing T-cell-derived Jurkat cells to deliver oncolytic adenovirus to the core and infiltrative zones of intracranial glioma in mice, thereby opening prospective for future autologous T-cell-mediated oncolytic virus delivery.

Although clear advances are being made toward oncolytic adenoviruses that can be used systemically, clinical implementation of systemic cancer treatment with oncolytic viruses is not yet being considered in the Netherlands. The Dutch ongoing and planned clinical trials with oncolytic

viruses treat locally recurrent prostate cancer or glioblastoma multiforme. In the latter case, the virus is administered by convection-enhanced delivery (CED) to locally enhance vector distribution. In this method the virus is slowly infused via catheters implanted in the brain, thus bypassing the blood-brain barrier. In a rat model, Idema *et al.* (2011) demonstrated that distribution of adenovirus particles delivered via CED is at least partially volume dependent when the virus was infused in white matter tracts, but not in the gray matter. Distant tumors could be reached, but penetration into these tumors was limited (Idema *et al.*, 2011). Therefore, the CED method seems particularly useful for delivery of oncolytic adenoviruses to infiltrative brain tumors in which cancer cells migrated into the surrounding brain parenchyma.

While various groups have been evaluating replication-competent adenovirus vectors in preclinical studies, so far only two replicating oncolytic viruses have reached the stage of clinical evaluation in the Netherlands. In a Rotterdam-Amsterdam collaborative study, the expanded-tropism tumor-selective adenovirus Ad5-Delta24RGD is evaluated for treatment of glioblastoma. So far, approximately 20 patients have been enrolled in this phase I/II study. Administration of the virus by CED was found to be safe, with no interruption of the phase I dose escalation because of patient safety issues (C. Dirven, Rotterdam, The Netherlands, personal communication). In addition, Bangma's group in Rotterdam studies the safety of the conditionally replicative adenovirus vector Ad[I/PPT-E1A] in a phase I dose escalation trial in patients with localized prostate cancer who are scheduled for curative radical prostatectomy. So far, no data have been published from either of these clinical studies. Both studies have benefited from the dedicated grant support of the ZonMw Translational Gene Therapy Research Program subsidized by the Dutch Health Ministry.

In parallel, other viruses too have been exploited in preclinical studies. Van den Hoogen explored unmodified Newcastle diseases virus (NDV) as an oncolytic agent in a panel of human pancreatic carcinoma cell lines in order to assess the heterogeneous properties of human pancreatic carcinoma (Buijs *et al.*, 2014). The Gerritsen and Rottier labs experimented with targeting nonhuman coronaviruses to human tumor cells using a bispecific single-chain antibody (Wurdinger *et al.*, 2005). Along the same lines, these groups redirected the coronavirus murine hepatitis virus (MHV), which is normally unable to infect human cells, to human tumor cells via a soluble receptor fused with an epidermal growth factor receptor (EGFR)-targeting moiety. Inclusion in the MHV genome of an expression cassette encoding the fusion protein allowed infection of EGFR-expressing tumor cells. Administration of this virus in mice bearing lethal intracranial EGFR-expressing gliomas could prolong the survival of the treated mice compared with mock-infected or control virus-infected mice (Verheije *et al.*, 2009).

Kranenburg exploited reovirus and studied the mechanisms of the reovirus' preference for lysing tumor cells rather than normal nontransformed cells (Smakman *et al.*, 2005). These studies provided evidence that the RAS signaling in tumor cells may facilitate reovirus infection at various levels. It stimulates reovirus entry by stimulating virus uncoating, reovirus gene expression, genome replication, and reovirus egress. To further develop reoviruses as oncolytic agents, Hoebe's lab used both genetic modification and bioselection

of spontaneous mutants to obtain reoviruses with an amended tropism. Genetically targeted viruses were generated that could use an artificial receptor on cells that lack expression of the canonical reovirus receptor JAM-A on the cell surface (van den Wollenberg *et al.*, 2008). The bioselection approach yielded tropism- and host-range-expanded reoviruses capable of infecting cells independent of JAM-A, purportedly via interaction via sialic acids (van den Wollenberg *et al.*, 2012). These viruses may be useful as oncolytic agents in tumors that do not express JAM-A on their cell surface. Although promising, it remains to be established how important JAM-A expression is for wild-type reovirus infection of tumor cells *in vivo*.

Recently, it was demonstrated that reoviruses can recruit an alternative high-affinity receptor, that is, the Nogo receptor NgR1, to infect neurons in the murine central nervous system (Konopka-Anstadt *et al.*, 2014). Moreover, in spheroid cultures of glioma cells, the reoviruses can enter via a route that is distinct from the route used in monolayer cultures of the same cells. Whereas in monolayer cultures of U118MG glioma cells the wild-type reovirus relies on JAM-A binding for entry, JAM-A expression is dispensable on the same cells grown in multicellular spheroids (Dautzenberg *et al.*, 2014). This could be attributed to the action of extracellular factors as incubation of wild-type reoviruses with conditioned medium from spheroid cultures converted the viruses to particles that are infectious also in monolayer cultures of JAM-A-deficient U118MG cells. Presumably, the acidified environment and the high amounts of cathepsins allow conversion of the viruses to partially uncoated infectious subviral particles (ISVPs). These ISVPs can associate directly with the plasma membrane and penetrate the membrane, allowing JAM-A and endosome-independent entry into the cytoplasm (Dautzenberg *et al.*, 2014). Such mechanisms may contribute to the varying efficiencies of reovirus transduction that was observed in cultures of resected glioma cells (van den Hengel *et al.*, 2013). These data illustrate that while monolayer cultures are extremely useful for studying biological parameters of oncolytic viruses, care should be taken in extrapolating these data to predict the performance of the oncolytic viruses in tumors.

From Virotherapy to Immunotherapy

For many years, the principal mode of action for oncolytic viruses was believed to be the direct lytic effect on tumor cells. Based on this concept, which was adopted by most Dutch researchers, numerous strategies were developed to enhance the virus entry, replication, spreading, and cytolytic activity as described above. These strategies also included the inhibition of the antiviral immune responses to prevent rapid viral clearance and loss of oncolytic activity such as co-treatment with cyclophosphamide (Lamfers *et al.*, 2006).

In oncolytic virus therapy, the immune system is both a foe and a friend. While it protects the host by containing viral infections, it may also frustrate efficient infection, replication, and spread of the therapeutic viruses, thereby limiting antitumor efficacy. Moreover, it is becoming increasingly apparent that oncolytic viruses are also capable of initiating an immune reaction that can lead to the development of tumor-specific T-cell responses. In fact, terminology for this form of cancer treatment is shifting from oncolytic virus therapy to

viro-immunotherapy, immunovirotherapy, or cancer/oncolytic vaccine treatment.

Some of the first evidence for this aspect of oncolytic efficacy came from the field of herpes simplex virus (HSV) therapy. Preclinical studies performed in the laboratories of Fraser (Philadelphia, PA) and Martuza (Washington, D.C.), using HSV1- and HSV2-based oncolytic viruses, respectively, demonstrated systemic antitumor immunity upon treatment of immune-competent animals. This was associated with an elevated cytotoxic T lymphocyte (CTL) activity specific to the tumor cells (Todo *et al.*, 1999; Miller and Fraser, 2000, 2003; Nakano *et al.*, 2001). Comparison of different HSV-based oncolytic viruses in a breast cancer model revealed variable levels of virus replication and clearance from the tumor. However, the therapeutic activity was related to levels of danger-associated molecular patterns (DAMPs), intratumoral antigen-presenting cells, and circulating antigen-specific CD8⁺ T cells, suggesting that the initial stages of HSV-induced activation of antitumor immunity are more important than viral persistence within the tumor (Workenhe and Mossman, 2014).

Studies into the antitumor immune stimulatory role of adenoviruses have lagged behind other oncolytic viruses because of the species specificity of human adenovirus and the hence consequential limitation to xenograft models in immune-deficient mice. Moreover, the relative ease of genetic modification of the adenoviral genome facilitated the development of a range of oncolytic adenoviruses armed with immune-stimulating transgenes (Alemany, 2012). This strategy has yielded promising results, but the intrinsic immune-stimulating activity of the adenovirus itself still remains to be fully charted.

Work on telomerase-dependent replication-competent adenoviruses demonstrated that upon tumor cell infection, danger signaling molecules are released, and dendritic cells (DCs) produce IFN- γ and IL-12, yielding tumor-directed CD8⁺ cytotoxicity that also inhibits distant metastases (Endo *et al.*, 2008; Edukulla *et al.*, 2009). In the Netherlands, Lamfers and colleagues demonstrated that the oncolytic adenovirus Ad5-Delta24RGD is capable of eliciting an effective T-cell-mediated antitumor response and long-lasting immunity in an orthotopic model for glioma. Co-treatment with dexamethasone completely abrogated this effect, further supporting the role of the antitumor immune response in oncolytic adenovirus therapy (Kleijn *et al.*, 2014). Interestingly, even in the absence of viral replication, adenovirus has been shown to induce an immune response against the tumor cells in which it resides. This was demonstrated to occur upon administration of adenovirus vectors in a syngeneic rat model for colorectal liver cancer (Geutskens *et al.*, 2000). Another approach in which an adenovirus is employed as an effective vaccine vehicle is the CD40-mediated DC-targeted vaccine in a murine B16 melanoma model. CD40 targeting of Ad-gp100, a melanoma-associated tumor antigen, significantly enhanced antitumor efficacy by induction of tumor-specific CD8 T cells (Hangalapura *et al.*, 2011).

The limited host range is much less a problem in the mammalian orthoreovirus T3D. With reoviruses, the antitumor efficacy can be evaluated in immune-competent animal models. This allows studies on the contribution of the immune system to antitumor efficacy (Smakman *et al.*, 2006). The immune response to reovirus treatment is well characterized in a number of studies from the laboratories of Melcher (Leeds, United Kingdom), Vile (Rochester, MN), Harrington

(London, United Kingdom), and Pandha (Guildford, United Kingdom).

In melanoma, prostate cancer, and ovarian carcinoma models, it was shown that reovirus-induced tumor cell death leads to cytokine and chemokine production and the display of tumor-specific immunogenic peptides on DC. The DCs produce a range of cytokines and chemokines, undergo maturation, and migrate into the tumor microenvironment along with CD8⁺ T cells and NK cells (Errington *et al.*, 2008; Prestwich *et al.*, 2008; Gujar *et al.*, 2010; Steele *et al.*, 2011). Reovirus treatment was also shown to decrease the frequency of immune-suppressive cells, such as myeloid-derived suppressor cells, and regulatory T cells (Gujar *et al.*, 2013). Also in clinical studies of intratumoral and intravenous reovirus administration, significant CD8⁺ T-cell infiltration into the reovirus-injected tumors was observed, as well as increases in circulating CD8⁺ perforin/granzyme⁺ T-cell numbers (White *et al.*, 2008; Thirukkumaran *et al.*, 2010). Similar data have been obtained with various other oncolytic viruses, including measles virus, vaccinia virus, vesicular stomatitis virus (VSV), parvovirus, and Newcastle disease virus.

Altogether, these data suggest a common mechanism of action in oncolytic virus-induced antitumor immunity. This response is initiated by the intratumoral replication of the virus leading to tumor cell lysis and the release of pathogen-associated molecular patterns (PAMPs; e.g., viral proteins and nucleic acids), DAMPs (e.g., high-mobility group protein B1 [HMGB1] and uric acid) (Guo *et al.*, 2013), and tumor-associated antigens (TAAs). Immune cells recognize the PAMPs and DAMPs through conserved receptors of the innate immune system known as pattern recognition receptors (e.g., toll-like receptors) driving the immediate innate immune response leading to the production of type I interferons (IFNs) (Guillermie *et al.*, 2013; Sieben *et al.*, 2013). These cytokines enhance the expression of MHC class I and II and of costimulatory molecules. Moreover, an increase in the production of additional cytokines and chemokines, including IL-1 β , IL-6, IL-12, TNF- α , RANTES, and MCP-1, is observed (Diaconu *et al.*, 2012; Donnelly *et al.*, 2013; Kleijn *et al.*, 2014). Antigen-presenting cells take up TAAs released by the tumor cells and effectively induce antigen-specific T-cell activation. This cytokine-driven proinflammatory response compromises the immunosuppressive tumor microenvironment by enhancing the expression of MHC as well as co-stimulatory molecules involved in antigen presentation.

Role of Immunogenic Cell Death

So far, the mechanisms involved in virus-induced cell killing received relatively little attention in Dutch oncolytic virus research. Studies abroad demonstrated that the mechanism of cell death plays an essential role in eliciting antitumor immunity. Understanding these mechanisms may contribute to the development of more efficient agents for viral oncolysis and immunotherapy.

Cell death can proceed through either immunogenic cell death or nonimmunogenic cell death (Tesniere *et al.*, 2008; Green and Kroemer, 2009). Various types of cell death processes have been recognized, including apoptosis, necrosis, autophagic cell death, necroptosis, and pathogen-triggered pyroptosis (Kroemer and Levine, 2008; Labbe and Saleh, 2008). Apart from apoptosis, all these cell death processes

are considered to be proinflammatory and immunogenic, while also for apoptosis an immunogenic form has been documented (Obeid *et al.*, 2007; Boozari *et al.*, 2010). Immunogenic cell death provokes the release of TAAs in conjunction with DAMP and PAMP molecules, as well as inflammatory cytokines (Tang *et al.*, 2012). Together, this creates an environment that aids eliciting antitumor immunity.

For most oncolytic viruses, immunogenic cell death mechanisms have been described. HSV, NDV, and reovirus have been reported to induce both apoptotic and autophagic cell death pathways in various tumor cells (Szeberenyi *et al.*, 2003; Stanziale *et al.*, 2004; Colunga *et al.*, 2010; Thirukkumaran *et al.*, 2013). Adenovirus-mediated cell killing has been reported to be apoptosis independent and may be associated with autophagy (Abou El Hassan *et al.*, 2004; Ito *et al.*, 2006; Jiang *et al.*, 2011). Measles virus has been shown to induce an apoptotic mechanism of syncytial death, which is associated with an proinflammatory process (McDonald *et al.*, 2006; Donnelly *et al.*, 2013). Also indirect cell killing, by targeting of the tumor vasculature, for example, contributes to the immunogenicity of the tumor because of release of DAMPs by necrotic cells (Liu *et al.*, 2008; Jing *et al.*, 2009; Breitbach *et al.*, 2011).

Combining Oncolytic Viruses with Immune Stimulatory Strategies

It is now clear that the antitumor immune response elicited by oncolytic viruses contributes significantly to the therapeutic benefit, and several strategies to enhance these antitumor responses are currently under investigation. Oncolytic viruses can be genetically engineered to express an immune-stimulatory transgene or to silence immune-suppressive signals in tumors. Alternatively, oncolytic treatment can be combined with more conventional immunomodulatory treatments to boost anticancer efficacy.

The insertion of transgenes encoding proinflammatory cytokines such as interleukin (IL)-2, IL-12, IL-15, and granulocyte-macrophage colony-stimulating factor (GM-CSF) has been extensively studied in preclinical and in clinical studies (Vacchelli *et al.*, 2013). Also, the insertion of T-cell costimulatory molecules such as 4-1BB ligand, CD80, and CD40 ligand has been evaluated (Atherton and Lichty, 2013). The insertion of TAA expression cassettes has been shown to enhance antitumor efficacy in preclinical models as well (Dave *et al.*, 2014). The insertion of GM-CSF cDNA in oncolytic viruses has been explored in a number of clinical trials. JX-594 (a.k.a. Pexa-vec) is a tumor-selective vaccinia virus carrying the *GM-CSF* gene (Heo *et al.*, 2013). In phase I dose escalation studies, patients with metastatic solid tumors were treated by intravenous injection of JX-594 (Park *et al.*, 2008; Breitbach *et al.*, 2011). Treatment was well tolerated and a dose-related antitumor activity was found. Interestingly, the levels of the proinflammatory cytokines IFN- γ , tumor necrosis factor (TNF)- α , and IL-6 increased in a dose-dependent manner (Breitbach *et al.*, 2011). In a study by Kim *et al.* (2013), functional antitumor immunity of Pexa-vec administration was assessed in a rabbit VX2 tumor model and in sera from treated patients. These data show that antibody-mediated complement-dependent cytotoxicity (ADCC) plays an important role in the induction of a selective antitumor

immune response. Furthermore, the ability to induce ADCC correlated with an improved survival of treated patients. Another clinical trial, in which an oncolytic adenovirus was armed with *GM-CSF*, showed a correlation between antiviral and antitumor immunity, suggesting that antiviral immunity is capable of breaking tumor-associated immune tolerance (Kanerva *et al.*, 2013). Treatment with Talimogene laherparepvec (or T-VEC, formerly known as OncoVEX^{GM-CSF}), a *GM-CSF*-armed oncolytic herpes virus, resulted in clinically stable disease in 3/26 treated patient in a phase I study (Hu *et al.*, 2006). Immunologic analysis in a subsequent phase II study demonstrated the presence of both local and distant antitumor immune responses in patients with advanced melanoma (Senzar *et al.*, 2009; Kaufman *et al.*, 2010). Although these studies present promising data in combining oncolytic viruses with *GM-CSF*, the actual therapeutic benefit needs be confirmed in larger clinical trials.

The recent groundbreaking results of immune checkpoint blockade with anti-CTLA-4 and anti-PD-1 antibodies (ipilimumab and nivolumab, respectively) in metastatic melanoma (Wolchok *et al.*, 2013) have surged the interest in immunotherapeutic approaches to cancer therapy and may offer a new combinatorial strategy for viro-immunotherapy. Immune checkpoints play an important role in the prevention of tissue damage by activated T cells and contribute to the immune-suppressive state of the tumor microenvironment (Chen and Flies, 2013). Reversal of this tumor-induced immune suppression either by blockade of immune checkpoints or by activation of costimulatory molecules leads to activation of T cells and long-term antitumor immunity (Pardoll, 2012).

The combination of virotherapy with immunotherapy, in theory, provides for effective virus-induced tumor cell lysis and release of antigens. The costimulatory agent efficiently enhances antitumor immune responses elicited by the oncolytic modality. Combined, these modalities have the potential to overcome immunosuppression by tumor cells and enhance antitumor efficacy.

Oncolytic viruses have been combined with CTLA-4 blockade in various preclinical models. In a murine B16 melanoma model, the oncolytic Newcastle disease virus was combined with systemic administration of anti-CTLA-4 antibodies (Zamarin *et al.*, 2014). This resulted in the tumor rejection and the formation of a protective antitumor memory. In a model of peritoneal mouse mammary tumors, combined treatment with recombinant VSV (rVSV) and anti-CTLA-4 antibodies resulted in 80% of cured animals with a protective long-term memory response, whereas treatment with rVSV alone was ineffective (Gao *et al.*, 2009). Depletion of the CD4 and CD8 T-cell subsets abrogated the antitumor efficacy of rVSV and CTLA-4 blockade, which highlights the importance of T-cell-dependent mechanisms of viro-immunotherapy. These data demonstrate that the combination of oncolytic virotherapy and immunotherapy with immune-checkpoint inhibitors can produce robust T-cell-mediated immune responses and a protective antitumor memory.

Toward Personalized Viro-Immunotherapy

For oncolytic virus therapy to be successful, effective virus-induced oncolysis, TAA, cytokine, and danger signal

release are probably all required, as well as (co)stimulation of T cells. With this approach, oncolytic virus therapy can generate an effective tumor vaccine *in situ* (Li *et al.*, 2012; Bartlett *et al.*, 2013). However, the data from clinical trials suggest variable responses to oncolytic virus treatment even in patients bearing similar tumor types and grades.

It has become evident that intertumoral variability in response to any given anticancer therapy reflects underlying (epi)genetic heterogeneity within tumor type. This supports the notion that treatment strategies should be tailored to specific molecular traits. As viruses modulate and exploit the host cell machinery to produce viral progeny and induce cell death, mutations and alterations in the underlying pathways may influence the efficiency of this process. Indeed, already a marked heterogeneity in the susceptibility of patient-derived glioblastoma stem cell cultures to reovirus-induced cytolysis was demonstrated, which was not directly correlated with the efficiency of cell entry and infection (van den Hengel *et al.*, 2013). In addition to the efficacy of viral replication and cell lysis, also the danger signals and the amounts of cytokines and chemokines released by the infected cells can strongly influence the ensuing antitumor immune response. Thus far, limited data are available on the intertumoral variation in this innate tumor response. Donnelly *et al.* (2013) described measles virus-induced variability in cytokine and chemokine release between melanoma cell lines, although specific patterns of expression did emerge.

Studies using panels of patient-derived tumor cell cultures or tissue samples can help to chart tumor-specific oncolysis and innate responses. With the availability of molecular data of the tumors, such an approach may aid identifying molecular correlates that predict antitumor efficacy of a particular virus. Finally, the success in mounting an antitumor immune response also depends on the efficacy of antigen presentation and T-cell activation. Mapping of expression patterns of immune-related molecules in patient-derived tumor specimens may offer leads to enhancing T-cell activation strategies on a per-patient basis, including oncolytic viruses armed with immune stimulatory transgenes or co-treatment with immune checkpoint inhibitors. Together, these approaches should ultimately delineate tumor profiles predicting the optimal oncolytic virus and optimal immune-stimulating strategy for a particular tumor, and help guide future individualized viro-immunotherapy treatment. It is this ambition that drives current oncolytic virus research in the Netherlands and abroad.

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