Newcastle Disease Virus and its Application in Oncolytic Viro-immunotherapy for Treatment of Pancreatic Cancer Patients

Joanna Fréderique de Graaf

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Newcastle Disease Virus and its Application in Oncolytic Viro-immunotherapy for Treatment of Pancreatic Cancer Patients

Newcastle Disease Virus en zijn applicatie in oncolytische viro-immunotherapie voor de behandeling van patiënten met alvleesklierkanker

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Co-promotor:	Dr. B.G. van den Hoogen

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“Doe je ogen eens dicht, wat je dan ziet
is van jou.”
(Elsie Eleanor de Graaf)

Preface

Lisianthus

A flower, bringer of love and hope
A treatment for mind and soul
But a flower never walks alone
Or blooms by itself
It shares its successes with everyone else

I am grateful to all who have giving me their time, faith and wisdom

Bernadette, Ron, Casper, Anja, Marloes, Linde, Tanja, Hugo, Daphne, Kevin, Pau, Susma, Anne, Marco, Jasmin, Adinda, Miruna, Theo, Dennis, Pascal, Oahn, Sander, Mathilde, Monique, Mark, Shanty, Sacha, Stefan, Rachel, Mathis, Marjolein, Jocynthe, Dirk, Youssra, Lisanne, Mirjam, Lex, Willemijn, Djenolan, Victor, Shirley, Rory, Thijs, Bri, Peter, Marion, Petra, Debby, Claudia, Miranda, Debby, Pieter, Barry, Bart, George, Reina, Werner, Brenda, Gijs, Wigdagdo, Fasa, Nisreen, Noreen, Stalin, Bas, David, David, Felicity, Laurine, Mart, Danny, Jurre, Seyar, Katie, Daryl, Simone, Thomas, Muriel, Danny, Laura, Nele, Ray, Carolien, Anna, Simone, Lonneke, Peter, Marco, Tamana, Nisreen, Wesley, Stephanie, Noreen, Peter Paul, Maria, Loubna, Simone, Talitha, Tamana, Vincent, Vincent, Dennis, Ingeborg, Henk, Frans, Jeroen, Clemens, Nadine, Eveline, Martine, Effy, Rob, Vera, Diana, Iris, Shweta, Ferry, Ramon, Marcel, Iris, Giulia, Felecia, Tamara, Koen, Elena, Mariska, Tsolere, Michiel, Jeroen, Gerben, Sijme, Iris, Bram, Kedar, Ed, Wing Yan, Wahwah, Emil, Benjamin, Victoria, Vera, Rianne, Delia, Maikel, Demi, Wouter, Marleen, Calum, Rachel, Wim, Elora, Muamer, Noralie, Sophia, Arjen, Nick, Lisa, Rianne, Robin, Marlies, Claire, Sanne, Sanne, Marjolein, Ariën,

Mariska, Robin, Lisanne, Alex, Ria, Kees, Carien, Yvonne, Herman, Mariëtte, Ton, Anneke, Dies, Elsie, Roel, Laurens,
Stefan

"The daylight is for the brain and the senses, the darkness is for the heart and the wits. Never, never be afraid. Your brain may fail you one day, but your heart won't."
(Fynn, *Mister God, This is Anna*, 1974)

Chapter 1

General introduction: the determinants of efficacy in clinical trials assessing viro-immunotherapy

Adapted from

Determinants of the efficacy of viro-immunotherapy: a review

J. Fréderique de Graaf[a], Stefan van Nieuwkoop[a], Theo Bestebroer[a], Daphne Groeneveld[a], Casper H.J. van Eijck[b], Ron A.M. Fouchier[a] and Bernadette G. van den Hoogen[a]

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a. Viroscience department, Erasmus Medical Centrum, Rotterdam, The Netherlands
b. Department of Surgery, Erasmus Medical Centrum, Rotterdam, The Netherlands

Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease, as the mortality rate almost equals the incidence rate. The disease has an incidence of 2.4 to 8.6 cases per 100.000 people globally each year.[1,2] Patients are often diagnosed late due to a late onset of symptoms such as weight loss, abdominal pain and jaundice. As a result, 50% of the patients is already in a state of progressing disease involving liver metastasis and/or invasion of adjacent organs at the time of diagnosis. Therefore, the disease is characterized by a low overall 5-year survival rate of only 6%.[3] Standard of care treatment consists of resection of the primary tumor followed by adjuvant chemotherapy, which can increase the survival rate of the patients with resectable tumors up to 20%.[4] However, in case of metastases, resection of the primary tumor is often not performed due to progressing disease. At this final stage of disease, the only treatment option left is palliative adjuvant chemotherapy to improve quality of life.[5,6] Current chemotherapies, such as FOLFIRINOX and gemcitabine/nab-paclitaxel, improve median survival only by 2-4 months.[7] New therapies, such as immunotherapy, are clearly needed.

Immunotherapy

In the last decade, it has become clear that the immune system, upon infiltration of tumor tissue, provides protection against tumor formation by killing unhealthy cells. However, in some cases tumor cells and the tumor micro environment (TME) start to express factors that lead to attraction of immune suppressive cells including T regulatory cells (Tregs) and myeloid derived suppressor cells (MDSCs), which themselves in turn secrete immune suppressive factors and hence contribute to the development of an immune suppressive TME.[8,9] Consequently, tumors are no longer infiltrated by immune stimulating cells resulting in non-inflamed or cold tumors as is often observed in PDAC. Immunotherapy is one of the relatively new strategies in anti-cancer therapy, in which the immune system is (re-) activated to target and kill tumor cells. Different approaches have been developed to induce strong antitumor immune responses, such as the use of cancer vaccines, adoptive T-cell transfer, monoclonal antibodies against tumor antigens, checkpoint inhibitors and oncolytic viruses (OVs).[10,11]

Oncolytic viro-immunotherapy

Oncolytic viro-immunotherapy, based on oncolytic viruses (OVs), is rapidly gaining interest in the field of immunotherapy against cancer. OVs are viruses that selectively infect and kill tumor cells without damaging healthy cells. In general, tumor cells are deficient in anti-viral responses, which makes these cells more susceptible to (oncolytic) virus infections and therewith virus induced cell death. Infection with oncolytic viruses results in direct oncolysis, which subsequently induces different types of immunogenic cell death such as necrosis and apoptosis. Recently, it has become clear that virus induced activation of anti-tumor immune responses (indirect

oncolysis), may be even more important.[12,13] Virus infection and virus induced lysis of tumor cells induces the secretion of immune stimulating cytokines and/or novel tumor antigens, such as tumor associated antigens (TAAs) (**Figure 1**). These TAAs are derivatives of tumor proteins which are now immunogenic and able to activate anti-tumor immune responses.[14,15] As a result, the reactivated immune system overcomes the immunosuppressive environment created by the tumor cells. The minimal toxicity upon treatment and the dual activity of direct oncolysis and immune activation make therapy with OVs an interesting treatment modality. OVs are either naturally occurring viruses, such as Newcastle disease virus (NDV) and reovirus, or have been genetically engineered to make them cancer specific, such as Adenovirus (AdV) and herpes simplex virus (HSV). The first two oncolytic virus accepted in OV therapy treatment were a modified picorna strain (RIGVIR) to treat melanoma in Latvia[16] and AdV (H101) to treat nasopharyngeal carcinoma in China.[17] In 2016, the first OV therapy for melanoma patients was approved in the USA, EU and Australia and was based on intratumoral injection of a genetically modified HSV expressing granulocyte macrophage colony stimulating factor (GM-CSF), Talimogene Laherparepvec (T-VEC/IMLYGIC).[18–21] In 2021, a second OV therapy, using another HSV strain (Delytact/Teserpaturev), was approved for intracranial treatment of malignant neuroblastoma after a phase II clinical trial in Japan.[22,23] Several other OV candidates are or have been evaluated in phase I and II trials.[24] However, the positive results observed in the preclinical phase are often lost in translation to clinical phase resulting in somewhat disappointing observations in clinical trials. Thus, although these studies demonstrated the potential of oncolytic viro-immunotherapy, improvement of the efficacy is necessary.

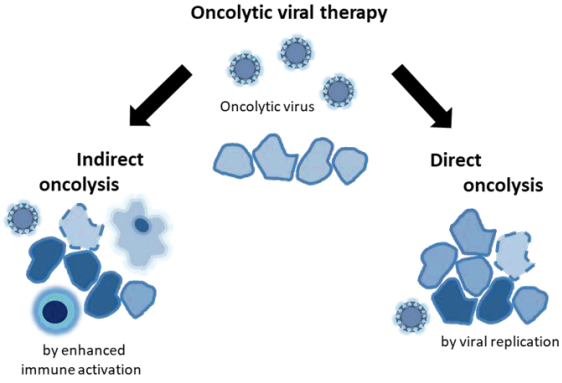

Figure 1. Schematic representation of oncolytic viral therapy using NDV. The oncolytic virus can infect the tumor cells, which eventually results in direct oncolysis induced by viral replication or indirect oncolysis by enhanced activation of the adaptive immune system.

Newcastle Disease virus

Newcastle Disease virus (NDV) is a negative sense single stranded RNA virus belonging to the family of Paramyxoviridae. The genome of NDV consists of eight genes encoding six structural proteins: the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase protein (HN) and the polymerase protein (L) (**Figure 2A-B**).[25] It encodes two non-structural proteins, which share an overlapping open reading frame with that of P, namely the avian specific interferon (IFN) antagonist (the V protein)[26,27] and a protein of which its function still remains unclear (the W protein).[28]

NDV strains are categorized in three pathotypes based on their virulence for chickens: non-virulent (lentogenic), intermediately virulent (mesogenic) and highly virulent (velogenic). Infections of chickens with velogenic strains may cause severe nervous and respiratory signs with up to 90% mortality. Infections with mesogenic strains may cause coughing and affect egg quality and production with up to 10% mortality. Upon infection with lentogenic strains, chickens generally show only mild signs of disease with negligible mortality. Lentogenic strains are enzootic in wild aquatic birds such as ducks.[25] Important drivers of virulence are the avian specific IFN antagonist (V)[29,30], hemagglutinin-neuraminidase (HN)[31] and especially the fusion (F) protein.[31,32] The F protein is translated as precursor protein F_0 and subsequently proteolytically cleaved into the activated protein F_1 and F_2 after which the virus becomes infectious. The differentiation in virulence is primarily determined by the number of basic residues at the cleavage site in the fusion protein. In lentogenic strains, containing a mono-basic cleavage site, the cleavage site can only be cleaved by extracellular host enzymes present in the respiratory tract. In mesogenic and velogenic viruses, containing a multi-basic cleavage site (MBCS), the F protein is cleaved by more abundant subtilisin-like proteases, such as furin (**Figure 2C**).[33] The presence of an MBCS and a phenylalanine at residue 117 in the fusion protein defines a virus strain as virulent according to the World Organisation for Animal Health (OIE).[25,34,35]

As of 2018, the OIE only discriminates between virulent and non-virulent strains instead of lentogenic, mesogenic and velogenic. The intracerebral pathogenicity index (ICPI) index is the formal standard used to determine whether an NDV strain is a risk for poultry.[25] In the ICPI assay, virus is inoculated intracerebrally in 10 one-day-old chicks and the index is the mean score per bird per 24 hour observation over 8 days when each bird is scored 0 if normal, 1 if sick and 2 if dead. An NDV strain classifies as virulent when the virus has an ICPI of 0.7 or greater or has an MBCS at position 113-116 of the C terminus of the fusion (F2) protein, plus a phenylalanine (F) at residue 117 of the F1 protein.[25,35] An alternative method used to determine the virulence of an NDV strain is the measurement of the mean death time (MDT) of embryonated chicken eggs upon allantoic inoculation with virus. An MDT of chicken embryos upon NDV inoculation below 90 hours has been shown to be typical for virulent strains.[36]

The differences in virulence of NDV strains in poultry do not translate to differences in virulence in humans as NDV strains are not known to infect humans under natural conditions and do not cause substantial disease in humans. Human infections are occasionally seen during the mass vaccination campaigns of poultry, when veterinarians apply large amounts of nebulized spray containing live attenuated NDV strains to poultry flocks. Such infections with NDV may result in a mild clinical picture of acute conjunctivitis and laryngitis in humans, which clears up rapidly and spontaneously.[37,38] There is no evidence that infections with virulent viruses result in disease in humans and there are no reports of human-to-human transmission of NDV.

NDV as oncolytic virus

NDV is thus known to be an avian specific pathogen. One exception on the species specificity, however, is that NDV can effectively infect and lyse human and murine tumor cells, which makes NDV a functional and effective OV. In the previous century and the beginning of this 21st century, several clinical trials were performed with NDV as an oncolytic agent. The first clinical studies using NDV as an OV were performed with oncolysates. An oncolysate is a vaccine containing a mixture of allogeneic or autologous tumor cells infected with an OV dedicated to enhance the immunological response against the tumor (reviewed in Schirrmacher, 2015).[39,40] Upon application of these oncolysates inpatients suffering from different types of cancers, an increased disease free rate and overall survival rate was observed.[41] Eventually, natural occurring replicating NDV strains were used as OVs in viro-immunotherapy in studies with patients with a variety of tumors.[42–44] The minimal adverse effects induced by the therapy were related to cytokine induction upon injection. These adverse effects reduced over the duration of treatment and seemed to correlate with the expression of anti-NDV antibodies in patients' sera.[42] However, limited effects on tumor growth were observed in treated patients, in which only one patient with an advanced solid tumor had a complete response.[45] These disappointing results of the clinical trials demonstrated the need for further improvements of oncolytic NDV therapy to improve antitumor efficacy and patient survival.

Improving NDV as oncolytic virus

Preclinical studies have demonstrated that more virulent strains of NDV were more oncolytic. At first, more virulent strains than the non-virulent NDV-HUJ and PV701 such as the Beaudette C strain[46], Italien strain[47] or MTH-68 strain[48] were used to demonstrate that a virulent strain induced more cell death in tumor cells. The advent of recombinant DNA technology and the ability to generate recombinant viruses provided opportunities to improve the efficacy of non-virulent NDV strains. For instance, to generate more virulent viruses, the mono-basic cleavage site in the F protein of lentogenic NDV strains were changed to an MBCS.[49,50] Several studies

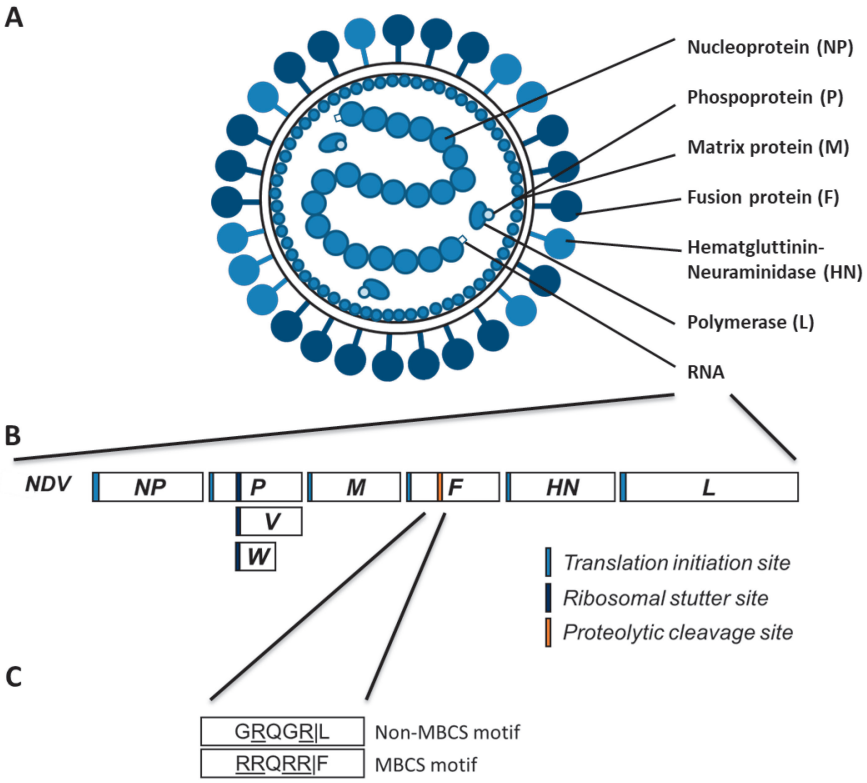

Figure 2. Schematic representation of the virus particle and genome of NDV. (A) Schematic presentation of the NDV viral particle and all structural proteins. (B) Schematic presentation of the RNA genome of NDV, including the translation initiation site or ribosomal stutter site for each open reading frame. (C) The amino acid sequence of the proteolytic cleavage site of the fusion protein with a non-virulent and virulent motif in which the basically charged amino acids are underlined.

have subsequently demonstrated that treatment with NDV containing an MBCS resulted in more tumor cell death in *in vitro* assays and reduced tumor growth in *in vivo* models.[50–54] Our previous study in macaques demonstrated that intravenous injection with NDV containing an MBCS (NDV F3aa) did not lead to clinical symptoms or pathological abnormalities.[55] In addition, haematological parameters remained stable and a basic serum chemistry profile demonstrated that expression levels of tissue damage proteins, such as c-reactive protein or creatine, did not differ between animals inoculated with non-virulent NDV F0 or NDV F3aa. These studies suggested that the use of NDV with an MBCS is effective and safe when used in preclinical models. However, NDV with an MBCS has an higher virulence in avian

species than non-virulent NDV F0, raising potential safety issues for the poultry industry upon use of viro-immunotherapy and environmental spills.[31,32]

Another approach to improve NDV as an oncolytic agent is arming NDV with immune modulatory protein transgenes, which can enhance the effect of indirect oncolysis.[56] These transgenes might encode for cytokines, chemokines or checkpoint inhibitors, which are often already used as monotherapy in immunotherapy to treat cancer patients.[57] These immune modulatory proteins, expressed by the virus upon infection of tumor cells, should then reactivate the immune system in the immune suppressive TME and hence improve the indirect oncolysis by NDV therapy with or without the combination of other immunotherapies.

Mechanism of action of oncolytic NDV

Several studies with oncolytic NDV in murine models for a wide variety of cancers have provided insights on the mechanism of action of oncolytic NDV.[58–63] To induce direct oncolysis, NDV enters the tumor cell via binding of the HN protein to sialic acids. These sialic acids are abundantly expressed on all cell types, including healthy cells. The exact features of cancer cell specificity of NDV remain disputable even today, but are likely dependent on the different tumor acquired mutations. Several studies have demonstrated that NDV effectively infects cancer cells that have defects in anti-viral pathways, such as those related to type I IFN[58–60], apoptotis,[61] cell stress[62] or aberrations in the mitogen-activated kinase (MAPK) pathways.[63] It has been suggested that the dysfunction of these pathways in tumor cells affect activation of both the intrinsic and extrinsic apoptosis pathway upon NDV infection.[64–67] Similar to what is observed upon infection with other OVs, caspases are activated and cellular proteins are cleaved leading to the release of pathogen-associated molecular patterns (PAMPs), consisting both of viral antigens, but also TAAs.[68] Simultaneously, immune stimulating cytokines are secreted by infected cells, such as type I and II IFN, tumor-necrose factor alpha (TNF) and interleukin(IL)-1β, but also chemokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF).[69–73] Together, both the excreted PAMPs, cytokines and TAAs result in activation of the innate and sequentially the adaptive immune system, initiating the indirect oncolysis. As a result of the secreted cytokines, innate immune cells, such as macrophages, dendritic cells (DCs), natural killer (NK) cells and neutrophils infiltrate the TME.[74] The cytokine excretion also leads to maturation of antigen presenting cells (APCs) and hence presentation of TAAs and viral antigens, which activate the adaptive immune system. Subsequently, cytotoxic T lymphocytes (CTLs) infiltrate the TME, resulting in an inflamed tumor. In addition, memory T cells are formed, which protect against new tumor challenges.[68]

The determinants of efficacy in clinical trials assessing viro-immunotherapy

Numerous clinical and preclinical studies have reported promising antitumor potential for viro-immunotherapy. Compared to chemotherapies, less toxicities and

adverse events were reported in clinical trials with viro-immunotherapy, demonstrating the applicability of the therapy. The reported mild adverse effects are often described as flu-like symptoms.[75–77] Modified recombinant HSV (T-VEC/IMLYGIC and Delytact/Teserpaturev) are the only OVs that have been successfully tested in phase II or phase III trials.[21,22] The results from the clinical trial resulted in the application of the therapy in the USA, EU and Australia.[18–20] This application illustrates that viro-immunotherapy is suitable for implementation in daily clinical practice.[21,78] However, while preclinical studies reported positive results, including enhanced immunological antitumor responses, tumor shrinkage and even complete clearance, viro-immunotherapy often resulted in a poor antitumor efficacy in clinical trials. The approval for only one OV to be used in viro-immunotherapy suggests that efficacy is lost in translation from preclinical trials to the clinic. Determinants of antitumor efficacy in murine models are often limited to increased infiltration of T cells into the tumor. However, virological or immunological parameters to predict a positive response to treatment in clinical trials are scarcely evaluated and vary between studies. Differences in administration strategies and clinical observations between studies make it difficult to draw conclusions on the efficacy of different viro-immunotherapies.

Administration strategies

The safety and efficacy of several viro-immunotherapies have been assessed in a number of Phase I clinical trials, including those using AdV, HSV, NDV, vaccina virus (VV), measles virus (MV), parvovirus, reovirus, and Seneca Valley virus (SVV) (summarized in Table 1). Results from these studies demonstrated that the chosen strategies for administration influenced the virological and immunologic parameters and the safety and efficacy of the therapy.

Route, dosage and schedule of treatment affecting efficacy

Administration strategies vary in administration route, dosage and schedule. In clinical trials, primarily intratumoral (IT) and intravenous (IV) injections have been applied. IT administration has often been preferred based on the assumption that IT administration, in contrast to IV injection, provided a better control of viral distribution, increased virus concentrations within the tumor and hence a better therapeutic effect. In two cohort studies with AdV (Enadenotucirev)[79] and Parvovirus (ParvOryx)[80], for treatment of resectable primary tumors or primary glioblastoma multiforme, the IT route and IV route were directly compared. In these studies, viral DNA was found in the tumors independent of the administration route, suggesting that IV administration can result in successful targeting of primary and metastatic tumor tissues. This suggestion is further supported by similar observations in other IV injection based studies using VV (Pexa-Vec, vvDD)[81,82], reovirus (Reolysin)[83,84] and SVV (SVV-001).[81,84,85] While IV injected viruses infected primary tumors as effective as metastatic tumors, IT administrated HSV (Oncovex) also infected

metastatic lesion, indicating subsequent systemic spread of the virus upon IT administration.[86] The results of these studies suggest that differences in administration routes do not substantially affect viral spreading. However, no direct comparison of overall survival between IV and IT administration has been made yet in clinical trials to show the effect of different administration routes on treatment efficacy. In addition to the administration routes, different dosages and schedule options have been investigated in phase I and II trials. Studies using oncolytic AdV (ICOVIR-5) or VV (Pexa-Vec) demonstrated that the use of a higher dosage improved the overall response rate significantly compared to low dosages.[87,88] Similar results were reported in two studies, respectively with AdV and HSV, in which treatment with serial dosages was compared to single treatment.[89,90] Thus, the use of higher dosages and/or serial treatment schedules improved efficacy compared to a single low dosage without affecting adverse events in the patients of these studies.

Administration strategy affecting adverse events

The route, dosage or schedule of treatments do not only influence the efficacy, but are also expected to have an effect on adverse effects and thus patient's health. Most clinical trials reported adverse events ranging from grade I to III and occasionally grade IV (**Table 1**), but there was no direct evidence that the administration strategy directly affected these events. Initially, the IT route was considered the safest route, as this route would provide more control over viral distribution, reducing systemic viral replication and hence should result in less adverse events. However, this assumption did not always hold true for every OV. The two cohort studies using AdV (Enadenotucirev)[79] and Parvovirus (ParvOryx)[80], directly comparing the IT and IV route, reported no additional toxicities due to I.V administration compared to I.T administration.[89,90] Similar to IV administration, higher dosages were thought to result in more adverse events compared to using low dosage. However, this correlation was not found in studies in which patients with melanoma or liver tumors were treated with AdV (ICOVIR-5) and VV (Pexa- Vec), where treatment with high and low dosages were compared.[87,88] In addition, dose-escalation studies with OVs such as SVV (NTX010) and reovirus (Reolysin), have demonstrated that for those OVs adverse effects were not dose dependent.[91,92] Although these studies suggest that the use of high dosage or serial dosages does not necessarily result in increased severe adverse effects, these parameters need to be evaluated for each OV, to improve therapy efficacy and prevent adverse events.

Short-term elevation in cytokine levels, such as IL-6, TNF and IFN-γ, upon viro-immunotherapy correlated with adverse events and the administration regimen could affect this type of adverse effects. [81,87,93,94] For example, one patient had high levels of IL-6 (200 pg/ml) upon IV injection with AdV (ICOVIR-5), which was found to account for the occurrence of the grade III adverse events that this person accrued.[92] In another study, grade I-II acute flu-like symptoms were associated with post-

treatment elevation of IL-6 and IL-8 levels, which rose after IV inoculation of poxvirus (vvDD).[82] To lower these severe adverse effects, longer infusion times or desensitization steps have been evaluated in different studies. In a study with NDV (PV701), the use of bolus dosing resulted in severely elevated cytokine levels and high frequency of adverse effects.[95] In contrast, in a different phase I trial, one hour infusion with the same virus resulted in only minor elevations of TNF and IFN-α expression levels.[96] Similarly, the use of a two-step desensitization strategy, with a smaller dosage followed by an higher dosage, reduced the occurrence of high-grade adverse events.[43] The reduction of adverse events after multiple cycles was also observed in patients treated with oncolytic reovirus.[97] The results of these studies suggest that adverse events are, at least in these clinical trials, temporary and linked to the start of therapy. Unfortunately, none of the studies using the different strategies with NDV (PV701) investigated the effect of the reduced induction of cytokine production on efficacy of the therapy. Several other studies have shown a beneficial effect of increased cytokine levels on therapy efficacy.[98] Future studies should evaluate the effect of administration strategies on cytokine responses, adverse effects and therapy efficacy.

Determinants of efficacy: virological and immunological parameters

The direct oncolysis induced by OVs and the indirect effects of the activated immune system influence various virological and immunological parameters. In clinical trials, these parameters consist of anti-viral antibodies, virus replication, systemic immune responses, immune cell influx and antitumor antibodies.

Anti-viral antibodies

Upon OV therapy, the immune system responds to the virus by producing antiviral antibodies, including neutralizing antibodies, which could affect the efficacy of viro-immunotherapy. In a study using AdV (Onyx-015), pre-existing neutralizing antibodies against AdV were associated with lower efficacy.[99] To avoid this neutralizing effect, animal viruses, such as SVV or NDV, were considered more beneficial as treatment modality in viro-immunotherapy. However, the presence of neutralizing antibodies did not seem to effect viral replication in clinical trials using oncolytic Reovirus and HSV.[100,101] Adair et al. and Roulstone et al. demonstrated that reovirus used virus neutralizing antibodies bound by monocytes to target tumors, indicating that these neutralizing antibodies did not necessarily limit viral distribution.[83,102] In addition, in a dose-escalation study with oncolytic HSV (OncoVEX) pre-existing antibodies reduced adverse events, as in seronegative patients, toxicities seemed to be more frequent compared to patients with pre-existing immunity.[89] The induction of an early antibody response was associated with a complete response of one patient who was treated with oncolytic NDV (HUJ).[103] A similar observation was made in a study with oncolytic VV (Pexa-Vec) in which responding patients had an increased antibody response compared to non-

responders.[104] These studies suggest that an anti-viral immune response could be a predictive parameter for treatment efficacy. However, in studies using the FDA approved HSV (T-VEC)[89], NDV (PV701)[43] or HSV (NV2010)[105], no correlation was observed between increased antiviral antibody titers and efficacy. Therefore, further investigations are necessary to understand the effects of the induction of antiviral antibodies and the potential correlation with efficacy of the therapies.

Virus replication

In principle, the efficacy of viro-immunotherapy is based on virus induced oncolysis and stimulation of the antitumor immune response. Direct oncolysis is a result of virus replication in tumor cells, which thus could be an important determinant of efficacy. Several studies have reported the presence of viral genomes, proteins or even infectious virus in tumor tissues.[80,82,87,97,106–112] In some studies, infectious virus was even detected in the tumor of patients 130 days after treatment with NDV (HUJ) and viral antigens 318 days after treatment with HSV (HF10) after the start of therapy.[45,100] Also in studies with the FDA approved HSV (T-VEC)[78], virus replication in tumor tissues was observed. However, the contribution of virus replication in the tumor to therapeutic efficacy is uncertain due to the lack of correlative evidence on efficacy in clinical studies. For instance, no correlation was observed between virus detection in tumors and the response to treatments with MV (MV-CEA)[109] or VV (JX-594).[111] Therefore, the contribution of the direct oncolytic effect by virus replication and the indirect oncolytic effects induced by the immune system still needs to be established.

Immune cell influx

Induction of an antitumor immune response is one of the most important objectives in clinical trials with immune therapies and can be divided in local and systemic responses. Viro-immunotherapy studies have shown that increased infiltration of cytotoxic T- and B-cells into the tumor is indicative for positive patient responses. For example, tumor influx of immune cells was observed in both a responding and a non-responding patient after treatment with NDV (PV701).[95] The tumor tissue of the responding patient contained lymphoid follicles with germinal centers consisting of infiltrated immune cells. However, the tumor tissue of the non-responding patient had multiple areas of necrosis and inflammatory mononuclear infiltrating cells. Furthermore, in a study comparing administration of serial versus single dosage of AdV (CGT-102) in a cohort of patients with different types of tumors, an increased T cell infiltration was found after serial treatment, which was not observed after single treatment.[90] This serial treatment resulted in a median overall survival of 269 days versus 128 days in the single treatment group and suggested the beneficial effects of the tumor infiltration of immune cells. Similar observations were made in a study with the FDA approved HSV (T-VEC) in which low counts of T helper and cytotoxic T cells in blood and tumors correlated with disease progression.[113,114] Therefore, tumor infiltration of T cells remains one of the most important objectives of viro-

immunotherapy, which has often been demonstrated in preclinical models, but is not always achieved in clinical trials.

In addition to inducing T cell infiltrations, the reduced presence of immune suppressive regulatory T cells (Tregs) in tumors is considered a prognostic value for survival of cancer patients in general.[115] For instance, in a phase I study with HSV (T-Vec) for patients with melonama, reduced Tregs infiltration was observed into the tumors.[113] In addition, in a study using AdV (Ad5/3-D24-GMCSF) in combination with cyclophosphamide, patients treated with only the virus or only with cyclophosphamide had less Tregs in their tumors compared to patients treated with combination therapy.[116] Improved clinical outcomes were reported for the patients receiving the combination therapy, but a correlation with the decreased Tregs levels was not mentioned. Thus, the importance of reduced amounts of Tregs in tumor tissues as an objective in clinical trials should be further investigated.

Systemic immune responses

The potential prognostic determinants of efficacy upon treatment could perhaps most easily be determined by evaluation of the systemic immune responses upon treatment. Studies conducting blood analyses often demonstrated that patients did respond systemically to the therapy by having short-term and/or long-term elevated cytokine levels and/or increased immune cell counts (Table 1). In case of short-term elevated cytokine levels, such as those of IL-6, IL-8 and TNF-α, it was already mentioned that this increased the prevalence of adverse effects, but little is known about the influence on treatment efficacy. In addition to short-term elevated cytokine levels, long-term elevated cytokines levels, such as those of IL-2, IL-10 and IFN-γ, are also often observed. For instance, in patients treated with serial dosages of AdV (CGTG-102), long-term elevation of IL-10 levels was observed and an improved overall survival was reported in patients treated with a serial dosage in contrast to patients treated with a single dosage. However, a correlation between increased long-term cytokine levels and survival was not investigated in detail.[90] Similarly, increased counts of granulocytes, such as neutrophils and eosinophils, and cytotoxic ($CD8^+$) and helper T ($CD4^+$) cells upon viro-immunotherapy are often observed, but potential correlations have not been investigated.[108,113,114,117,118] Thus, the effect of systemic immune responses on overall survival remains to be evaluated as prognostic value for treatment efficacy.

Tumor-associated antigen specific antibodies

Another important systemic immune response is the production of TAA specific antibodies (TAA-Ab), which are produced by B cells and can induce antibody dependent cellular cytotoxicity by NK cells. However, B cells themselves are often negatively associated with tumor development, because they secrete pro-tumorigenic factors and immune suppressive cytokines.[119] In addition, the expression of TAA-Abs often correlates with decreased overall survival in cancer

patients.[120] For example, patients that responded to treatment with ADV (CGTC-602) displayed decreased antibody titers against tumor antigens CEA, NY-ESO-1, survivin and MUC-1, whereas antitumor T cell responses were increased.[121] These results suggest that inhibition of B cell responses could be important for an effective therapy and reduced TAA production upon treatment could be a good prognostic value for the efficacy of viro-immunotherapy.

Thus, oncolytic viro-immunotherapy is establishing itself as an immunotherapy. However, in clinical trials, the overall responses have often been limited, whereas in preclinical trials the therapy looked promising. Differences in administration strategies between studies and immunological responses between mouse strains make it difficult to translate these promising preclinical observations to the clinic.[122] The host range of the virus strain used as OV might impact the observations on anti-tumor efficacy even more, because virus-specific immune responses in a mouse will most likely differ from the responses in humans, for instance, upon injection with NDV, a avian pathogen, or HSV, a human pathogen. These differences in host specific immune responses make the translation of preclinical studies complex indicating more research in different models is crucial to reach the clinic. Therefor, the translation of NDV as oncolytic agent is still a few steps away before the OV therapy can be applied to PDAC patients. A couple of these steps will be taken as addressed in this thesis in which the emphasis is laid on environmental safety and anti-tumor efficacy of recombinant modified NDV LaSota.

Outline of this thesis

NDV as an oncolytic agent has been evaluated in several clinical trials, but antitumor efficacy of these natural occurring strains in patients with advanced solid tumors was limited. Previously, we have shown that the virulent NDV F3aa had higher oncolytic potential than the non-virulent NDV F0 *in vitro* and in immune deficient *in vivo* models for pancreatic cancer. Here, we investigated the optimal administration route in relation to antitumor efficacy by comparing the effect of intravenous and intratumoral injections with NDV F3aa in an immune deficient murine PDAC model (**Chapter 2**). As NDV F3aa may pose a threat for the environmental safety, as defined by a potential risk to chickens, we aimed to generate a virus that had reduced virulence for avian species, while maintaining its oncolytic efficacy. To this end, we generated a virus which lacked expression of the V protein. As the IFN antagonistic function of the V protein is host specific, NDV lacking V protein expression was expected to infect and lyse human tumor cells, but not avian cells.[26,27] The oncolytic efficacy and the virulence of NDV F3aa lacking V protein expression was assessed in human pancreatic tumor cells and first proofs of attenuated virulence for poultry are shown in **Chapter 3**. To further validate the risk of these mutant variants, the pathogenesis induced by this NDV F3aa variant was studied upon inoculation via the natural route in chickens as well as by the determination of the ICPI value (**Chapter 4**). As an approach to generate NDV with higher oncolytic efficacy, we report on a NDV strain

with two mutations in the vicinity of the cleavage site of the F protein of NDV F0 (NDV F0-M/S) (**Chapter 5**). NDV containing these mutations can be produced in mammalian cells without the requirement of passage in embryonated chicken eggs. This is of importance because egg-generated NDV is neutralized relatively efficiently by the human complement system, and using cell-grown NDV in viro-immunotherapy could reduce this neutralization and hence might improve the oncolytic efficacy. Another approach to improve the efficacy of NDV based viro-immunotherapy involves arming of NDV with immune modulatory protein genes. In order to determine which immune modulator would serve best as a transgene in oncolytic NDV, a broad literature search was performed in which all studies regarding immunotherapies in combination with oncolytic viruses were summarized (**Chapter 6**). Recombinant viruses were generated expressing murine GM-CSF, CD70, soluble CD70, tumor necrosis factor superfamily member 14 (TNFSF14, also known as LIGHT), and IL-1 receptor antagonist (IL-1RA). In addition, viruses were armed with human TIR-domain-containing adapter-inducing IFN-β (TRIF), CD40L, LIGHT and IL-15. For all these viruses, expression of these proteins was demonstrated. The improved anti-tumor immune responses induced by these viruses can only be addressed in immune competent murine models for pancreatic cancer. Preliminary data were obtained on the oncolytic efficacy and safety of NDV F3aa, NDV F0 and NDV F0 expressing CD70 in an immune competent PDAC model (**Chapter 7**). The findings presented in this thesis in relation to the progress in the field of oncolytic viro-immunotherapy is summarized and discussed in **Chapter 8**.

Table 1. Reported oncolytic virus clinical trials

Virus	Disease	Regiment		Parameters				Clinical Outcome
		→	Schedule	Adverse events (grade)	Cytokine elevation serum	Viral shedding and replication	Immune responses	
AdV (CGTG-102)[90]	Advanced solid tumors	IT	Single	1-2 & 4	↑IL-6, IL-10	Viremia	↑ TILs	OS: 111 days
			Serial	=Single	>Single	Viremia	>Single	OS: 277 days
AdV (CGTG-602)[121]	Advanced metastatic tumors	IT	Serial	1-3	N.A.	Viremia	↑ NAbs, ↓TAA-Abs, ↑TILs	OS: 80-135 days
AdV (Ad5/3-Δ24)[123]	Recurrent ovarian cancer	IP	Serial	1-2	N.A.	Viremia, viruria, saliva, replication	↑ NAbs	SD: 6/8
AdV (Ad5/3-Δ24)[124]	Advanced recurrent refractory solid tumors	IT	Dose-escalation	1-3	↑GM-CSF, = TNF, IL-6	Viremia	↑ NAbs	PD: 7/7
AdV (Ad5/3-Δ24+GM-CSF)[124]					↑GM-CSF, = TNF, IL-6	Viremia	↑ NAbs, ↑ T-cells	SD: 4/7
AdV (Ad5/3-Δ24+GM-CSF)[116]	Advanced solid tumors	IT	Single	1-3	N.A.	Replication	↑ NAbs	OS: 120 days
			Single + CP	1-3	N.A.	Replication	↑NAbs, ↓Tregs	OS: 376 days
AdV (Temolysin)[125]	Advanced solid tumors	IT	Single	1-4	↑IL-6, IL-7 IL-10	Viremia	↑ NAbs	OS: 10 months SD: 7/10
AdV (H103)[126]	Advanced solid tumors	IT	Dose-escalation	1-4	N.A.	N.A.	↑ T cells, ↑ NAbs	ORR: 11.1%
AdV (Ad5/3-Δ24+GM-CSF)[127]	Advanced solid tumors	IT	Dose-escalation	1-3	-	Viremia	↑ T-cells, ↑ NAbs	OS: 200-400 days
AdV (ICOVIR-7)[94]	Advanced solid tumors	IT	Dose-escalation	1-3	↑IL-6, IL-8 IL-10, TNF	Viremia	↑ NAbs	PR+SD: 9/17
AdV (Ad5-Δ24-RGD)[128]	Recurrent gynaecologic tumors	I.P.	Dose-escalation	1-3	N.A.	Viremia, viruria, saliva, replication	↑ NAbs	SD: 15/21
AdV (DNX-2401)[129]	Recurrent malignant glioma	IT	Dose-escalation	1-4	N.A.	Replication	↑TILs	OS:9.5 months CR: 12%
AdV (Enadenotucirev)[79]	Resectable primary tumors	IT	Single	1-2	-	Faecal shedding, replication	↑NAbs, ↑TILs	N.A.
		IV				Replication, viremia		
AdV (ICOVIR-5)[87]	Cutaneous and uveal melanoma	IV	Serial (low)	1-3	↑IL-6, IL-10	-	↑ NAbs	SD: 2/7
			Serial (high)			Shedding, replication, viremia		SD: 5/6, OS: 73-271 days
HSV (T-VEC)[21,78]	Stage IIIB-IV melanoma	IT	Serial	1-4	N.A.	N.A.	N.A.	OS: 23.3 months
		IT	Serial		N.A.	Replication	N.A.	
HSV (NV1020)[107,130]	Metastatic colorectal cancer	I.A.	Single	1-2	= IL-1, IL-2, TNF-α, IFN-γ	Shedding and replication	N.A.	OS: 25 months SD: 7/10
HSV (NV1020)[93,105]	Metastatic colorectal cancer	I.A.	Single	1-2	↑IL-6, IFN-γ, TNF	-	↑ NAbs	OS: 12.4-11.8 months
HSV (G207)[131]	Recurrent malignant glioma	IT	Single + radiation	1-3	N.A.	Saliva	↑ CD8+/ CD4+ T cells, ↑ NAbs	OS: 7.5 months PR+SD: 6/9
HSV (HF10)[100]	Non-resectable pancreatic cancer	IT	Dose-escalation	-	-	Replication	↑ NAbs, ↑ NK cells, ↑TILs	OS: 180 days PR+SD: 4/6
HSV (T-Vec)[114]	Stage IIIB-IV melanoma	IT	Serial + Ipilimumab	1-4	N.A.	N.A.	↑ CD8+ T cells	DRR: 8 months CR+PR+SD: 13/18

HSV (T-Vec)[113]	Stage IIIB-IV melanoma	IT	Serial + Pembrolizumab	1-2	↑IFN-γ	N.A.	↑ CD8+ T cells, ↑TILs	CR+PR+SD: 16/21
HSV (Oncovex)[86]	Squamous Cell Cancer of the Head and Neck	IT	Serial + Cisplatin + radiation	1-4	N.A.	Shedding, viremia	↑ NAbs	OS: 82.4% up to 29 months
HSV (Oncovex)[89]	Refractory metastases from breast, GI, epithelial of the head and neck caner or melanoma	IT	Single	1-2	-	Viremia, viruria, replication	↑NAbs, ↑TILs	SD: 1/13
			Serial	=single	-	Viremia	↑ NAbs	SD: 2/17
HSV (HSV1716)[112]	Relapsed, refractory extracranial cancers	IT	Single (low)	1-3	N.A.	Viremia	↑ VAbs	OS: 2.25 months
			Single (high)					OS: 7 months
HSV (Oncovex)[132]	Stage IIIc-IVM1c melanoma	IT	Serial	1-3	N.A.	-	↑ VAbs	OS: 52% up to 24 months
MV (MV-CEA)[109]	Recurrent ovarian cancer	I.P.	Single	1-3	N.A.	Viruria, saliva	=Vabs	OS: 12.15 months
MV (MV-EZ)[110]	Cutaneous T-cell lymphoma	IT	Dose-escalation	1	↑IFN-γ, IL-12, IL-2	Syncytia formation	TILs: ↑ CD8+, ↓ CD4+	CR+PR+SD: 5/6
MV (MV-NIS)[133]	Refractory myeloma	IV	Dose-escalation	1-4	N.A.	Viruria, saliva, viremia	↑ NAbs	CR: 1/32
Parvovirus (ParvOryx)[80]	Progressive primary or recurrent glioblastoma multiforme	IT	Dose-escalation	1-4	↑ IL-12, IL-2	Viremia, shedding	↑TILs, ↑ a-viral T cells	OS: 464 days
		IV				Replication, shedding		
Reovirus (Reolysin)[83]	Metastatic Colorectal cancer	IV	Serial	1-2	↑ IFN type I	Replication	↑ Nabs, ↑ NK cells	N.A.
Reovirus (Reolysin)[134]	Metastatic breast cancer	IV	Serial + paclitaxel	1-3	N.A.	N.A.	N.A.	OS: 17.4 months
			Paclitaxel					OS: 10.4 months
Reovirus (Reolysin)[135]	Recurrent ovarian, tubal or peritoneal cancer	IV	Serial + Docetaxel or Pemetrexed	1-4	N.A.	N.A.	N.A.	OS: 7.8 months
Reovirus (Reolysin)[136]	Advanced solid tumors	IV	Serial + Paclitaxel	1-4	N.A.	N.A.	N.A.	OS: 12.6 months
			Paclitaxel					OS: 13.1 months
Reovirus (Reolysin)[97]	Advanced solid tumors	IV	Single + Docetaxel	1-3	N.A.	Viruria, viremia, saliva, replication	↑ NAbs	CR+PR+SD: 14/16
Reovirus	Recurrent malignant gliomas	IT	Single	1-2	N.A.	Saliva, feaces	↑ NAbs	OS: 21 weeks
Reovirus (Reolysin)[106]	Recurrent ovarian cancer	IV	Serial	1-4	N.A.	Replication	↑ NAbs	OS: 165 days
Reovirus (RT3D/Reolysin)[76]	Squamous Cell Carcinoma of the Head and Neck	IT	Serial + radiation	1-2	N.A.	No shedding, replication	↑ NAbs	PR: 7/14, SD: 7/14
Reovirus (RT3D/Reolysin)[91]	Advanced solid tumors	IV	Serial + carboplatin & paclitaxel	1-4	N.A.	Shedding	↑ NAbs	OS: 7.1 months
Reovirus (RT3D/Reolysin)[137]	Recurrent malignant glioma	IT	Single	1-3	N.A.	Viruria, viremia, saliva	N.A.	OS: 140 days
Reovirus (Reolysin)[101]	Relapsed extracranial solid tumors	IV	Single	2-3	N.A.	Viremia	↑ NAbs	SD: 3/24
			Single + CP					

Reovirus (Reolysin)[138]	Metastatic melanoma	IV	Single + carboplatin & paclitaxel	1-3	N.A.	N.A.	N.A.	OS: 10.9 months
Reovirus (REO-001)[139]	Advanced solid tumors	IT	Single Serial	1-4	N.A.	Viremia	↑ NAbs	CR+PR+SD: 7/17
Reovirus (RT3D/Reolysin)[102]	Advanced solid tumors	IV	Serial	1-3	N.A.	Viremia	↑ Nabs	N.A.
Reovirus (Reolysin)[84]	Relapsed myeloma	IV	Single Serial	1-3	N.A.	Replication	↑ NAbs	SD: 3/12
SVV (SVV-001)[85]	Neuro-endocrine based tumors	IV	Single	1-3	N.A.	Replication	↑ NAbs	OS: 780 days
SVV (NTX-010)[92]	Neuro-related tumors	IV	Single Serial	1-3 1-4	N.A.	Viremia, Feacal shedding	↑ NAbs	SD: 6/12 SD: 4/6
VV (PexaVec/JX-594)[81]	Advanced solid tumors	IV	Single	1-2	↑IFN-γ, TNF, IL-6, IL-10, =IL-1	Replication	↑ NAbs	PR+SD: 12/ 20
VV (PexaVec/JX-594)[140]	Hepatocellular carcinoma, neuroblastom aand Ewing sarcoma	IT	Single	1-4	↑IFN-γ	Pustules vorm van replication?	↑ α-viral T cells	SD: 4/6
vvDD (JX-929)[82]	Advanced solid cancers	IV	Single	1-3	↑IFN-γ, TNF, IL-6, IL-10, IL-7, IL-8, GM-CSF	Saliva, replication, viremia	↑ Nabs	OS: 4.8 months
VV (PexaVec/JX 594)[88]	Liver tumors	IV	Single (low) Single (high)	1-2 1-4	↑GM-CSF	Viremia	↑ Nabs, ↑ Neutrophils +Eosinophils, ↑ α-viral T cells	OS: 4.3 months OS: 13.6 months
VV (TG4023)[141]	Primary or metastatic liver tumors	IT	Single + F-FC/5-FU	1-3	N.A.	N.A.	↑ Nabs	SD: 8/15
VV (PexaVec/JX-594)[117]	Stage IV melanoma	IT	Serial	1-3	N.A.	Viremia	↑Nabs, ↑Neutrophils+Eosinophils	OS: 7.1 months
VV (GL-ONC1)[142]	Advanced head and neck cancer	IV	Single + Cisplatin Serial + Cisplatin	1-4	N.A.	Skin rash, Replication	N.A.	2 year OS: 69.2%
VV (Pexa-Vec/JX-594)[104]	Refractory primary or metastatic liver cancers	IT	Single	IT	↑TNF, IL-6, IL-10	Replication, Viremia	↑ Eosinophils ↑ Nabs	PR+SD: 9/10
VV (Pexa-Vec/JX-594)[118]	Colorectal cancer	IV	Serial	1-3	↑TNF, IL-6, IL-8, MIP-1α/β, MCP-1, ↑↑ IL-2, IL-10, IFN-γ	Viremia, Saliva	↑ Neutrophils	OS: 10.3 months
VV (PVSRIPO)[143]	Glioblastoma multiforme	IT	Dose-escalation	1-5	-	N.A.	-	OS↑
VV (vvDD)[143]	Advanced solid tumors	IT	Single	1-2	↑ CCL5, CXCL9 & CXCL10	Viremia, replication	↑T cells, =TILs	-
NDV (NDV-HUJ)[45]	Glioblastoma Multiforme	IV	Serial	1-3	N.A.	Viruria, viremia, replication	↑Nabs	OS: 32 weeks
NDV (PV701)[43]	Advanced solid tumors	IV	Serial (desensitization)	1-3	N.A.	Viruria	↑Nabs	SD: 5/8
NDV (PV701)[95]	Advanced solid tumors	IV	Serial (desensitization)	1-4	↑ IFN type 1, IFN-γ, Il-6, TNF	Saliva, Viruria	↑Nabs, ↑TILs	CR+PR: 2/62
NDV (PV701)[44]	Advanced solid tumors	IV	Serial	1-3	↑ IFN type 1, TNF	Viruria	↑ Nabs	CR+PR: 6/18 SD: 9/18

OS: overall survival, PD: progressing disease, CR: complete responder, PR: partial responder, SD: stable disease, Nabs: neutralizing antibodies, Vab: anti-virus antibodies, TILs: tumor infiltrating lymphocytes

Used mesh terms: (-Desjarinds and Lang) ("oncolytic viruses"[MeSH Terms] OR ("oncolytic"[All Fields] AND "viruses"[All Fields]) OR "oncolytic viruses"[All Fields] OR ("oncolytic"[All Fields] AND "virus"[All Fields]) OR "oncolytic virus"[All Fields]) NOT ("review"[Publication Type] OR "review literature as topic"[MeSH Terms] OR "review"[All Fields]) AND (Clinical Trial[ptyp] AND ("2008/12/01"[PDAT] : "2018/12/01"[PDAT])

Part I

The environmental safety and toxicity of modified recombinant NDV

"Looking at these stars suddenly dwarfed my own troubles and all gravities of terrestrial life. I thought of their unfathomable distance, and the slow inevitable drift of their movements out of the unknown past into the unknown future"
(H.G. Wells, *The Time Machine*, 1895)

Chapter 2

Comparison between intratumoral and intravenously administered oncolytic virus therapy with Newcastle disease virus in a xenograft murine model for pancreatic adenocarcinoma

J. Fréderique de Graaf[a], Marco Huberts[a], Daphne Groeneveld[a], Stefan van Nieuwkoop[a], Casper H.J. van Eijck[b], Ron A.M. Fouchier[a] and Bernadette G. van den Hoogen[a*]

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[a] Viroscience department, Erasmus Medical Centrum, Rotterdam, The Netherlands
[b] Department of Surgery, Erasmus Medical Centrum, Rotterdam, The Netherlands

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a poor clinical prognosis and is usually a metastatic disease. In the last decades, oncolytic viro-immunotherapy has shown a promise as treatment strategy with encouraging results for a variety of tumors. Newcastle Disease Virus (NDV) is an oncolytic virus which selectively infects and damages tumors either by directly killing tumor cells or by promoting an anti-tumor immune response. Several studies have demonstrated that NDV strains with a multi-basic cleavage site (MBCS) in the fusion protein (F) have increased anti-tumor efficacy upon intratumoral injection in murine tumor models. However, intravenous injections, in which the oncolytic virus spreads systemically, could be more beneficial to treat metastasized PDAC in addition to the primary tumor. In this study, we compared the oncolytic efficacy and safety of intratumoral and intravenous injections with NDV containing an MBCS in F (NDV F3aa) in an immune deficient murine xenograft (BxPC3) model for PDAC. In this model, both intratumoral and intravenous injections with NDV F3aa induced anti-tumor efficacy as measured at 10 days after the first injection. Upon intravenous injection virus was detected in some of the tumors, indicating the systemic spread of the virus. Upon both treatments, mice did not display weight loss or abnormalities and treated mice did not secrete virus to the environment. These data demonstrate that intravenous injections of NDV F3aa can be applicable to treat metastasized cancers in immune deficient hosts without inflicting adverse effects.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a cancer with a poor clinical prognosis and high mortality rate. Current approved standard of care of treatment is the resection of the primary tumor if possible, followed by adjuvant chemotherapy with gemcitabine or radiotherapy. [4,144] However, the diagnosis of pancreatic cancer is often made at a late stage of the disease when the primary tumor is often already metastasized.[5] In the case of metastatic spread, the only treatment option left is adjuvant chemotherapy as a palliative therapy to improve quality of life. The use of immunotherapies, such as those with checkpoint inhibitors, has been investigated for treatment of PDAC but has not led to improvement of the current treatment options so far.[145] Oncolytic viro-immunotherapy using oncolytic viruses (OVs) has been explored as a promising new treatment option for of wide variety of cancers.[12,146]

OVs selectively infect and damage tumors either by directly killing the cells or by promoting an anti-tumor immune response towards them [147]. Several oncolytic viruses have been tested in clinical trials with promising results, including Newcastle Disease Virus (NDV). NDV belongs to the family *Paramyxoviridae* and has an avian host range under normal conditions.[148] NDV has shown promise as an oncolytic virus for treatment of a wide range of tumors.[42,43,96,149–154] However, studies have also shown that the efficacy of viro-immunotherapies with wild type NDV strains needs improvement.[42,43,96] We, and others, aimed to increase the efficacy of treatment with NDV by substitution of the mono-basic cleavage site by a multi-basic cleavage site (MBCS) in the fusion protein (F) of the virus (NDV F3aa).[51–53,155] This cleavage site alteration results in trypsin independent activation of F and hence an improved replication in multiple cell types, including tumor cells.[148,155]

Numerous studies have shown that NDV F3aa had increased oncolytic efficacy compared to wild type NDV when administered intratumorally (IT) in murine models for different cancers.[51–53] The IT route of administration is often used to increase the local viral dose in the tumor and hence obtain improved oncolytic efficacy.[146] In addition, IT injections are hypothesized to be safer than intravenous (IV) injections if the virus is restricted to the tumor. In contrast, IV injections are expected to be more effective in treating metastasized tumors, but the systemic delivery might form a health risk for the patient and its environment. These different opinions about the optimal administration route have resulted in a variety of administration strategies in viro-immunotherapy studies. Two clinical studies have compared safety and efficacy between IV and IT injections using oncolytic Adenovirus (Enadenotucirev)[79] and Parvovirus (ParvOryx).[80] These studies demonstrated safety and efficacy of the therapy in humans, independent of the route of administration. In addition, these clinical trials showed that application by both administration routes resulted in the presence of viral genomes in the tumor, suggesting that IV injections also result in successful targeting of tumors. This was confirmed in a clinical study applying IV

injections with the wild type NDV strain PV701 in patients suffering from advanced chemo refractory cancers, where virus was also detected in the tumor.[42]

Our previous study in a PDAC xenograft model demonstrated that IT injections with NDV F3aa induced more tumor regression than injection with wild type NDV, however shedding and viral dissemination to different tissues and the subcutaneous tumor upon treatment was not studied. In that study, tumor growth was assessed during 40 days, after which viral genomes are likely not detected anymore.[155] Studies with experimental infections of chickens and cynomolgus macaques with NDV have demonstrated that NDV is only detected in tissues during the first week after inoculation and not at later time points.[55] Here, we aimed to address the question whether IT or IV injections lead to systemic viral distribution. To this end, we compared IT and IV injections, given at days 0, 4 and 8, with NDV F3aa in a PDAC xenograft model for virus distribution and anti-tumor efficacy at day 10 after the first, and 2 days after the last, injection.

Materials and methods

Cell lines

The PDAC BxPC3 cell line was obtained from American Type Culture Collection (ATTC) and was cultured at 37 º C in Roswell Park Memorial Institute (RPMI) 1640 (Lonza, Switzerland) media supplemented with 100 U ml $^{-1}$ penicillin, 100 U ml $^{-1}$ streptomycin, 2 mM L-glutamine (PSG) and 10 % Hyclone Characterized Fetal Bovine Serum (FBS) (Thermo Fischer Scientific, The Netherlands). BSR-T7 (kind gift of K. Conzelmann) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, The Netherlands) supplemented with PSG and 10 % FBS at 37 º C. Vero cells, obtained from ATCC, were cultured in Iscove Modified Dulbecco Media (IMDM) (Lonza, Switzerland) with the same supplements. Periodically, cells were tested and confirmed to be *mycoplasma* free.

Virus preparation

The plasmid containing the full-length cDNA of lentogenic NDV strain La Sota (pNDV F0) and expression plasmids for the NP, P and L proteins were kindly provided by Prof. B. Peeters from the Central Veterinary Institute of Wageningen, The Netherlands.[156] To create the NDV cDNA clone of mesogenic NDV F3aa, the amino acid sequence of the protease cleavage site was changed from ^{112}GRQGR↓L^{117} (lentogenic) to ^{112}RRQRR↓F^{117} using site-directed mutagenesis as described earlier [155]. Recombinant NDVs were rescued using an adapted method from the one described previously.[156] Briefly, BSR-T7 cells were transfected with 5 µg full length pNDV, 2.5 µg pCIneo-NP, 1.25 µg pCIneo-P and 1.25 µg pCIneo-L using 8nM calcium phosphate. For NDV F0, three days later, 200 µL BSR-T7 supernatant was injected into the allantoic cavity of 10-day-old specified pathogen free (SPF) embryonated chicken eggs. After incubation in a humidified egg incubator at 37 ° C for three days, allantoic fluid was harvested and stored at -80 ° C. For F3aa 200 µL

BSR-T7 supernatant was used to inoculate Vero cells. Five days later cells and supernatant were harvested and stored at -80 ° C. The titer of the virus stock were determined by end-point titration in Vero cells and calculated using the method of Reed & Muench and expressed as $TCID_{50}$ ml $^{-1}$.[157]

Virus stocks were generated after the second passage in Vero cells using a multiplicity of infection (MOI) of 0.01, based on the titer of the virus stocks generated during passage 1 and the number of cells plated out in the flasks. Five days after inoculation the virus was harvested and subsequently titrated by end-point titration in Vero cells. In case of NDV F0, 2 µg ml $^{-1}$ TPCK-treated Trypsin (T1426, Sigma-Aldrich, The Netherlands) was added to the media during infection. Virus batches were stored in 25 % sucrose (w/w) at -80 º C. Virus stocks for *in vivo* studies were concentrated by centrifugation over an Amicon® Ultra – 100 kDa NMWCO (Merck Millipore, UFC9100, Germany) and purified by filtration over a low protein binding filter membrane of 0.45 µM (Merck Millipore, LHV033RS). Mock treatment consisted of supernatant from non-inoculated cells that was purified and concentrated as described for the virus batches. Stocks were store at -80 º C.

Replication curves

One million cells were seeded in 6-well plates (Corning, The Netherlands). The next day, cells were inoculated at an MOI of 0.05 and one hour after inoculation cells were washed three times with phosphate buffered saline (PBS) after which media without FBS or trypsin was added. At indicated time points, 100 µl sample was collected and stored in 25 % sucrose (w/w) at -80 º C. Subsequently, samples were titrated by end point dilution assay in quadruplicate in Vero cells.

Cytotoxicity assay

Quadruplicates of 2 × 10^4 cells per well in 96-well plates (Greiner, The Netherlands) were either mock inoculated or inoculated with at the indicated MOI. One hour after inoculation, cells were washed once with PBS and fresh media without FBS or trypsin was added. Five days after inoculation, a lactate dehydrogenase assay (LDH, Cytotox 96 Non-Radioactive Cytotoxicity Assay, Promega, The Netherlands) was used to determine cell viability following the manufacturer's instructions as described before.[158]

Animals and experimental design

In total 32 eight-weeks old, athymic male mice (strain NMRI-*Foxn1^{nu}*, Charles River, Sulzfeld, Germany) were used. The mice, with an average weight of 30 gram, were acclimated to the housing conditions for 7 days, were fed chow and water ad libitum and were housed under specific pathogen-free conditions. Group sizes were determined as suggested by Charan et al.[159] In brief, a 2-sided t-test was used with a power of 80 %, a significance of 0.05 and expected difference between experimental groups of 25 with a standard deviation of 15, leading to the use of 8

animals per group. After acclimatization, groups of 8 mice, randomly divided over 4 groups based on weight, were inoculated subcutaneously with 5 x 10^6 human PDAC BxPC3 cells, suspended in culture medium without FCS, while under isoflurane anesthetics. Five weeks later, mice with tumor sizes >15 mm^3 were selected in each group, resulting in the following treatment groups: (1) 7 animals IT injection with mock; (2) 6 animals IT injection with NDV; (3) 6 animals IV injection with mock and (4) 6 animals IV injection with NDV. IT injections were given under isoflurane anesthetics and intravenous injections were given without anesthetics under a heating lamp. Mice were treated three times every four days with either mock or 1x10^5 $TCID_{50}$ virus by intravenous injection via the tail vein (100 µl) or intratumorally (50 µl). The inoculum was titrated in Vero cells to confirm the injected dosage. Weight was measured until the mice were euthanized. Tumor growth was measured every two days by using a digital calliper. Volume was calculated by the following formula: $width^2$ x length/2, as described before [155]. Swabs from the urinal duct and approximately one gram of fresh feces (fecal sample) from the bottom of the cage were collected every two days, and a throat swab was taken during necropsy. All samples were collected in 1 ml virus transport media consisting of Hanks balanced salt solution containing 0.5 % lactalbumin, 10 % glycerol, 200 U ml^{-1} penicillin, 200 µg ml^{-1} streptomycin, 100 U ml^{-1} polymyxin B sulfate, 250 µg ml^{-1} gentamicin, and 50 U ml^{-1} nystatin (ICN, The Netherlands).

Ethics statement

All experiments involving animals were conducted strictly according to the European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The animal study was reviewed and approved by an independent animal experimentation Dutch ethical review committee (DEC consult number: AVD101002017867).

Collection, processing and storage of tissue and environmental samples

Swabs and feces were collected in virus transport media (500 µl).[160] Blood samples were collected in blood collection tubes (Minicollect, Greiner Bio-one, 450533, The Netherlands), centrifuged for 10 minutes at 250 x *g* and the serum was stored at -20 º C. All organs collected during necropsy were snap frozen and stored at -80 º C. Organs and tumor tissues were supplemented with Dulbecco's Modified Eagle's Medium (DMEM, Lonza, The Netherlands) and PSG and subsequently homogenized using a FastPrep 24 tissue homogenizer (MP Biomedicals, The Netherlands). Homogenized samples were centrifuged for 10 minutes at 2000 x *g* and supernatant was stored at -80 º C or 200 µl was used for Ribonucleic acid (RNA) isolation.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from the urine, fecal and throat samples by adding 60 µl sample to 90 µl Magnapure 96 external lysis buffer (6374913001, Roche Diagnostics, The

Netherlands) as described before.[161] Subsequently, the lysed sample was added to 60 µl Agencourt AMPure XP magnetic beads (A63880, Beckman Coulter, The Netherlands) and incubated 15 min at room temperature. Magnetic beads were washed three times with 70 % ethanol using the DynaMag-96 magnet (12027, Invitrogen, The Netherlands) and subsequently air-dried. RNA was eluted by 6 minutes of incubation in bidest H_2O. NDV-specific quantitative reverse transcription-PCR was performed using 5 µl RNA in an ABI PRISM 7000 Sequence Detection System using TaqMan Fast Virus 1-Step Master Mix (both from Thermo Fischer) in a total volume of 30 µl. The NDV-specific primers used were described by Wise *et al*..[162] The reverse transcriptase step was 5 min at 50 ° C, followed by 95 ° C for 20 s. Cycling consisted of 40 cycles of 3 s denaturation at 95 ° C, 5 s annealing at 54 ° C and 31 s extension at 60 ° C.

NDV serology

Sera were tested for the presence of NDV specific antibodies by hemagglutination inhibition assay (HI) using turkey erythrocytes as described before.[55] Chicken polyclonal NDV antibody (ab34402, Abcam, UK) was used as positive control.

Histopathology and Immunohistochemistry

Formalin-fixed, paraffin-embedded, 3-µm-thick sections of the same tissues examined histopathological and were stained using an immunoperoxidase method as described before [55]. Tissue sections were mounted on coated slides, deparaffinized and rehydrated. Sections were incubated in 3 % H_2O_2 in PBS for 10 min at RT to block endogenous peroxidase. Subsequently,
antigen was retrieved by incubation of the section in Tris–EDTA buffer (pH 9) for 15 min, washed with PBS containing 0.05% Tween 20 and subsequently incubated in PBS with 0.1 % Bovine Serum Albumin (BSA) for 10 min at RT. Next, sections were incubated in PBS with 0.1 % BSA with a monoclonal mouse antibody IgG2a to NDV (dilution 1:100, MAb 6H12, specific to ribonucleoprotein; La Sota strain, Hytest Ltd, Turku, Finland) or with a negative control isotype mouse monoclonal antibody (dilution 1:100, MAb 003, R&D System, Minneapolis, USA) for 1 hour at RT. After washing, sections were incubated with goat anti-mouse antibody (dilution 1:400, Southern Biotech, Birmingham, AL, USA) labeled with horseradish peroxidase (HRP) for 1 hour at RT. In the next step, the sections were incubated in 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, USA) in *N*,*N*-dimethylformamide (Sigma Chemical Co.) solution for 10 min at RT, resulting in a red precipitate, which represents HRP activity. The sections were counterstained with hematoxylin. Brain tissue sections from a cormorant (*Phalacrocorax auritus*) known to be infected with NDV were used as a positive control and tissue sections of a mock inoculated mice were used as a negative control. Slides for histopathology were stained with hematoxylin and eosin (H&E) and examined by light microscopy.

Results

NDV induced cell death of human pancreatic adenocarcinoma (PDAC) cells *in vitro*

Human PDAC BxPC3 cells, to be used in the subcutaneous xenograft model, were first assessed for susceptibility to infection with NDV F3aa. Infection with recombinant NDV F0 was taken along to compare the cell killing to that induced by infection with NDV F3aa and for comparison with results of our previous experiments.[155] Upon inoculation of the BxPC3 cells, NDV F3aa replicated significantly more efficient than NDV F0 (**Figure 1A**). In addition, virus induced cell death was significantly higher upon inoculation with NDV F3aa than upon inoculation with NDV F0 (**Figure 1B**). These data demonstrated that NDV F3aa replicated to high titers in BxPC3 cells and, at 5 days after inoculation, this replication resulted in cell death of more than 80% of the cells which was in line with our previous *in vitro* studies.[155]

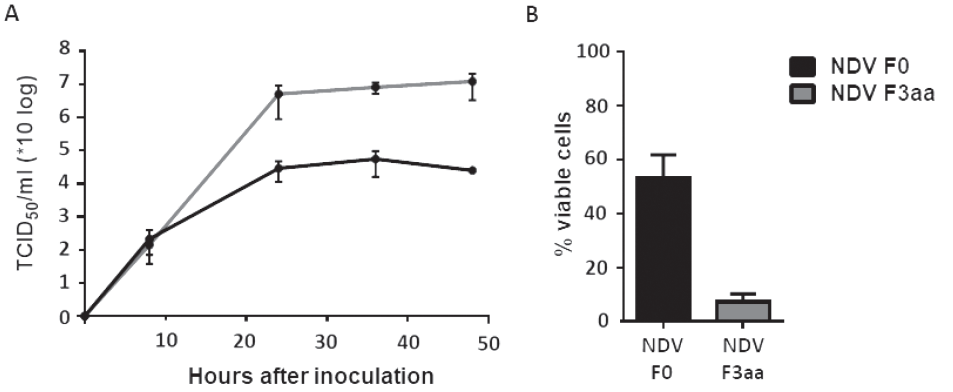

Figure 1. In vitro evaluation of BxPC3 cells for susceptibility to NDV infection. (A) Replication kinetics of NDV F0 and NDV F3aa in human pancreatic tumor cells BxPC3. Cells were inoculated at an MOI of 0.05 in triplo. At the indicated time points samples were taken and titrated in Vero cells. The experiment was conducted two times in triplo. Means and standard deviations of triplicates of a representative experiment are plotted. Black: NDV F0, dark green: NDV F3aa. (B) Virus induced cell death of BxPC3 cells upon inoculation with NDV F3aa and NDV F0. BxPC3 cells were inoculated at an MOI of 1 in triplo with indicated viruses. Results are represented as percentage viable cells compared to mock, which were considered as 100% viable. The experiment was conducted two times. Means and standard deviations of triplicates of a representative experiment are plotted.

Anti-tumor efficacy induced by IT and IV injection with NDV-F3aa in immune deficient mice subcutaneously inoculated with BxPC3 cells

The anti-tumor efficacy induced by IT or IV injections with NDV F3aa was evaluated in athymic mice subcutaneously inoculated with human PDAC BxPC3 cells. The animals were treated at day 0, 4, and 8 and were euthanized at day 10 after the first injection. This is two days after the final injection, and ten days after the first injection,

a time point at which viral genomes could potentially still be detected in the tissues of the treated animals. At day 10 after the first IT injections, and two days after the last injection, with mock, all mice had increased tumor volumes compared to day 0. In contrast, four out of six mice that had tumors IT injected with NDV F3aa showed decreased tumor volumes at day 10 (**Figure 2A-B**). On average, the tumors in all seven mice IT injected with mock had a higher fold change in tumor volume than the tumors in the six mice IT injected with NDV F3aa (**Figure 2C**). At day 10 after the first IV injection with mock, and two days after the last injection, all mice had increased tumors volumes compared to day 0, while in only two out of six animals IV injected with NDV F3aa the tumors increased in volume. In the other four animals IV injected with NDV F3aa, the tumor volumes slightly decreased or remained similar in size (**Figure 3A-B**). On average, fold change in tumor volume at 10 days after treatment was statistically significant smaller in animals IV injected with NDV F3aa than in animals IV injected with mock (**Figure 3C**). These data show that both IV and IT injection with NDV F3aa have anti-tumor efficacy in immune deficient mice subcutaneously inoculated with PDAC cells, at least at ten days after the first injection.

Virus dissemination upon IT or IV treatment

To compare viral dissemination between IV and IT injection with NDV F3aa in immune deficient tumor bearing mice, the presence of viral RNA in tumors, lungs, spleen, liver and kidneys at day 10 after the start of treatment, and two days after the last injection, was determined. Upon three IT injections with NDV F3aa, viral RNA was detected in most 4 out of 6 injected tumors, while upon IV injection viral genomes were only detected in the tumor of one treated animal. Upon the IT injections with NDV F3aa, viral genomes were not detected in any other organ than the tumor in 5 out of 6 mice. In the sixth IT injected animal, viral genomes were also detected in the spleen and lung, but not in the liver and kidney. In contrast, upon three IV injections with NDV F3aa, viral RNA was detected in the spleen and lung of all animals in a significant increase compared to those of IT injected animals, and in the kidney of 5 animals and the liver of one treated animal (**Figure 4**). Subsequently, immunohistochemistry was used to detect viral protein in those organs positive for viral RNA as indication for viral replication. Using brain tissue sections from a cormorant (*Phalacrocorax auritus*) known to be infected with NDV as a positive control and tissue sections of a mock inoculated mice as a negative control, viral protein expression was not observed in any of the RNA-positive organs nor were any pathological abnormalities observed (data not shown). The average weight of the virus treated mice, either IT or IV injected, did not differ significantly from the average weight of the mock treated mice and none of the mice showed weight loss during the 10 days of treatment (**Figure 5**). In addition, viral RNA was not detected in urine, throat and feces samples, collected from the IT and IV injected animals, indicating that both IT and IV virus injected mice did not shed detectable amounts of virus in the environment. None of the IV or IT treated mice showed seroconversion.

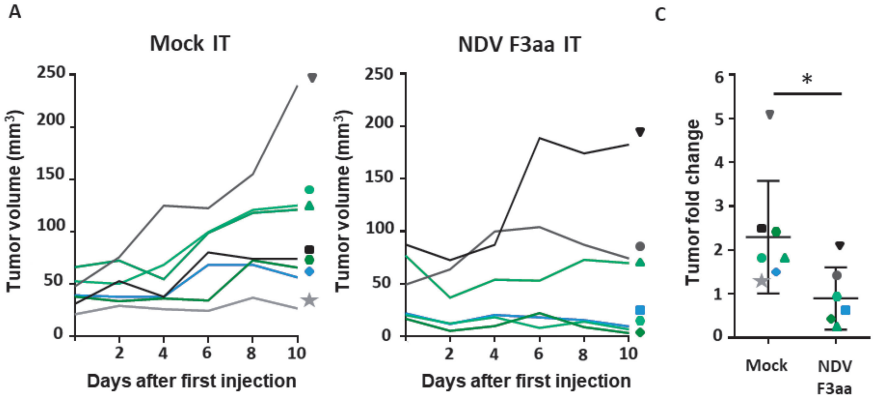

Figure 2. Tumor growth upon intratumoral injection with NDV F3aa in immune deficient tumor bearing mice. (A-B): Tumor volumes per individual mice during 10 days after three intratumoral (IT) injections with (A) mock and (B) NDV F3aa. (C) Mean fold change in tumor volume and standard deviation between day 0 and 10 per group of treated animals. Each colour and symbol correspond with the individual mice within the mock group or within the NDV F3aa group. Data was compared between groups using a paired T test. P values below 0.05 were considered statistically significant and are represented by a *.

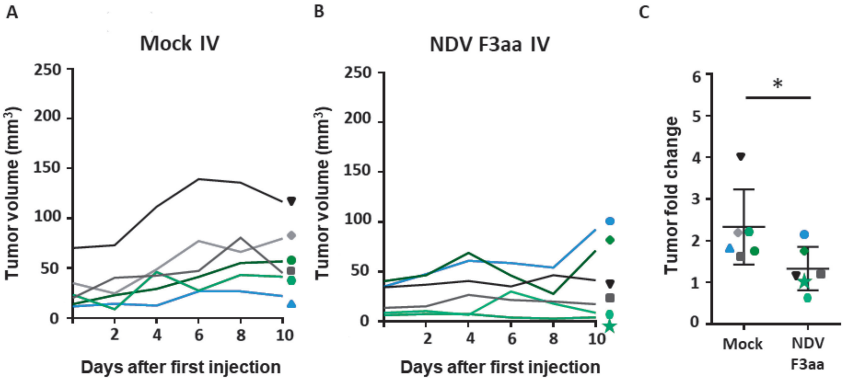

Figure 3. Tumor growth upon intravenous injection with NDV F3aa in immune deficient tumor bearing mice. (A-B): Tumor volumes per individual mice during 10 days after three intravenous (IV) injections with (A) mock and (B) NDV F3aa. (C) Mean fold change and standard deviation in tumor volume between day 0 and day 10 per group of treated animals. Each colour and symbol correspond with the individual mice within the mock group or within the NDV F3aa group. Data was compared between groups using a paired T test. P values below 0.05 were considered statistically significant and are represented by a *.

These data suggests that IV and IT injections with NDV F3aa did not induce any adverse effects in these immune deficient animals even though viral RNA was detected in several organs in IV virus injected animals.

In conclusion, IV and IT injection with NDV F3aa in this xenograft murine model for PDAC resulted in reduced tumor growth during ten days after start of the treatment and IV injection did result in targeting tumors systemically in one of the animals. In addition, both IV and IT injections with NDV F3aa in this model did not lead to adverse effects in the mice and neither to shedding of virus to the environment.

Discussion

Oncolytic viro-immunotherapy is a form of cancer immunotherapy for which research has shown promising results in preclinical and clinical studies for treatment of a variety of tumors.[10,11] Previously, we have shown that IT injections with NDV F3aa had higher oncolytic efficacy than injections with NDV without an MBCS in this same xenograft model, as measured at 40 days after start of the treatment.[155] Numerous preclinical and clinical studies for oncolytic viro-immunotherapy have been reported, but the routes of administration, such as IV and IT injections, vary greatly and are sparsely compared side by side.[146] Patients with PDAC are often diagnosed at a time point that the disease is already metastatic. The systemic spread of tumor cells would argue for IV administration of viro-immunotherapy, in addition to the fact that application of IT injection of pancreas tumors would be challenging. However, upon IV administration the oncolytic virus might not reach the tumor, which would reduce anti-tumor efficacy. In addition, IV administration of NDV F3aa could induce adverse effects upon replication in healthy tissues and the systemic spread of the viruses might cause a problem for the environmental safety. Some reports have suggested that locoregional viral administration, via hepatic arterial infusion or via a split-spleen reservoir, offers a more tumor specific treatment modality, without the need for large systemic viral loads thereby minimizing systemic toxicity. However the full potential of locoregional application of virotherapy has yet to be investigated.[69,163,164] So far, IV and IT injections with NDV F3aa have not been compared side by side to evaluate the differences between anti-tumor efficacy and safety, such as viral dissemination and virus shedding. Here, we made this comparison with NDV F3aa in an immune deficient subcutaneous xenograft model for PDAC, during 10 days of treatment.

For this study, we used the NDV susceptible human PDAC BxPC3 cell line to induce tumor growth in an immune deficient murine model.[155] In the mice IT injected with NDV F3aa, reduced tumor growth was observed compared to mock treated mice, at 10 days after the first, and 2 days after the last, injection. As IT injection might have caused an initial local inflammatory response, the reduction in tumor volumes might have been underestimated. These observations were in line with other studies in which NDV F3aa was IT injected in xenograft murine models for different tumors,

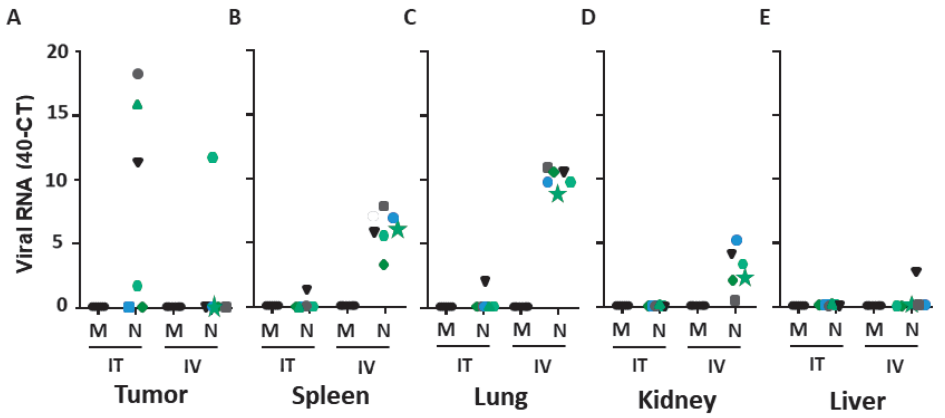

Figure 4. Dissemination of viral RNA and weight changes in immune deficient tumor bearing mice upon intratumoral or intravenous injection with NDV F3aa. Detection of viral genomes in (A) tumor, (B) Spleen, (C) Lung, (D) Liver and (E) Kidney collected from mice at day 10, after three intratumoral (IT) or intravenous (IV) injections with mock (M) or NDV F3aa (N). Values are shown in ΔCT (40-CT): higher values for ΔCT indicate the presence of higher levels of viral genomes in the samples. Each colour and symbol correspond with the individual mice represented in Figure 2 and 3.

such as head and neck cancer, ovarian cancer and mesothelioma models.[51–54,155] In these studies, tumor growth was followed for more than ten days allowing to address anti-tumor immune responses, but virus dissemination and shedding was not addressed.

Similar as upon IT treatment, IV injection with NDV F3aa in our xenograft model reduced the growth of subcutaneous tumors compared to treatment with mock during the 10 days of treatment, indicating that systemically applied virus is effectively targeting tumors in the flank of the animals. Although these results were obtained in immune deficient mice, similar results have been reported for an immune competent glioma murine model in which IV injections improved survival of mice bearing glioma tumors.[68] In this study, a dose of $1x10^5$ $TCID_{50}$ NDV generated in Vero cells was used. A dose of $1x10^5$ $TCID^{50}$ has been shown to be effective upon application in a syngeneic model for pancreatic adenocarcinoma.[165] It has previously been shown that egg-produced NDV is partially neutralized by the mammalian complement system,[166,167] hence the effective dose used in the present study may be higher than reported by Schwaiger et al. Future studies have to show whether the efficacy indeed increased with mammalian-derived NDV. Upon IT injections, viral genomes were only detected in the tumors and not in other tissues, while IV treatment resulted in the presence of viral RNA in several organs, but to a lesser extent in the tumor tissues. That IV injection leads to viral expression spleens, in contrast to IT injection, might be beneficial for activation of the anti-tumor immune response, but this has to be confirmed in an immune competent tumor model. These

data show that, in contrast to IT injection, IV treatment results in systemic delivery of the virus and hence potential effective targeting of metastatic tumor sites**.** These data suggest that detection of virus in the tumors is not indicative for oncolytic efficacy. The mechanism of action for viro-immunotherapy relies not only on direct oncolysis but also on activation of the innate and adaptive immune system.[146] The NDV treated animals did not develop an anti-viral antibody response as the duration of the experiment was too short for the animals to develop such a response. In addition, the used athymic mice lack T cells and as a result have B cells with a reduced function. However, it has been demonstrated that NK cells can still function in these immune deficient mice.[168] Virus induced activation of the NK cells could be the mechanism resulting in the observed reduced tumor growth in our model, but more detailed research is necessary to prove this mechanism of action in immune deficient mice.

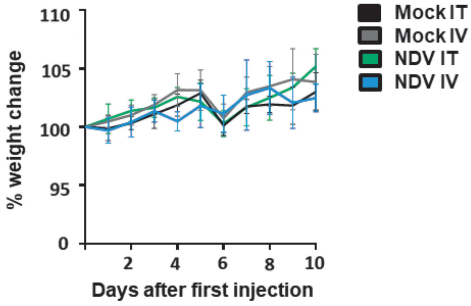

Figure 5. Average weight per group and standard deviation. Weight of each animal was measured and depicted as normalized average weight per group. Black line: average of animals IT injected with mock, grey line: average of animals IV injected with mock, green line: average of animals IT injected with NDV F3aa, blue line: average of animals IV injected with NDV F3aa.

IV injections with NDV F3aa significantly decreased tumor growth compared to mock treated mice, however tumor growth was only evaluated until day ten post inoculation. Further studies should be conducted to determine the effect of IV injections with NDV F3aa on the health of mice and tumor regression at later time points in both immune deficient and immune competent murine models for PDAC. Different studies in immune competent murine tumor models have shown that IV injections with NDV strain La Sota (a wildtype nonvirulent strain) or strain Italien (a virulent strain) resulted in virus expression in subcutaneous breast cancer tumors or in metastatic sites in the liver.[54,169] The detection of viral RNA in only one out of six tumors in our study could be explained by the fact that the subcutaneous BxPC3 tumors are less perfused than those in the liver or breast cancer model, but also by the immune status of the mice. In contrast to these reported studies in immune competent mice, the immune deficient mice in our study did not induce an antiviral antibody response. Several studies have shown that the presence of anti-viral antibodies may increase the efficacy of oncolytic viro-immunotherapy.[83,102,151] Altogether, our study demonstrates that IV injections with NDV F3aa results in anti-tumor efficacy in an immune deficient subcutaneous murine model, within 10 days of treatment, and suggest this administration route is applicable to treat metastasized tumors. However, additional studies are needed to show the effect of IV injections

with NDV F3aa on tumor regression at later time points in both immune deficient and immune competent murine models for PDAC.

The absence of any tissue pathologies or weight loss upon IT or IV injections with NDV F3aa in this model is in agreement with results from other studies using oncolytic NDV in immune deficient and immune competent murine models.[51–54,63,169–171] In addition, these data agree with data obtained from our study in cynomolgus macaques. This study in immune competent cynomolgus macaques demonstrated that IV injections with wild type NDV or NDV F3aa did not cause abnormalities in the animals.[55] All together, these data indicate that IV injections with NDV F3aa does not cause health implications in these mammalian hosts.

However, systemic spread of the virus upon IV injections with NDV F3aa could potentially result in shedding of virus by the patients, which poses a potential threat to wild birds or the poultry industry.[172] Here, we did not observe viral shedding in the saliva, urine or feces upon IV and IT injections of immune deficient tumor bearing mice. However, small amounts of viral RNA were observed in the lungs and kidneys of IV injected mice, and it cannot be ruled out that the amount of viral RNA that was shed was below the detection limit of the qRT-PCR assay. The detection limit of the assay was previously determined at 10^2-10^4 copies.[162] In our assays the limit of detection was determined to be 0.5 $TCID_{50}$. Previous studies have shown that low amounts of viral RNA can be detected in clinical specimens collected in virus transport medium from cynomolgus macaques, mice and chickens, including fecal samples and cloacal, nose and throat swabs. In clinical trials, where patients suffering from incurable solid tumors were IV injected with wildtype NDV, viral shedding in saliva was observed.[43] Upon IV injection of cynomolgus macaques with NDV F3aa viral shedding was also observed, however there was no effective viral transmission to naive animals and these animals did not demonstrate any sign of viral infection.[55] These data indicate that IV injections with NDV in a mammalian host leads to minimal amounts of shedding. Whether this limited amount of shedding poses a threat to the environment needs further evaluation in clinical studies.

In conclusion, both IT and IV injections with NDV F3aa resulted in anti-tumor effects in a xenograft athymic murine model for PDAC during 10 days after the first injection. The efficacy needs confirmation in an immune competent model for PDAC, as well as at later time points. However, this study shows that both IT and IV treatment with NDV F3aa were safe in this immune deficient mammalian model, indicating that oncolytic viro-immunotherapy using NDV F3aa as oncolytic virus can be safely used to treat PDAC patients.

"A certain type of perfection can only be realized through a limitless accumulation of the imperfect"
(Haruki Murakami, *Kafka on the shore*, 2002)

Chapter 3

Optimizing environmental safety and cell-killing potential of oncolytic Newcastle Disease virus with modifications of the V, F and HN genes

J. Fréderique de Graaf[a], Stefan van Nieuwkoop[a], Theo Bestebroer[a], Daphne Groeneveld[a], Casper H.J. van Eijck[b], Ron A.M. Fouchier[a] and Bernadette G. van den Hoogen[a]

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a. Viroscience department, Erasmus Medical Centrum, Rotterdam, The Netherlands
b. Department of Surgery, Erasmus Medical Centrum, Rotterdam, The Netherlands

Abstract

Newcastle Disease Virus (NDV) is an avian RNA virus, which was shown to be effective and safe for use in oncolytic viral therapy for several tumor malignancies. The presence of a multi basic cleavage site (MBCS) in the fusion protein improved its oncolytic efficacy in vitro and in vivo. However, NDV with a MBCS can be virulent in poultry. We aimed to develop an NDV with a MBCS but with reduced virulence for poultry while remaining effective in killing human tumor cells. To this end, the open reading frame of the V protein, an avian specific type I interferon antagonist, was disrupted by introducing multiple mutations. NDV with a mutated V gene was attenuated in avian cells and chicken and duck eggs. Although this virus still killed tumor cells, the efficacy was reduced compared to the virulent NDV. Introduction of various mutations in the fusion (F) and hemagglutinin-neuraminidase (HN) genes slightly improved this efficacy. Taken together, these data demonstrated that NDV with a MBCS but with abrogation of the V protein ORF and mutations in the F and HN genes can be safe for evaluation in oncolytic viral therapy.

Introduction

In the last few decades, the use of oncolytic viruses (OVs) as therapy in cancer patients has shown to be a promising treatment strategy with encouraging results for a variety of tumors. OVs selectively infect and damage tumors either by directly killing the cells or by promoting an anti-tumor immune response towards them. Several oncolytic viruses have been tested in clinical trials with promising results, including Newcastle Disease Virus (NDV).[43,95,96,103] NDV is a replication competent oncolytic virus belonging to the family *Paramyxoviridae* with an avian host range under normal conditions. Several studies have shown that OV therapy using NDV resulted in promising responses in several cancer types both *in vitro* and *in vivo* and was even capable of inhibiting metastases and inducing prolonged protection to tumor reoccurence.[68,173,174] The direct oncolytic effect was a result of cell lysis due to viral replication, of which the efficacy is dependent on the NDV strain used.[50,174] Similar to other OVs, a part of the therapeutic effect of oncolytic NDV is based on the induction of immune responses against the virus and the cancer cells. The secretion of pro-inflammatory cytokines, such as type I interferon (IFN), leads to counter-acting immune suppressive cells and hence enhances the anti-tumor response resulting in an indirect oncolysis.[73]

NDV strains are categorized in three groups based on disease severity in chickens: non-virulent (lentogenic), intermediately virulent (mesogenic) and highly virulent (velogenic). An intracerebral pathogenicity index (ICPI) in chickens is determined as a marker for virulence.[175,176] The cleavage site in the fusion (F) protein of NDV was shown to be a major determinant of these differences in virulence.[31,32] Previously, we have shown that a lentogenic LaSota strain in which a multi-basic amino acid sequence at the cleavage site of the F protein was engineered (NDV F3aa), making this a mesogenic virus, had significant higher efficiency in killing tumor cells *in vitro* and in murine tumor models compared to the lentogenic strain NDV F0.[50] Our study in non-human primates showed that NDV F3aa did not result in high pathogenicity, suggesting it is safe to use as oncolytic therapeutic in humans.[55] However, mesogenic and velogenic strains can cause outbreaks of severe disease in poultry and are defined as select agents in the USA.[177] Therefore, NDV F3aa has not been applied in clinical trials yet.[96]

We aimed to generate a mutant NDV F3aa with reduced replication capacity in avian cells and eggs, while maintaining killing potential in human tumor cells. Park and colleagues showed that the V protein of NDV is a determinant of host range restriction.[26] Multiple studies have confirmed that mutations in the open reading frame (ORF) of the V protein led to increased susceptibility to IFN responses and reduced virulence in embryonated chicken eggs.[29,178] Nevertheless, the effect of abrogating the V protein ORF in the viral genome on virus induced tumor cell killing and environmental safety is not known yet.

In this study, we generated NDV F3aa mutants in which expression of the V protein was abolished by introducing mutations in the stutter site and throughout the V protein ORF (NDV F3aa-$_{STOP}$V). The mutations introduced in the V protein ORF affected all of the third positions of the codons of the essential Phosphoprotein (P) gene which did not result in any amino acid substitutions in the P protein but resulted in 15 stop codons in the V protein ORF. We investigated the effect of these modifications on avian-specific virulence. To improve the replication efficiency and oncolytic efficacy of these NDV F3aa mutants, additional mutations were introduced in the MBCS (F-117-S) and the intracellular domain (ICD) of the F protein (Y-524-A), which were suggested to improve F protein expression.[179,180] In addition, the translation initiation site (TIS) of the F and HN genes was mutated to an optimized mammalian Kozak consensus sequence, aimed to obtain improvement of protein expression and hence viral replication.[181] These mutant viruses were evaluated for replication efficiency and cell-killing potential in human pancreatic adenocarcinoma cells (HPACs).

Methods

Cell lines

The avian cell lines DF-1 and QT6 were both obtained from the American Type Culture Collection. DF-1 cells were cultured at 39 ºC in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, The Netherlands) supplemented with 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin, 2mM L-glutamine (PSG) and 10% Hyclone Characterized Fetal Bovine serum (FBS HC, Thermo Fischer Scientific, The Netherlands). QT6 cells were cultured at 37 ºC in Medium 119 (Lonza) supplemented with PSG, 5% FBS HC, 1% chicken serum (Sigma-Aldrich, Germany) and 5% tryptose phosphate broth (MP Biomedicals, Belgium). BSR-T7 (kind gift of K. Conzelmann) were cultured in DMEM supplemented with PSG and 10% FBS FC at 37 ºC. A549 were cultured in HAM's F-12 (GIBCO, Life Technologies, The Netherlands). Vero cells and human pancreatic adenocarcinoma cell lines were cultured as previously described.[158] In case of virus infection experiments, 2% FBS was used in all media (infection media).

Cloning of recombinant viruses

The reversed genetics system for the NDV LaSota strain has been described before and was kindly provided by Ben Peeters from the Central Veterinary Institute of Wageningen, The Netherlands.[156] To introduce mutations in the V ORF a subclone was generated, using the unique digestion sites Sac-II and Not-I. The V protein with introduced mutations coding for stop codons (NDV F3aa-$_{STOP}$V1 or $_{STOP}$V2, Fig 1B) was ordered at Integrated DNA Technologies (IDT, Iowa) and inserted via PCR-cloning using the V protein specific insertion primers (**Table 1**) as described in Liu, 2008.[182] Additional mutations were introduced by side-directed mutagenesis as described before using specific primers (**Table 1**).[50] Subsequently, the different

subclones were cloned back to the full-length construct and sequenced for their correctness using a 3130xL Genetic Analyzer (Life Technologies).

Table 1: Primers used for cloning

Construct	Primer	Sequence 5'- 3'
Delete V	F	GCAATAAATCGTCCAATGCTGGTTGACTATCAGCTAGATC
	R	GATCTAGCTGATAGTCAACCAGCATTGGACGATTTATTGC
Insert ΔV1	F	GCAATAAATCGTCCAATGCTAAGAAGGGCCCCTGGTCTAG
Insert ΔV2	F	GCAATAAATCGTCCAATGCTAAGAAAGGCCCCTGGTCTAG
Insert ΔV1/ ΔV2	R	GATCTAGCTGATAGTCAACCTTACTAACCCTTTGCGAAATAG
***SDM F117S**	F	AATAATGGCGCCTATAGAGCGCCTCTGTCTCCG
	R	GGAGACAGAGGCGCTCTATAGGCGCCATTATT
SDM ICD	F	CTAGCATGCGCCCTAATGTAC
	R	GTACATTAGGGCGCATGCTAG
SDM TIS F	F	CGCCCTCCAGGGCCACCATGGGCTCCAGACC
	R	GGTCTGGAGCCCATGGTGGCCCTGGAGGGCG
SDM TIS HN	F	CACCGACAACAGTCCGCCACCATGGACCGCGC
	R	GCGCGGTCCATGGTGGCGGACTGTTGTCGGTG
Sequence analysis V	F	GGGTAAACCAGCAGAG
	R	CTTGCTTAGGAGCTTGGC
Sequence analysis MBCS	F	GGCCAAGATACTCTGGAG
	R	GTAAAGTGCCTGAATAG

*SDM: site directed mutagenesis

Sequencing

RNA was extracted from the virus stocks using the High Pure RNA isolation kit (Roche Diagnostics, The Netherlands) according to manufacturer's instructions. cDNA was produced as described before using the Superscript III Reverse Transcriptase kit (Invitrogen, Thermo Fischer).[183] Primers (**Table 2**) were used to amplify seven overlapping parts of the genome using Pfu Ultra II Fusion HS NA Polyemarase (600674, Agilent technologies, USA) followed by purification using the MinElute Purification Kit (Qiagen, Germany). Sequence primers were used to sequence the V gene and the MBCS of the F gene using a 3130xL Genetic Analyzer (Life Technologies) (**Table 1**).

Rescue and passaging of recombinant viruses

Recombinant NDVs were rescued by transfecting BSR-T7 cells with 5 µg full length NDV plasmid, 2.5 µg pCIneo-NP, 1.25 µg pCleo-P and 1.25 µg pCIneo-L using 8 nM calcium phosphate. Three days later, BSRT-7 cells were scraped and co-cultured with Vero cells. After 5 days, co-cultures were harvested and used to generate passage 1 in Vero cells. Titrations were done in Vero cells as described before.[50] Passage 2 was produced in Vero cells using an MOI 0.01, titrated and stored at -80

ºC. In case of NDV F0, BSRT-7 cells were scraped, and the cell suspension was then inoculated in to 10-day old eggs. Following passages were produced in Vero cells in the presence of 2 µg/ml TPCK-treated Trypsin (T1426, Sigma-Aldrich). For all experiments virus stocks from passage 2 were used unless indicated otherwise. The titer of the virus stocks was determined by end-point titration in Vero cells, calculated using the method of Reed & Muench and expressed as $TCID_{50}$ ml^{-1}.[157]

Table 2. Amplification primers

Function	Amplicon size	Primer	Sequence 5'- 3'
Amplicon 1	2232	F	ACCAAACAGAGAATCCGTGAGTTACG
		R	GTTGCTTGCTCCGGTCCTGAG
Amplicon 2	2340	F	CAGCATGGGAGAAGCATGGGAG
		R	GGATTGTATTTGGCAAGGGTGTGCC
Amplicon 3	2172	F	GCCAAGATACTCTGGAGTCAAACCG
		R	GCTTCACCGACAACAGTCCTC
Amplicon 4	2404	F	GTGTGAAAGTTCTGGTAGTCTGTCAG
		R	GGAAGCGGTAGCCCAGTTAATTTCC
Amplicon 5	2375	F	GTGGCAATGAGATACAAGGCAAAACAGC
		R	GGCTTGATGCAACTGTGTCAACACC
Amplicon 6	2421	F	GCCAGAAGCTATGGACAATGATCTC
		R	CTGCAAGTTGGTGTGATCCGTCATG
Amplicon 7	3298	F	GAAGTGCTCCTCGACTGTTCTTACC
		R	ACCAAACAAAGATTTGGTGAATGACGAGAC

Replication kinetics

For all cell types, one million cells were seeded in 6-well plates (Corning). The next day, cells were inoculated at the indicated multiplicity of infections (MOIs) and after 1h incubation cells were washed three times with phosphate buffered saline (PBS) and cell-specific media was added. In case of IFN pre-treatment, cells were pretreated for 24h with 30 µg/ml chicken IFN-β (abx067344, Abbexa, UK). At the indicated time points, 100 µl supernatant was collected and stored with 25% sucrose (w/w) at -80 ºC. Subsequently, collected samples were titrated in Vero cells. To this end, 24- well plates (Corning, The Netherlands) were seeded with 200.000 cells per well and inoculated with virus and supernatant was collected 48 hours after washing. All samples for the NDV F0 virus were titrated in media supplemented with 2 µg/ml TPCK-treated Trypsin (T1426, Sigma-Aldrich).

Replication kinetics *in ovo*
Embryonated chicken and duck eggs of various ages were inoculated in the allantoic cavity with 100 µl 1E6 $TCID_{50}$/ml virus. After 48 hours incubation at 37 ºC, the allantoic fluid was harvested and stored at -80 ºC until titration in Vero cells as described before, using infection media without trypsin supplementation.

Determination of virus induced IFN-β mRNA expression levels
DF-1 or A549 cells were seeded in 24-well plates (Corning) and inoculated at an MOI of 0.01 (DF-1) or 1 (A549). In case of A549 cells, spin-inoculation (800xg, 10 min) was applied.[184] After 1 hour, cells were washed once with PBS and fresh infection media was added. Subsequently, cells were incubated for 24 hours and RNA was isolated according to the manufacturer's instructions. In addition, cells were treated with trypsin-EDTA (Lonza) and fixed in Cytofix/Cytoperm (BD, The Netherlands) for flow cytometry analysis according the manufacturer's instructions. After fixation, cells were incubated in 1% normal goat serum (MP Biomedicals) and stained with 1:1000 anti-NDV (ab34402, Abcam, UK) and 1:1000 secondary FITC-labelled antibody (ab6749, Abcam) and analyzed by FACS Canto (BD) to determine the percentage of infected cells. qRT-PCR (45 cycles) was performed using 5 µl RNA in an ABI PRISM 7500 sequence Detection System (Life Technologies) using TaqMan gene expression assay for human IFN-β, chicken IFN-β and chicken β-actin (Hs00277188, Gg03344129, Gg03815934, Thermo Fischer Scientific). Human β-actin primers have been described before.[158] Results are shown as fold change of inoculated samples versus mock-inoculated samples both corrected for the household gene actin-β, calculated using the $2^{-\Delta\Delta_T}$ method.[185]

Cytotoxicity assay
HPAC cell lines were seeded in 96-well plates (Greiner) and inoculated at the indicated MOIs. After 1 hour, cells were washed once with PBS and fresh infection media was added. A lactate dehydrogenase assay (Promega, The Netherlands) was used to determine cell viability as described before.[158]

Fusion assay
One million cells were seeded in 6-well plates (Corning) and inoculated at an MOI of 1. After 1 hour, cells were washed once with PBS and fresh infection media was added. Subsequently, cells were incubated for 16 hours and fixed with 4% PFA. The cells were then stained with Giemsa (HX71780604, Millipore) and the fusion index was determined by averaging the number of nuclei of 30 fusion foci.

Mean death time assay
Ten-day-old embryonated specific pathogen free (SPF) chicken eggs were inoculated with 100 µl 1E6 TCID50/ml virus and incubated at 37 ºC for up to 160 hours. All eggs were candled every 8 hours to determine whether the embryo was still alive.

Results

Replication kinetics of NDV F3aa lacking V protein expression *in vitro*

The ORFs of the V and W proteins overlap with the ORF of the essential phospho (P) protein. The V protein ORF is only transcribed after a frameshift occurs as the result of stuttering by the viral polymerase protein.[27] To produce viruses that lack V protein expression, the stutter site was mutated at one or two nucleotide positions. In addition, every third nucleotide of the P protein codons was mutated, but only if this mutation did not result in a change of amino acid sequence of the P protein. In total 95 mutations were introduced, which resulted in the introduction of 15 stop codons in the V protein ORF, yielding the viruses NDV F3aa-$_{STOP}$V1 and NDV F3aa-$_{STOP}$V2, respectively (**Figure 1A-B**). The amino acid sequence of the P protein remained unchanged, because the mutations introduced into the ORF of the V protein only affected the third positions of the codons of the P protein gene. However, the modifications of the V protein ORF resulted in 40 amino acid substitutions in the W protein ORF including the deletion of the stop codon. Recombinant viruses were rescued in BSRT-7 cells and propagated further in Vero cells. The V protein gene of recombinant virus stocks from passage 2 were sequenced, which revealed that the viruses maintained the intended V protein sequence (data not shown).

To assess possible attenuation of the mutant viruses, replication kinetics of the $_{STOP}$V viruses were evaluated in two different avian cell lines and in human type II pulmonary epithelial cells (A549 cells). In chicken fibroblasts (DF-1 cells), the NDV F3aa-$_{STOP}$V viruses were slightly attenuated (**Figure 1C**), while no replication was observed in quail fibroblasts (QT6 cells) (**Figure 1D**). Compared to the F3aa virus, the F3aa-$_{STOP}$V viruses were severely attenuated in A549 cells (**Figure 1E**). Thus, the abolishment of V protein expression from NDV F3aa resulted in substantially attenuated virus replication in avian and human cells.

Increased interferon sensitivity of NDV F3aa lacking V protein expression

To evaluate the IFN sensitivity of the F3aa-$_{STOP}$V viruses *in vitro*, chicken fibroblasts were pretreated with chicken IFN-β before virus inoculation. In these IFN-β pretreated cells, NDV F3aa replication was not affected (**Figure 2A**). In contrast, replication of both NDV F3aa-$_{STOP}$V viruses was attenuated in IFN treated cells compared to untreated cells. These data indicate that abrogation of V protein expression increased the sensitivity of the viruses to IFN.

To examine whether the loss of V protein expression also led to increased IFN responses, IFN-β mRNA expression levels upon inoculation of chicken DF-1 cells and human A549 cells were determined. Inoculation of DF-1 cells with NDV-F3aa did not result in elevated IFN-β mRNA levels, whereas inoculation with both NDV F3aa-$_{STOP}$V viruses resulted in increased IFN-β mRNA expression levels compared to mock inoculation (**Figure 2B**). These results confirm that the V protein acts as species specific IFN antagonist in avian cells.[26] In human A549 cells, all viruses

induced increased IFN-β mRNA levels but differences were not observed between the viruses with and without an intact V ORF (**Figure 2C**).

Increased interferon sensitivity of NDV F3aa lacking V protein expression

To evaluate the IFN sensitivity of the F3aa-STOPV viruses *in vitro*, chicken fibroblasts were pretreated with chicken IFN-β before virus inoculation. In these IFN-β pretreated cells, NDV F3aa replication was not affected (**Figure 2A**). In contrast,

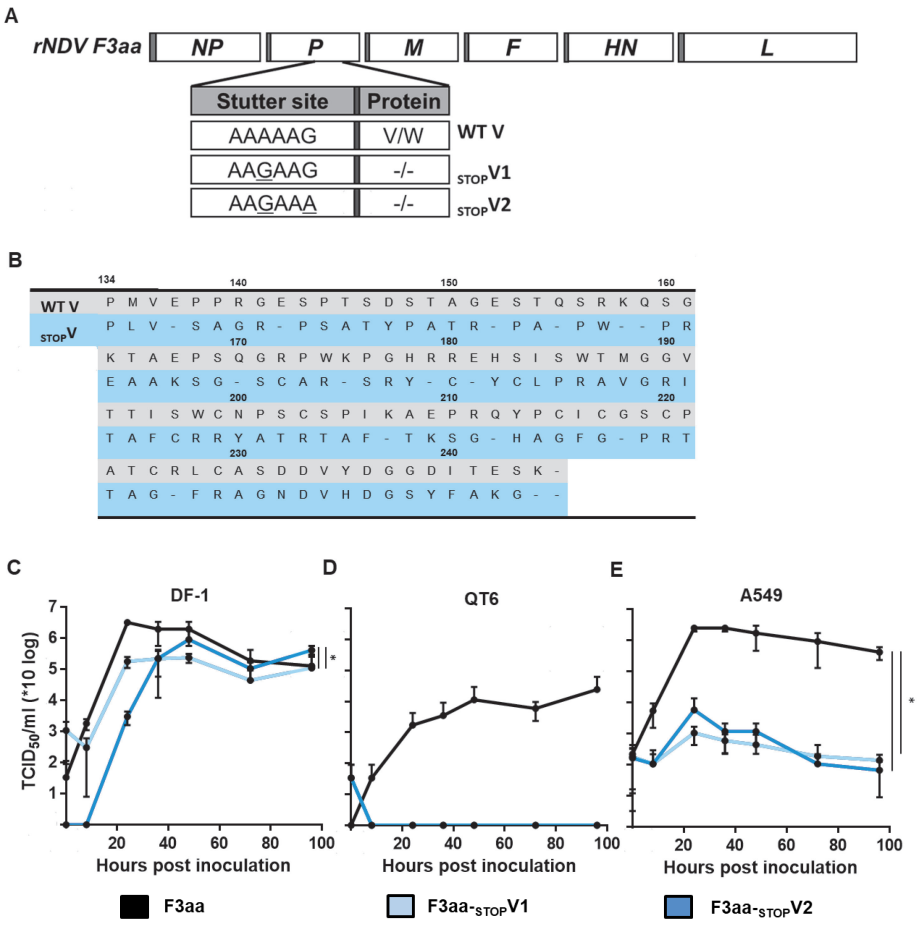


Figure 1. Characterization of NDV F3aa-STOPV viruses *in vitro*. (A) Schematic representation of the sequence of the stutter site of NDV F3aa and the NDV F3aa-STOPV mutants. (B) Amino acid sequence of V protein ORFs of NDV F3aa and F3aa-STOPV. (C-E) Cells were inoculated in triplo in (C) DF-1 at an MOI of 0.005 and at an MOI of 0.05 in (D) QT6 and (E) A549 cells. Supernatant samples were collected at the indicated time points and titrated in Vero cells (N=2). Means and standard deviations of triplicates of representative experiments are plotted. The area under the curve (AUC) was used for statistical analysis. * = p <0.05, ** = p<0.01, *** = p<0.001 (one-way ANOVA + paired t-test).

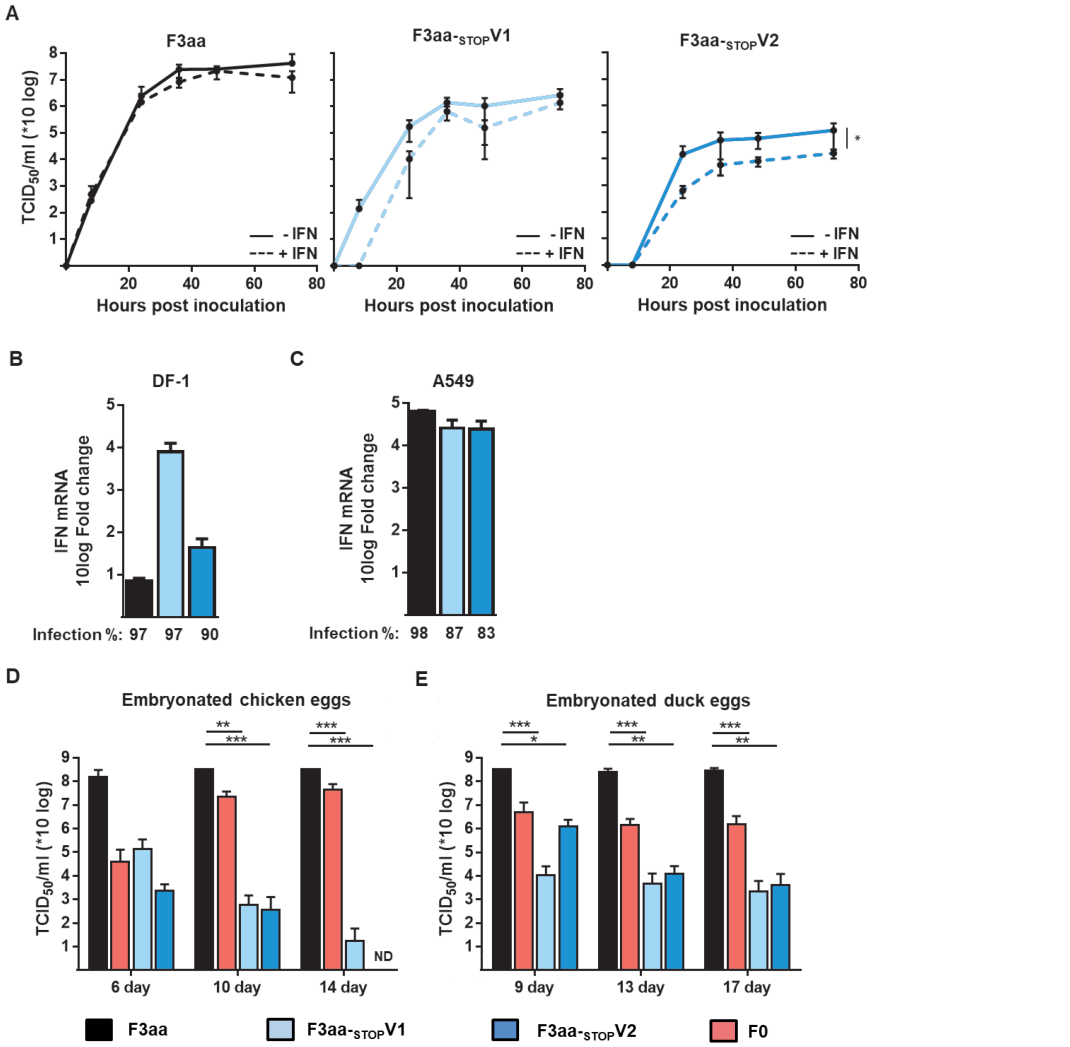


Figure 2. Characterization of NDV F3aa-STOPV viruses *in ovo*. (A) DF-1 cells were mock-treated (solid line) or pretreated with 0.4 µg/ml chicken IFN-β for 24 hrs (dotted line) and inoculated in triplo at an MOI of 0.005. Samples were taken at the indicated time points and titrated. The experiment was conducted two times. Means and standard deviations of triplicates of a representative experiment are plotted. The AUC was used for statistical analysis. * = p <0.05, ** = p<0.01, *** = p<0.001 (one-way ANOVA + paired t-test). (B) DF-1 or (C) A549 cells were inoculated with mock or at an MOI of 0.05 and harvested after 24h. The percentage of infected cells was determined by flow cytometry. Results are represented as fold change of IFN mRNA transcription in virus-infected cells versus mock. (D) Chicken and (E) duck eggs of different ages were inoculated and the allantoic fluid was harvested after 48 hours. The amount of infectious virus particles was determined by titration, with a maximal cut off of 8.3E8 TCID50/ml. * = p <0.05, ** = p<0.01, *** = p<0.001 (one-way ANOVA + Dunn's multiple comparison test). ND: not detected.

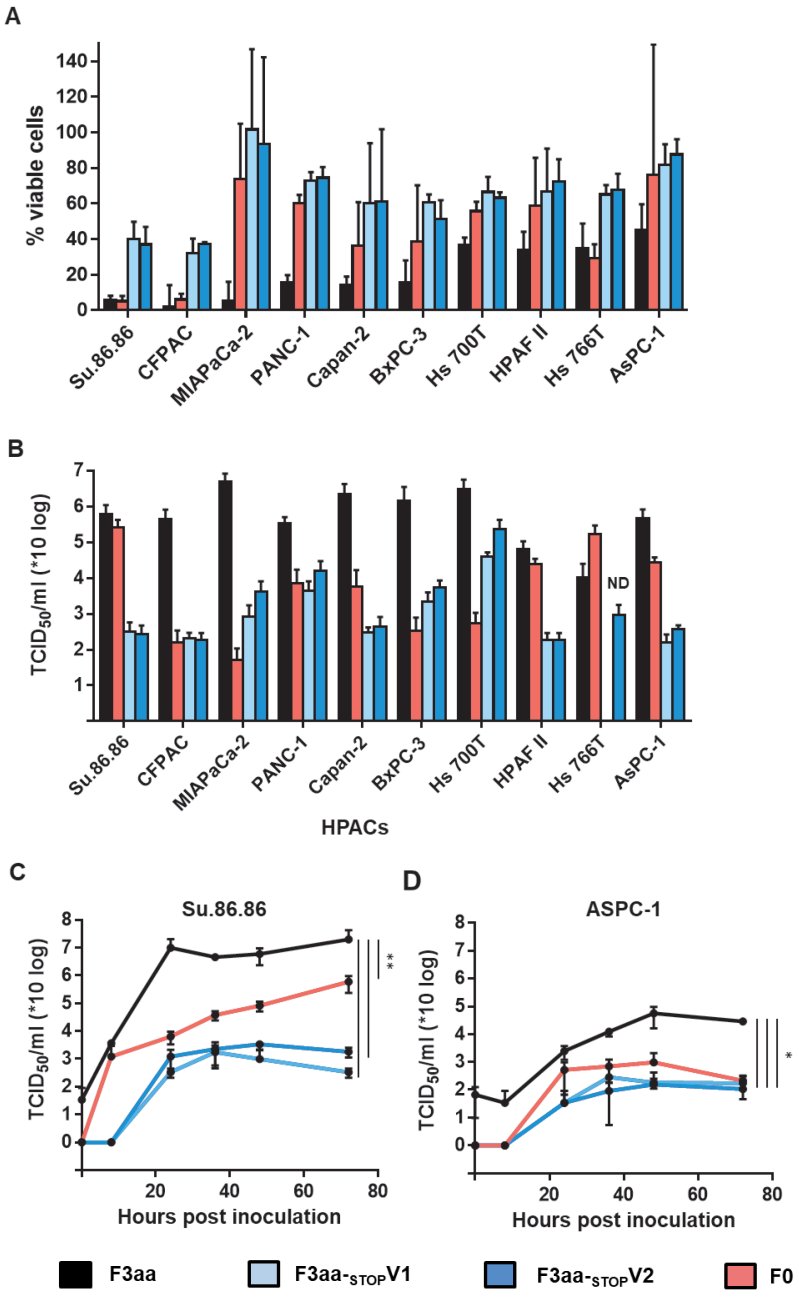
replication of both NDV F3aa-$_{STOP}$V viruses was attenuated in IFN treated cells compared to untreated cells. These data indicate that abrogation of V protein expression increased the sensitivity of the viruses to IFN.

To examine whether the loss of V protein expression also led to increased IFN responses, IFN-β mRNA expression levels upon inoculation of chicken DF-1 cells and human A549 cells were determined. Inoculation of DF-1 cells with NDV-F3aa did not result in elevated IFN-β mRNA levels, whereas inoculation with both NDV F3aa-$_{STOP}$V viruses resulted in increased IFN-β mRNA expression levels compared to mock inoculation (**Figure 2B**). These results confirm that the V protein acts as species specific IFN antagonist in avian cells.[26] In human A549 cells, all viruses induced increased IFN-β mRNA levels but differences were not observed between the viruses with and without an intact V ORF (**Figure 2C**).

To confirm that the NDV F3aa-$_{STOP}$V viruses were also attenuated in eggs due to increased IFN sensitivity, virus replication was assessed in embryonated chicken and duck eggs of different ages. Embryonated chicken eggs older than 10 days and duck eggs older than 13 days were described to produce IFN, in contrast to younger eggs.[186] Inoculation of chicken and duck eggs of all ages with NDV F3aa resulted in higher virus titers than inoculation with NDV F0 or F3aa-$_{STOP}$V viruses (**Figure 2D-E**). In 6-day old chicken eggs, which have been reported to lack IFN production, higher virus titers of NDV F3aa-$_{STOP}$V mutants were obtained than in older eggs. Upon inoculation of 14-day old chicken eggs which have been reported to produce IFN with NDV F3aa-$_{STOP}$V2, no virus titers were obtained at all. Surprisingly, inoculation with NDV F0 led to higher titers in older eggs than in younger eggs. Inoculation of duck eggs with NDV F3aa-$_{STOP}$V mutants resulted in similar virus titers in both young and older eggs, although inoculation of younger eggs with NDV F3aa-$_{STOP}$V2, resulted in slightly higher titers than inoculation of older eggs. These data indicate that abolishment of V protein expression in NDV F3aa-$_{STOP}$V resulted in an attenuated replication in both chicken and duck eggs, which in chicken eggs seems to be in part related to the age of the eggs, probably as a consequence of increased IFN sensitivity of the viruses.

Effect of NDV F3aa lacking V protein expression on tumor cell killing

The virus induced killing of tumor cells was assessed by determining the cell viability upon inoculation of ten HPAC cell lines. In general, inoculation of HPACs with NDV F3aa resulted in more cell death than inoculation with the other viruses (**Figure 3A**) and inoculation with NDV F3aa-$_{STOP}$V viruses induced a similar level of cell killing as inoculation with NDV F0 in most HPACS, but not in Su.86.86 and CFPAC. The virus induced cell killing did not always correspond with the peak virus titers obtained at 48 hours after inoculation (**Figure 3B**). For instance, upon inoculation of CFPAC cells, NDV F3aa-$_{STOP}$V induced less cell killing than NDV F0,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% viable cells
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PANC-1
Capan-2
BxPC-3
Hs 700T
HPAF II
Hs 766T
AsPC-1
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TCID50/ml (*10 log)
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MIAPaCa-2
PANC-1
Capan-2
BxPC-3
Hs 700T
HPAF II
Hs 766T
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Hours post inoculation
*
F3aa
F3aa-STOPV1
F3aa-STOPV2
F0

while peak virus titers were similar for these viruses. Similarly, although similar virus titers were obtained upon inoculation of CPFAC and HPAFII cells with F3aa-$_{STOP}$V, more cell killing was observed in CFPAC than in HPAFII cells. In two HPACS that were selected because they were most sensitive and least sensitive to NDV (**Figure 3A**), Su.86.86 and AsPC-1 respectively, replication of F3aa-$_{STOP}$V viruses was clearly attenuated compared to NDV F3aa (**Figure 3C-D**). Although the replication of NDV F3aa-$_{STOP}$V viruses was attenuated compared to NDV F3aa, the observed killing of tumor cells, being similar to that induced by NDV F0 in most cell lines, indicate that the viruses still have oncolytic potential.

Genomic stability of NDV F3aa-$_{STOP}$V viruses

During passaging of the recombinant viruses in mammalian and chicken cells seven times, all mutations introduced in both the stutter site and the open reading of the V protein were found to be genetically stable. However, in all virus stocks passaged in Vero cells, a phenylalanine to serine substitution occurred at position 117 in the F protein (F-117-S), located at the cleavage site of the protein. This substitution was not observed in any of the viruses passaged in chicken DF-1 cells. To evaluate the effect of the F-117-S substitution on virus production and cell death in HPACs, the substitution was introduced in the backbone of the NDV F3aa and NDV F3aa-$_{STOP}$V2 viruses, resulting in the viruses NDV F3aa-S and NDV F3aa-S-$_{STOP}$V2 (**Figure 4A**). Given that NDV F3aa-$_{STOP}$V1 and NDV F3aa-$_{STOP}$V2 induced similar responses in HPACs and embryonated eggs (Figure 1-3), we continued only with the virus with 2 mutations in the stutter-site as this virus has a smaller hypothetical chance of reversion. The F-117-S substitution did not result in substantial differences in virus induced cell killing for the NDV F3aa-S and NDV F3aa-S-$_{STOP}$V2 viruses upon inoculation of four different HPACs (**Figure 4B**). However, increased cytotoxicity was observed upon inoculation of Vero cells with viruses with F-117-S for both NDV F3aa-S as NDV F3aa-S-$_{STOP}$V2 (**Figure 4C**). In addition, slightly higher replication kinetics in Vero cells were observed for the NDV F3aa-S virus than for NDV F3aa virus and a larger increase in replication was seen for NDV F3aa-S-$_{STOP}$V2 as

Figure 3. Cell death of human cancer cell lines upon inoculation with NDV F3aa-$_{STOP}$V viruses. (A) Indicated HPACs were inoculated at an MOI of 10 in triplo with the indicated viruses. Cell viability was determined by an LDH cytotoxicity assay 120 hours after inoculation. Results are represented as percentage viable cells compared to mock, which were considered as 100% viable. Experiments were conducted two times. Means and standard deviations of triplicates of representative experiments are plotted. (B) Indicated HPACs were inoculated in duplo at an MOI of 0.1. After 48 hours, supernatant samples were collected and titrated in Vero cells. The experiment was conducted two times. Means and standard deviations of triplicates of a representative experiment are plotted. (C) Su.86.86 and (D) AsPC-1 cells were inoculated at an MOI of 0.05 in triplo. Supernatant samples were collected at the indicated time points and titrated in Vero cells. The experiment was conducted two times. Means and standard deviations of triplicates of a representative experiment are plotted. The AUC was used for statistical analysis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (one-way ANOVA + paired t-test). ND: not detected.

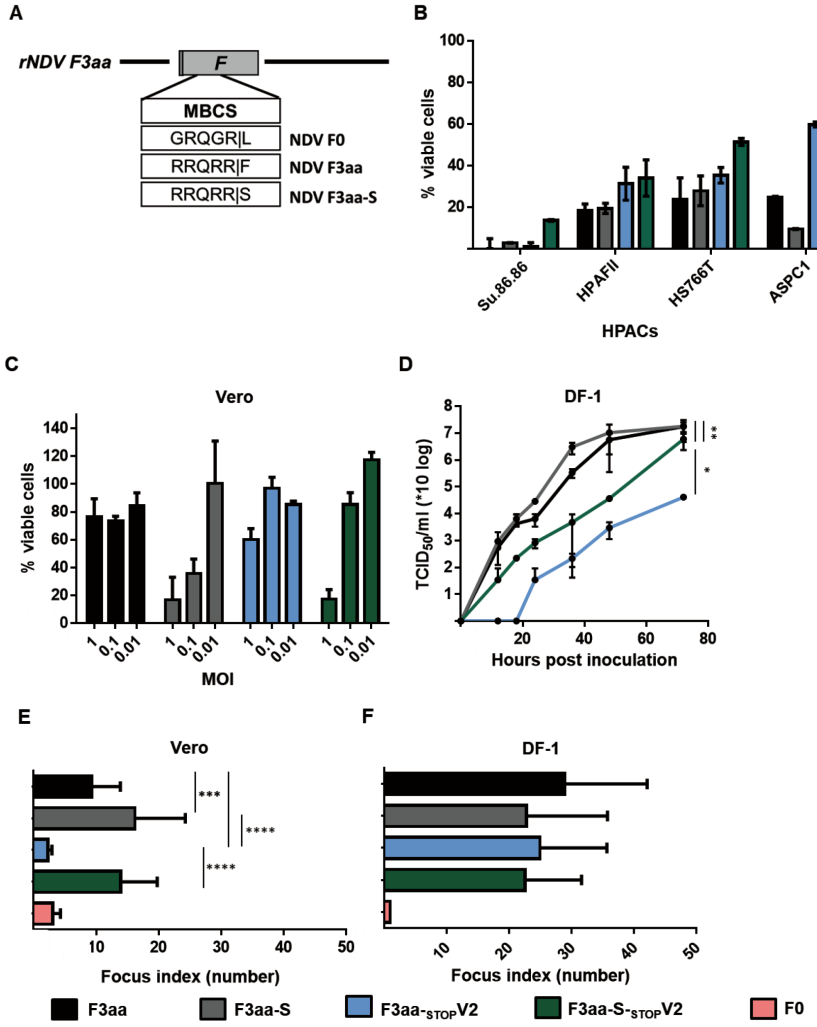


Figure 4. Characterization of F3aa-S mutant viruses. (A) Schematic representation of the amino acid sequence of the cleavage site in the F protein of recombinant viruses. (B) Indicated HPACs were inoculated at an MOI of 10 or (C) Vero cells at indicated MOIs (Vero cells) in triplo. The percentage viable cells was determined by an LDH cytotoxicity assay 120 hours after inoculation. Results are represented as percentage viable cells compared to mock, which were considered 100% viable. (D) Vero cells were inoculated at an MOI of 0.05 in triplo. Supernatant samples were collected at the indicated time points and titrated. The AUC was used for statistical analysis. * = p <0.05, ** = p<0.01, *** = p<0.001 (one-way ANOVA + paired t-test). (E) Vero and (F) DF-1 cells were inoculated and fixed 16 hours later and stained. The focus index was determined by counting the number of nuclei per foci for N=30 foci. Experiments were conducted twice. Means and standard deviations of triplicates of a representative experiment are plotted. *** = p<0.001, **** = p<0.0001 (one-way ANOVA + unpaired t test). NDV F0, taken along as control.

compared to NDV F3aa-$_{STOP}$V2 (**Figure 4D**). The improved replication of NDV F3aa-S-$_{STOP}$V in Vero cells could be beneficial for obtaining high virus titers during the production of oncolytic viruses for cancer therapy.

To investigate whether the improved replication in Vero cells was due to improved fusion activity as a consequence of the substitution in the F protein cleavage site, fusion assays were performed. In these assays, cells were inoculated with virus and fixed 16 hours later followed by Giemsa staining. Subsequently, the number of nuclei per foci were counted to determine the fusion activity of each virus. NDV F3aa-S and NDV F3aa-S-$_{STOP}$V2 viruses showed significantly increased fusion activity in Vero cells compared to viruses without F-117-S and compared to NDV F0 (**Figure 4E**). The beneficial effect of F-117-S on the fusion activity of NDV F3aa-S and NDV F3aa-S-$_{STOP}$V2 viruses was not observed in DF-1 cells (**Figure 4F**). These data indicate that the F-117-S mutation probably provides a specific adaptation towards Vero cells.

Reduced virulence of NDV F3aa-(S)-$_{STOP}$V2 viruses

To further assess the virulence of NDV F3aa-$_{STOP}$V2 and NDV F3aa-S-$_{STOP}$V2, the mean death time (MDT) of embryonated chicken and duck eggs upon inoculation with these viruses was determined as compared to viruses with an intact V protein ORF (**Table 3**). A MDT of chicken embryos upon NDV inoculation greater than 90 hours has been shown to be typical for nonvirulent strains that have an ICPI value smaller than 0.7.[187] Upon inoculation with NDV F3aa and NDV F3aa-S, chicken eggs reached an MDT below 60 hours and duck eggs below 100 hours. Inoculation with NDV F0 resulted in an MDT just above 100 hours for eggs of both origins. No MDT could be determined for both chicken and duck eggs inoculated with NDV F3aa-$_{STOP}$V2 and NDV F3aa-S-$_{STOP}$V2, because all embryos survived. These data indicate that NDV F3aa-$_{STOP}$V2 and NDV F3aa-S-$_{STOP}$V2 were nonvirulent for poultry embryos, in contrast to NDV F3aa and NDV F3aa-S.

Mutations in the F and/or HN gene improved replication of NDV F3aa-S-$_{STOP}$V2

The attenuation of NDV F3aa-(S)-$_{STOP}$V2 mutants in avian cells and eggs was accompanied by reduced virus replication and virus-induced cell death in most HPACs. To improve virus replication and virus-induced cell killing, we introduced a number of mutations in the F and HN genes of the NDV F3aa-S-$_{STOP}$V2 virus (**Figure 5A**). First of all, a substitution (Y-524-A) was introduced in the intracellular domain (ICD) of the F protein. This substitution was previously shown to cause increased virus replication, possibly due to improved stability of the F protein.[179] Introduction of this substitution in the genome of NDV F3aa-S-$_{STOP}$V2, yielded the virus NDV F3aa-S-$_{STOP}$V2-ICD. In addition, the translation initiation sites (TIS) of the F and HN proteins were changed to a mammalian optimal TIS sequence, the Kozak consensus sequence, with the aim to increase the translation and expression of both proteins.[181] This yielded NDV F3aa-S-$_{STOP}$V2-TIS (**Figure 5A**). The NDV F3aa-S-$_{STOP}$V2-ICD

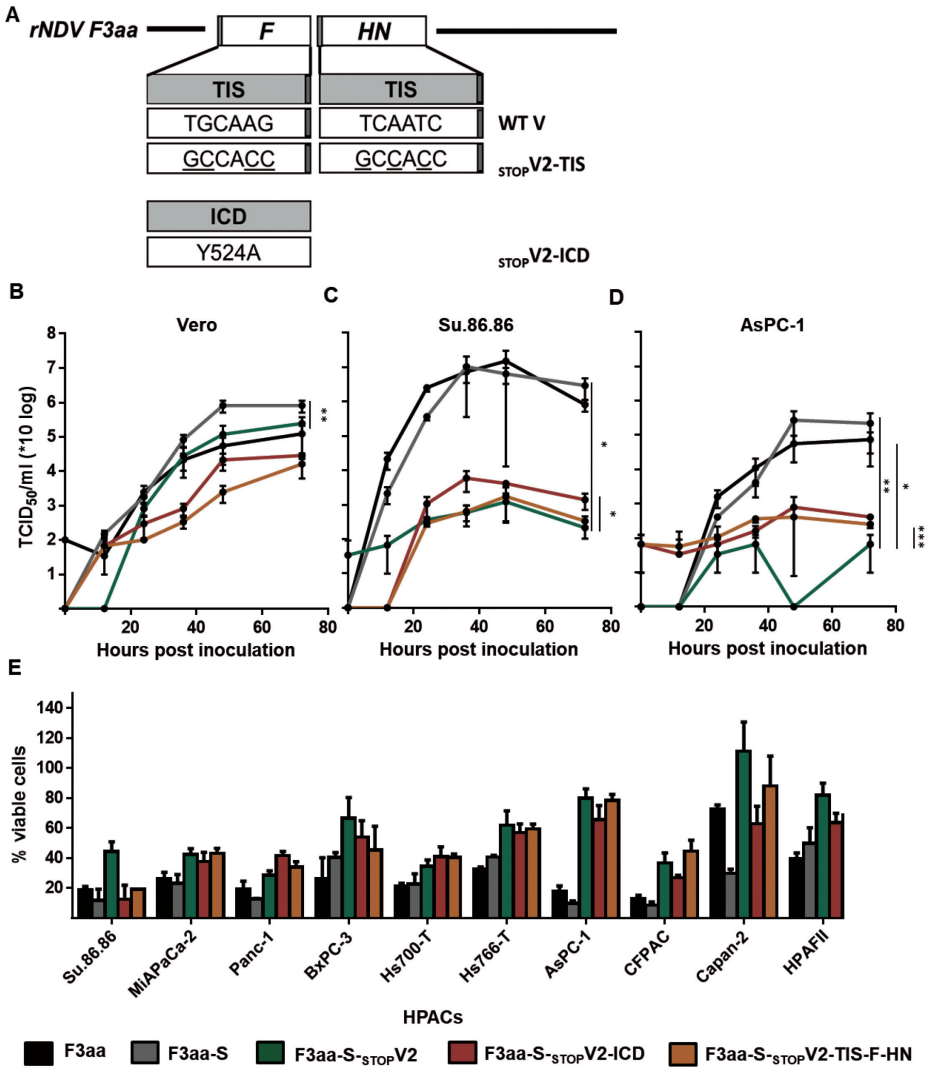


Figure 5. Characterization of NDV F3aa-(S)-STOPV mutants. (A) Schematic representation of the nucleotide sequence of the TIS and amino acid substitution of the ICD of the F and HN protein. (B) Vero cells, (C) Su.86.86 and (D) AsPC-1 were inoculated at an MOI of 0.05 in triplo. Supernatant samples were collected at the indicated time points and titrated. Means and standard deviations of triplicates of a representative experiment are plotted. The AUC was used for statistical analysis. * = p <0.05, ** = p<0.01, *** = p<0.001 (one-way ANOVA + paired t-test as compared to NDV F3aa-S-STOPV2). (E) The indicated HPACs were inoculated at an MOI of 10 in triplo. The percentage viable cells was determined by an LDH cytotoxicity assay. Results are represented as percentage viable cells compared to mock, which were considered 100% viable. The experiment was conducted two times. Means and standard deviations of triplicates of a representative experiment are plotted.

and NDV F3aa-S-STOPV2-TIS viruses were genetically stable until at least passage 4 in Vero cells.

In Vero cells, the replication of NDV F3aa-S-STOPV2-TIS and NDV F3aa-S-STOPV2-ICD was attenuated compared to NDV F3aa-S-STOPV2 but reached similar end titers (**Figure 5B**). In contrast, in the HPAC cell line Su.86.86, replication of NDV F3aa-S-STOPV2-ICD was slightly higher than that of NDV F3aa-S-STOPV2 and F3aa-S-STOPV2-TIS (**Figure 5C**). In AsPC-1 cells, replication of both NDV F3aa-S-STOPV2-TIS and NDV F3aa-S-STOPV2-ICD was higher than that of NDV F3aa-S-STOPV2 (**Figure 5D**). The higher replication of NDV F3aa-S-STOPV2-TIS and NDV F3aa-S-STOPV2-ICD in ASPC-1 cells as compared to NDV F3aa-S-STOPV2 did not result in increased cell death of these HPACs. Only in 2 of the HPACs (Su.86.86 and HPAFII), inoculation with NDV F3aa-S-STOPV2-TIS and NDV F3aa-S-STOPV2-ICD resulted in more cell death than inoculation with NDV F3aa-S-STOPV2. Inoculation of CFPAC and Capan-2 cells resulted in more cell death after inoculation with NDV F3aa-S-STOPV2-TIS than upon inoculation with NDV F3aa-S-STOPV2 or NDV F3aa-S-STOPV2-ICD (**Figure 5E**). In the other HPACS virus induced cell killing was similar for the three viruses. Thus, the introduced mutations in the F and HN genes of the NDV F3aa-S-STOPV2 virus improved virus replication and cell killing of NDV F3aa-S-STOPV2 somewhat, but not consistently across all cell lines tested.

Examination of the virulence in embryonated chicken eggs demonstrated that NDV F3aa-S-STOPV2-TIS and NDV F3aa-S-STOPV2-ICD did not kill chicken embryos (MDT >160 hours, **Table 3**). These observations were similar to those seen for the parental NDV F3aa-S-STOPV2 virus, indicative of the nonvirulent phenotype of NDV F3aa-S-STOPV2-ICD and NDV F3aa-S-STOPV2-TIS viruses. The virulence in duck eggs was not investigated for NDV F3aa-S-STOPV2-TIS and NDV F3aa-S-STOPV2-ICD.

Table 1. Mean death time of chicken and duck eggs upon inoculation with NDV and engineered mutants thereof.

Virus	Chicken egg MDT (h)*	Duck egg MDT (h)
NDV F0	107	32
NDV F3aa	52	87
NDV F3aa-S	58	96
NDV F3aa - $_{STOP}$V2	>160	>184
NDV F3aa-S - $_{STOP}$V2	>160	>184
NDV F3aa-S - $_{STOP}$V2-ICD	>160	N.D.
NDV F3aa-S - $_{STOP}$V2-TIS	>160	N.D.

* An MDT of chicken eggs >90h was shown previously to be typical for nonvirulent NDV strains.[29]

N.D. – Not done.

Discussion

Several studies have demonstrated the safety and efficacy of lentogenic NDV F0 in clinical trials[95,103], but the use of mesogenic NDV F3aa resulted in higher oncolytic efficacy *in vitro* and *in vivo*.[50] However, using such a strain in oncolytic viral therapy could theoretically pose a risk for poultry due to the possible shedding of virus by treated patients.[43,96] To avert any risks of outbreaks with NDV F3aa, we aimed to develop a virus that has oncolytic potential but does not cause disease in avian species. To this end, we abrogated the expression of the V protein, a specific avian IFN antagonist, by mutation of the stutter site and introduction of several stop codons in the ORF of the V protein, yielding viruses NDV F3aa-$_{STOP}$V1 and NDV F3aa-$_{STOP}$V2, and evaluated the genetic stability and their virulence *in vitro* and *in ovo*.

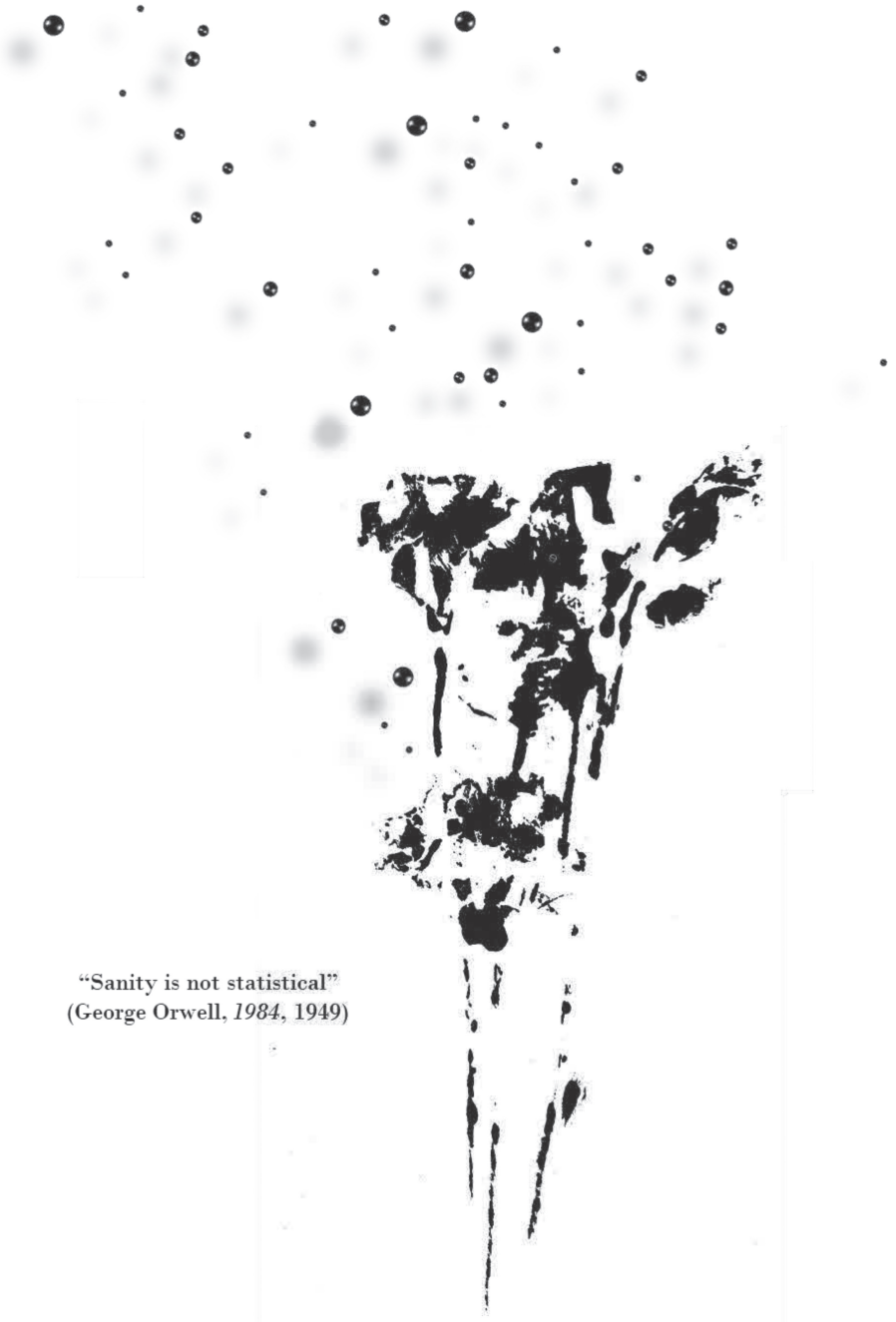
Analyses of the genetic stability upon passaging the mutant viruses in chicken and mammalian cells revealed that the mutations introduced in the V protein ORF were genetically stable. However, during passaging in Vero cells, a F-117-S substitution in the MBCS of the F protein was observed. Introduction of this substitution in NDV F3aa and NDV F3aa-$_{STOP}$V2 resulted in increased virus replication, fusion activity and cell death in Vero cells, but not in increased cell death in most HPAC cell lines. Our fusion experiment results are in agreement with previously reported data on the characterization of NDV with the F-117-S substitution in *in vitro* experiments showing that F-117-S resulted in higher fusion activity in HT1080 and Hela cells. However, in that study, the NDV 73 T strain with the F-117-S substitution had an intracerebral pathogenicity index (ICPI) of 1.05 in chickens, substantially higher than the ICPI of 0.13 for the wild type virus.[180] In contrast, in our study the LaSota variant of NDV F3aa-S had a slightly delayed MDT of chicken and duck embryos as compared to NDV F3aa, indicative for a slightly lower virulence. These observed differences in virulence might be due to the use of different methods for determining the virulence of NDV, e.g. MDT vs ICPI or due to differences between the NDV LaSota and NDV 73 T strains.

The NDV F3aa-$_{STOP}$V viruses displayed attenuated replication in avian and human cells. This attenuation was more pronounced in IFN-pretreated chicken cells. In addition, inoculation of chicken cells with the NDV F3aa-$_{STOP}$V viruses induced higher IFN mRNA levels than the virus with an intact V protein, but this difference was not observed in human cells. Multiple studies have reported comparable results on viruses with a single mutation in the stutter site or introduction of a single stop codon in the V protein ORF, which led to increased susceptibility to IFN.[26,27,188] Our results are in agreement with these studies and confirm that the disruption of the V protein ORF attenuates NDV, likely due to increased IFN sensitivity *in vitro*. Similar results were obtained upon inoculation of chicken eggs, in which reduced virus titers were observed for NDV without a V protein.[27] Our study demonstrated that NDV F3aa-$_{STOP}$V viruses were attenuated in both chicken and duck eggs. Virus titers for NDV F3aa-$_{STOP}$V were slightly higher in young eggs as compared to older eggs,

which is in agreement with the fact that older eggs produce IFN, suggesting that the attenuation *in ovo* is also based on increased IFN sensitivity. Based on the analyses of the MDT of eggs, both the NDV F3aa-$_{STOP}$V and F3aa-S-$_{STOP}$V should be considered as nonvirulent (lentogenic) viruses, compared to the mesogenic NDV F3aa and NDV F3aa-S viruses. Several studies have reported that eggs inoculated with viruses containing only a single stop codon in the V protein ORF had a similar or slightly higher MDT than eggs inoculated with the wild type virus.[29,189] This difference in MDT in our study might be the result of additional amino acid substitutions in the W protein of our NDV F3aa-(S)-$_{STOP}$V viruses. The function of the W protein has not been studied extensively, but it has been shown that the protein is expressed and does not function as an IFN antagonist.[28,29] The attenuation of the NDV F3aa-$_{STOP}$V viruses for poultry cells and eggs indicate that these viruses can be safely evaluated in oncolytic viral therapies.

Although the NDV F3aa-(S)-$_{STOP}$V viruses were slightly attenuated in mammalian cells, these viruses were still able to replicate in the HPACs and induced cell death. Mutating the ICD of the F protein or the TIS of the F and HN proteins slightly improved virus replication of the NDV F3aa-S-$_{STOP}$V2 virus in HPACs, but not to similar levels as those of NDV F3aa. The improved replication of the ICD mutant in AsPC-1 is in line with previously reported studies.[179] In addition, the introduction of the TIS mutation in NDV F3aa-S-$_{STOP}$V2 did increase virus induced cell death in some HPACs. Additional adaptations of these viruses, such as the incorporation of immune modulatory genes to increase anti-tumor responses, may further improve the therapeutic efficacy of our NDV mutants.[74]

Taken together, our data indicate that NDV F3aa(-S)-$_{STOP}$V viruses are attenuated for avian species but are perhaps over-attenuated to kill tumor cells as efficiently as NDV F3aa. This attenuation was not completely overcome with the introduced mutations (TIS, ICD) investigated in this study. Further adaptations and *in vivo* studies are necessary to confirm the safety and effectivity of NDV F3aa-$_{STOP}$V viruses in oncolytic viral therapies in the future.



"Sanity is not statistical"
(George Orwell, *1984*, 1949)

Chapter 4

Assessment of the virulence for chickens of Newcastle Disease virus with an engineered multi-basic cleavage site in the fusion protein and disrupted V protein gene

J.F. de Graaf, S. van Nieuwkoop, D. de Meulder, P. Lexmond, T. Kuiken, D. Groeneveld, R.A.M. Fouchier, and B.G. van den Hoogen

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Viroscience department, Erasmus Medical Centrum, Rotterdam, The Netherlands

Abstract

Newcastle Disease virus (NDV) has shown promise as an oncolytic virus for treatment of a wide range of tumors. NDV with a multi-basic cleavage site (MBCS) in the fusion (F) protein (NDV F3aa) has increased oncolytic efficacy in several tumor models, but also increased virulence in chickens compared to non-virulent NDV F0, raising potential environmental safety issues. Previously, we generated a variant of NDV F3aa with a disrupted V protein gene and a substitution of phenylalanine to serine at position 117 of the F protein (NDV F3aa-S-$_{STOP}$V). Compared to NDV F3aa this virus had decreased virulence in embryonated chicken eggs. In this study, the virulence of the virus was evaluated upon inoculation of six-week-old chickens through a natural infection route and by determination of the intracerebral pathogenicity index (ICPI). Based on these data NDV F3aa-S-$_{STOP}$V classified as a non-virulent virus. Although NDV F3aa was classified as a virulent virus based on the ICPI, the virus was also less pathogenic than NDV F0 upon inoculation of six-week-old chickens. These data indicate that NDV with a MBCS is not necessarily pathogenic in chickens. In addition, these data show that F3aa-S-$_{STOP}$V is safe to use in viro-immunotherapies without posing a threat for chickens upon accidental exposure.

Introduction

Oncolytic viro-immunotherapy is considered a new form of cancer immunotherapy for which studies have shown promising results for treatment of a variety of tumors. Oncolytic viruses replicate in tumor cells, resulting in direct cell death and in activation of an anti-tumor immune response. One of these promising oncolytic viruses is Newcastle disease virus (NDV), a replication competent virus, which belongs to the family *Paramyxoviridae* and has an avian host range under normal conditions. Several studies have shown the oncolytic efficacy of wild type non-virulent strains of NDV in *in vitro* assays and in murine tumor models, but more virulent strains had higher oncolytic efficacy.[49,55,56,155] However, virulent NDV could be very pathogenic for avian species, which raises concerns about the environmental safety when used in oncolytic viro-immunotherapy for cancer patients.[43,44,95,103]

NDV strains are categorized in three groups based on their virulence for chickens: non-virulent (lentogenic), intermediately virulent (mesogenic) and highly virulent (velogenic). Upon infection with lentogenic strains, chickens show only mild signs of disease with negligible mortality. Infections with mesogenic strains cause coughing, affect egg production and quality, and cause up to 10% mortality. Infections of chickens with velogenic strains induce severe nervous and respiratory signs and cause up to 90% mortality.[25,148] As of 2018, the World Organisation for Animal Health and EU guidelines only discriminates between virulent and non-virulent strains.The cleavage site in the fusion (F) protein of NDV is a major determinant of the virulence of the virus.[31,32] The F protein is translated as precursor protein F_0 which is subsequently proteolytically cleaved into the activated subunits F_1 and F_2 during infection. Non-virulent strains, such as the LaSota strain (NDV F0), are dependent on exogenous trypsin to cleave the F0 protein, whereas virulent viruses with a multi-basic cleavage site (MBCS) in the F protein are not.[25]

The internationally recognized standard to determine the virulence of NDV strains is based on determination of the intracerebral pathogenicity index (ICPI) in one-day old chickens [35]. For this assay, virus is inoculated intracerebrally in 10 one-day-old chicks and the index is the mean score per chick per 24-hour observation over 8 days when each bird is scored 0 if normal, 1 if sick and 2 if dead. Lentogenic viruses have an ICPI up to 0.7, mesogenic viruses have an ICPI 0.7 to1.4 and velogenic viruses have an ICPI higher than 1.4. According to the latest World Organisation for Animal Health and EU guidelines, an NDV strain classifies as virulent by either an ICPI of 0.7 or greater, or the presence of multi basic amino acids at position 113-116 of the C terminus of the fusion (F2) protein, plus phenylalanine (F) at residue 117 of the F1 protein.[25,35]

Another determinant used for virulence is the intravenous pathogenicity index (IVPI). This index is generated upon intravenous injection of the virus into 10 six-week-old chickens. Monitoring of the disease and calculation of the index is similar as

described for the ICPI.[190] A third method used for determining the virulence of an NDV strain is the induced mean death time (MDT) in embryonated chicken eggs. The MDT is determined by inoculation of groups of 10 nine-day-old chicken embryos with serial dilutions of virus. MDT is the mean time in hours for the minimal lethal dose (MLD) to kill embryos. The MLD is the highest virus dilution that causes all the embryos inoculated with that dilution to die. The result is reported in 'hours to kill' resulting in the following classification: virulent < 60 hours, mesogenic 60–90 hours and lentogenic > 90 hours. Although this interpretation is widely accepted and the test is convenient, the MDT has been criticised for lack of reproducibility.[35] Previously, we, and others, have shown that infection with a recombinant non-virulent LaSota strain containing an MBCS in the F protein (NDV F3aa) led to significantly higher efficiency killing tumor cells in *in vitro* assay*s* and in murine tumor models compared to the recombinant non-virulent NDV F0.[51,52,155] However, several studies have shown that based on the ICPI, the induced MDT of embryonated chicken eggs and the IVPI, recombinant NDV F3aa is classified as a virulent strain.[25,31,148,191]

In order to decrease the pathogenicity of recombinant NDV F3aa for poultry we generated a variant of NDV F3aa in which viral expression of the V protein was abrogated by introducing mutations throughout the V protein gene and in the stutter site that is essential for expression of the V protein (NDV F3aa-$_{STOP}$V). The mutations introduced in the V protein open reading frame (ORF) affected all of the third positions of the codons of the essential Phosphoprotein (P) ORF which did not result in any amino acid substitutions in the P protein but resulted in 15 stop codons in the V protein ORF.[192] The V protein has been reported to be a driver of virulence as the protein is a host specific antagonist of the innate immune response.[26,27,193,194] The replication efficiency and cell fusion capacity of NDV F3aa and F3aa-S-$_{STOP}$V upon infection was further enhanced by substitution of phenylalanine to serine at position 117 of the F protein (F-117-S) of these viruses resulting in NDV F3aa-S and F3aa-S-$_{STOP}$V (**Table 1**).[180,192]

Table 1. Amino acid sequence of the multi-basic cleavage site (MBCS) of generated recombinant NDV strains

Virus	MBCS
NDV F0	GRQGR|L
NDV F3aa	RRQRR|F
NDV F3aa-S	RRQRR|S

Previously, we reported on the decreased virulence of NDV F3aa-S-$_{STOP}$V compared to NDV F3aa-(S) in avian cells and in chicken and duck eggs [192]. Here we report on the virulence of these viruses as determined by ICPI assays in one-day old chicks and the pathogenicity in six-week-old chickens upon inoculation via a natural infection route. Our data demonstrated that oncolytic NDV F3aa-S-$_{STOP}$V was non-

virulent as determined in ICPI assays and did not cause disease in six-week-old chickens upon inoculation via the natural route. This indicates that NDV F3aa-S-$_{STOP}$V can be safely used in viro-immunotherapies in humans without posing a threat for chickens upon accidental exposure. In addition, our data show that the virulence of NDV F3aa, as determined in the ICPI assay, did not correlate with the pathogenicity observed after inoculation of six-week-old chickens via a natural route of infection. This suggests that this LaSota NDV strain with an engineered MBCS may also be considered safe to use in oncolytic viro-immunotherapies in humans with respect to potential disease in chickens upon accidental exposure of poultry.

Methods

Cell lines

Vero cells were obtained from the American Type Culture Collection and were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine (PSG) and 10% Hyclone Characterized Fetal Bovine serum (FBS-HC, Thermofischer) at 37 ºC. BSR-T7 cells (kind gift of K. Conzelmann) and chicken fibroblast (DF-1) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) supplemented with PSG and 10 % FBS-HC at 37 ºC.

Virus preparation

The reversed genetics system for the recombinant NDV LaSota strain has been described before and was kindly provided by B. Peeters from Wageningen Bioveterinary Research, The Netherlands.[156] Recombinant NDV F0 and F3aa and mutants thereof were cloned and rescued as described before.[155,192] Virus stocks were stored in 25% sucrose at -80 ºC. For ICPI assays, virus stocks were concentrated to 1x10^7 $TCID_{50}$/ ml and purified of residual waste from the cell culture by using 100K centrifugal filters (Amicon Ultra15, Merck Millipore, The Netherlands) and then stored at -80 ºC without sucrose. Before inoculation of the chickens, stocks were filtered through a 0.45 µm membrane (Merck Millipore, The Netherlands).

Virus titration

Virus stocks, samples from animal tissue homogenates and clinical specimens collected from chickens were titrated by end-point dilution in Vero cells in infection medium: IMDM supplemented with PSG and 0.2 mg/ ml TPCK-treated Trypsin (T1426, Sigma-Aldrich, The Netherlands). Seven days after inoculation, cells were fixed with 80% acetone in phosphate buffered saline (PBS). After washing with PBS and one hour incubation with 10% milk in PBS, the cells were stained with 1:1000 diluted anti-NDV (ab34402, Abcam, UK) followed by 1:1000 diluted secondary FITC-labelled antibody (ab6749, Abcam) both diluted in 1% milk in PBS. Stained cells were detected by fluorescence microscopy (Carl Zeiss Axio Vert. A1). The titer was

calculated using the method of Reed & Muench and expressed as $TCID_{50}$/ ml^{-}.[157] Only samples with a CT<35 in qRT-PCR assays were titrated.

Replication kinetics
One million DF-1 cells were seeded in 6-well plates (Corning) and were inoculated the next day at a M.O.I. of 0.005. One hour post inoculation the cells were washed three times with PBS after which infection medium was added. At indicated time points, 100 µl sample was collected and stored with 25% sucrose (w/w) at -80 ºC. Subsequently, samples were titrated in Vero cells.

Cytotoxicity assay
DF-1 cells were seeded in 96-well plates (Greiner Bio-One, The Netherlands) and inoculated with virus at the indicated M.O.I.s. One hour after inoculation, the cells were washed once with PBS and fresh infection medium was added. After 72 hours, cell viability was determined using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, The Netherlands) as described before.[155]

Animals and experimental design
Per virus group, 6 six-week-old specific-pathogen-free (SPF), unvaccinated, chickens (GD Animal Health, Deventer) were inoculated 100 µl with $1x10^5$ $TCID_{50}$ virus via the choanae. At 24 hours after inoculation, three naive animals were added per group to study virus transmission. Animal wellbeing and weight was observed daily throughout the experiment. Swabs from the conjunctiva, choanae, nasal cavity and cloaca were collected daily in 1 ml virus transport medium.[160] At days 3 and 7, three inoculated animals were euthanized for necropsy and at day 14 after inoculation, the contact animals were euthanized for necropsy. During necropsy blood was collected in blood collection tubes (Minicollect, Greiner Bio-one, 450533).

Sample preparations
After mixing swab samples in virus transport medium, 200 µl was used for RNA isolation. Each collection tube with collection media was weighted before and after collection of the different tissues. The weight differences between comparable tissue samples were consistent and it was decided that the unadjusted CT values were more informative than the CT values normalized to weight.
Blood samples were centrifuged for 10 minutes at 1000 RPM. Serum was stored at -20 ºC of which 200 µl was used for RNA isolation. At necropsy, samples from 19 organs (listed in Fig. 5) were collected in virus transport media and homogenized using a FastPrep 24 tissue homogenizer (MP Biomedicals). Subsequently, samples were centrifuged for 10 minutes at 2000 *g* and supernatant was stored at -80 ºC or 200 µl was used for RNA isolation.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)
RNA was isolated from collected samples with the High Pure RNA isolation kit (Roche Diagnostics, The Netherlands) according to the manufacturer's instructions. NDV specific qRT-PCR (45 cycles) was performed using 5 µl RNA and NDV specific primers in an ABI PRISM 7500 Realtime PCR system (Thermo Fischer Scientific) with the TaqMan Fast Virus 1-step Master Mix (Thermo Fischer Scientific) as described before.[162]

Sequencing
cDNA was produced from isolated RNA with the Superscript III Reverse Transcriptase kit according to the manufacturer's instructions (18080051, Invitrogen). The V and F protein regions were amplified by PCR assays using primers described before [192]. PCR products were sequenced using the 3130xL Genetic Analyzer according to the manufacturer's instructions with the following sequence primers: Fwd-MBCS: 5'-ggccaagatactctggag-3', Rev-MBCS: 5'-ggccaagatactctggag-3', Fwd-V: 5'-ggcactccccaatcg-3' and Rev-V: 5'-cttgcttaggagcttggc-3'.

NDV serology
Sera were tested for NDV specific antibodies by hemagglutination inhibition assay (HI) using turkey erythrocytes as described before.[55] Chicken polyclonal NDV antibody (ab34402, Abcam) was used as positive control.

Histopathology and Immunohistochemistry
Samples for histopathological analysis were fixed with 10% neutral-buffered formalin, embedded in paraffin and sectioned at 3 µm. Tissue sections were mounted on coated slides (Klinipath, Netherlands), deparaffinized and rehydrated. Slides for histopathology were stained with haematoxylin and eosin (H&E) and examined by light microscopy. Slides for immunohistochemistry were stained for NDV as described before [195]. The influx of immune cells was assessed by counting granulocytes in infected areas of the nasal conchae.

Intracerebral pathogenicity index (ICPI) assay
One-day old chickens were inoculated intracerebrally with $1x10^4$ $TCID_{50}$ virus in 0.05 ml, which was diluted in 10% allantoic fluid and PBS. Animals were observed daily for eight consecutive days. The ICPI was calculated as described before.[34]

Ethics statement
All experiments involving animals were conducted strictly according to the European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The experiment protocol (AVD101002017849) was reviewed and approved by an independent animal experimentation ethical review committee.

Results

Virulence assessment *in vitro*

We previously generated a variant of the recombinant non-virulent NDV LaSota strain in which we inserted an MBCS and interrupted the V protein open reading frame (ORF) by introduction of 15 stop codons in this ORF. To improve the replication efficiency and oncolytic efficacy of this virus, the phenylalanine in the MBCS was substituted by serine at position 117 of the F protein (F-117-S) resulting in NDV F3aa-S-$_{STOP}$V, resulting in increased fusion activity as previously reported.[192]

To compare virus replication efficiency of F3aa-S-$_{STOP}$V with NDV F3aa, F3aa-S and F0 *in vitro*, chicken fibroblasts (DF-1 cells) were inoculated. In these cells, NDV F3aa and F3aa-S replicated most efficiently and induced the most cell death, followed by NDV F3aa-S-$_{STOP}$V. The least efficient replication and induced cell death was observed upon inoculation with NDV F0. No significant differences were observed

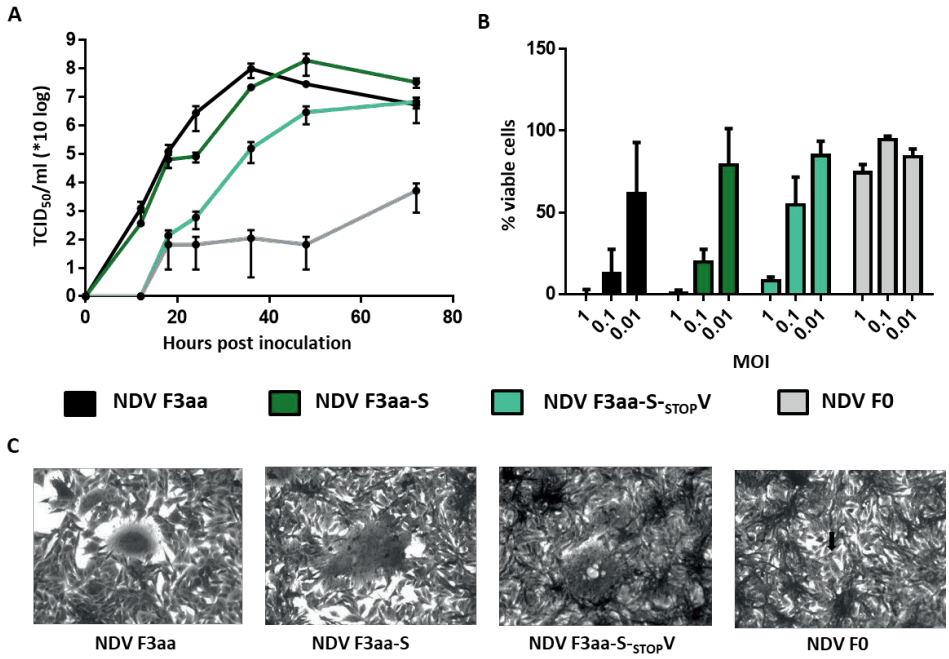

Figure 1. Virus replication and induced cell death upon inoculation of DF-1 cells with NDV F0, NDV F3aa and variants thereof. (A) Virus replication kinetics in DF-1 cells. Cells were inoculated at a multiplicity of infection (MOI) of 0.05 in triplo. At indicated time points samples were taken and titrated. Values are represented as average. (B) Virus induced cell death at five days post inoculation. DF-1 cells were mock-inoculated or inoculated at indicated MOI in triplo. The percentage viable cells was determined by a LDH cytotoxicity assay. Results are represented as percentage viable cells compared to mock, which were considered as 100% viable. (C) The morphology of infected cells analyzed by light microscopy after Giemsa staining. Cells were fixed 24 hours after inoculation. Representative images are shown.

between NDV F3aa and F3aa-S with regard to virus replication and induced cell death of DF-1 cells (**Figure 1A-B**). During replication of NDV F3aa, F3aa-S and F3aa-S-$_{STOP}$V in these cells, large syncytia were observed which were not present during replication of NDV F0 (**Figure 1C**). These data show that in DF-1 cells, NDV F0 spreads less efficiently than NDV F3aa and variants thereof, and that in these in *in vitro* assays the replication and cell killing efficiency of NDV F3aa-S-$_{STOP}$V was decreased compared to that of NDV F3aa-(S).

Pathogenicity in six-week-old chickens

To assess the pathogenicity of the virus infections in chickens, 6 six-week-old SPF chickens were inoculated via the choanal cavity. One day later three naive animals were placed in the same isolator as NDV F3aa-S, F3aa-S-$_{STOP}$V and F0 inoculated animals to monitor transmission (see scheme in **Figure 2A**). Due to insufficient available animals, transmission was not assessed for NDV F3aa. Monitoring of

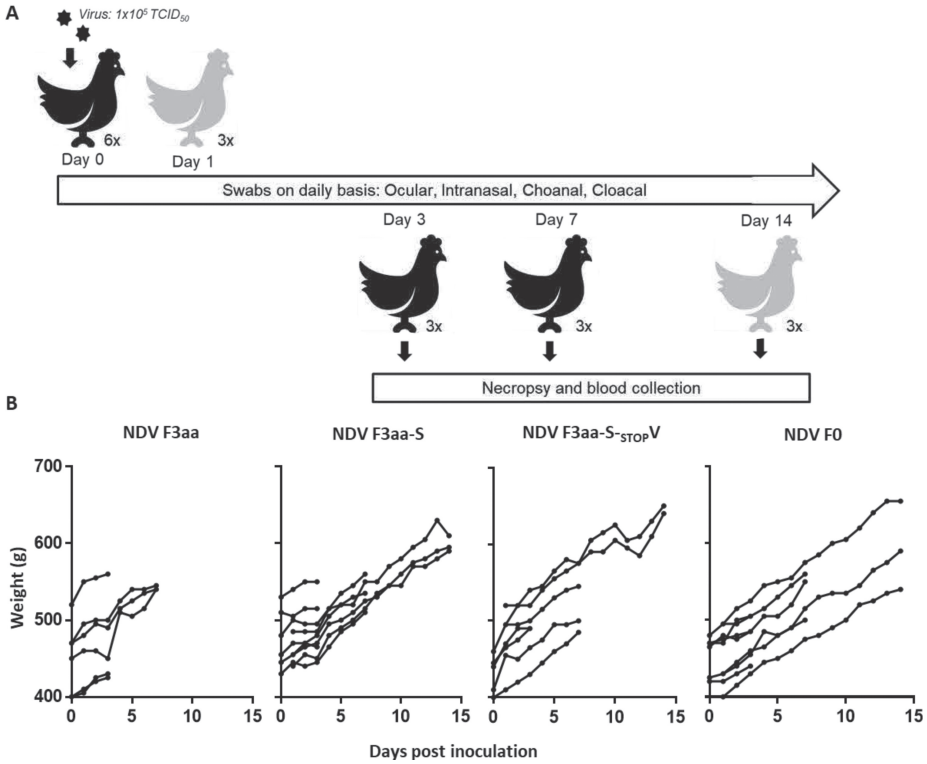

Figure 2. Inoculation of six-week-old chickens with NDV F0, NDV F3aa and variants thereof via the choanal route of infection. (A) Schematic representation of experimental setup indicating the day of inoculation, addition of contact animals, time points of sample collection and necropsy. Per virus group, 6 six-week-old chicken were inoculated with 1x10^5 TCID50 virus via the choanae. (B) Weight of all individual animals (all depicted in black lines) per group during the course of the experiment, including the three contact animals of NDV F3aa-S, NDV F3aa-S-$_{STOP}$V and NDV F0 inoculated animals.

transmission of NDV F3aa-S was chosen over that of NDV F3aa as the aim of this experiment was studying NDV F3aa-S-$_{STOP}$V, for which the best control would be NDV F3aa-S. After inoculation, none of the animals showed clinical signs or weight loss during the 14 days of the experiment (**Figure 2B**). Surprisingly, the most pronounced shedding, as determined by the presence of virus genomes in collected samples, was observed for the chickens inoculated with NDV F0, which was especially seen in the ocular and choanal samples (**Figure 3A-B**). The lowest amount of shedding was observed for the chickens inoculated with NDV F3aa-S-

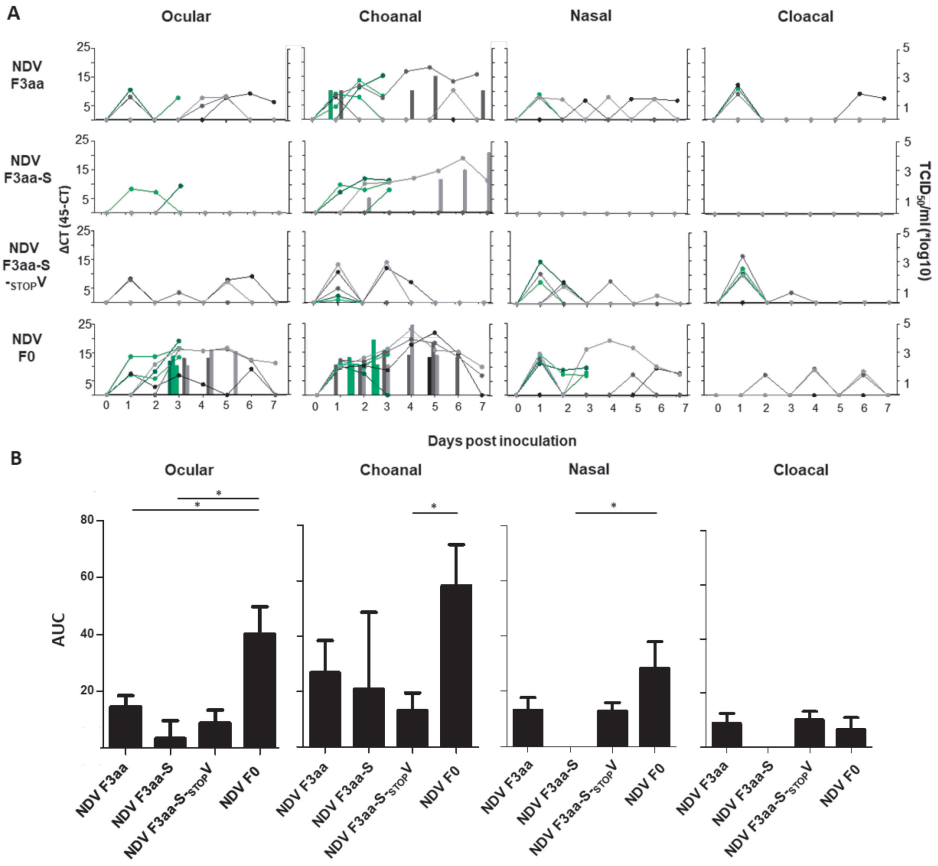

Figure 3. Virus shedding by six-week-old chickens inoculated with NDV F0, NDV F3aa and variants thereof. (A) Presence of viral genomes and infectious virus in collected clinical specimens. Ocular, nasal, choanal and cloacal samples were taken daily and the presence of NDV genomes was determined by qRT-PCR assays and infectious virus by titrations. Values for the presence of viral genomes are shown in ΔCT: higher values for ΔCT indicate the presence of more viral genomes in the samples (connected dots, left Y-axis). Virus titers are given as $TCID_{50}$/ml and depicted in bars (right Y-axis). Each of the six chickens in a group is represented by an individual colour. (B) The area under the curve (AUC) of the amounts of virus genomes depicted in panel A, per inoculated group per clinical specimen. * = $p < 0.05$ (one-way ANOVA + Dunn's multiple comparison test followed by paired t-test).

$_{STOP}$V, while the amount of shedding by chickens inoculated with NDV F3aa and F3aa-S was comparable. While all NDV F0 inoculated chickens shed infectious virus, none of the NDV F3aa-S-$_{STOP}$V inoculated chickens did, and only 1 or 2 out of six chickens inoculated with either NDV F3aa or F3aa-S shed infectious virus.

The more pronounced shedding by NDV F0 inoculated chickens was also reflected in the higher rate of transmission of this virus to the contact animals. Viral genomes were detected in samples collected from all contact animals of NDV F0 inoculated chickens, while only in a few samples from one or two contact chickens of NDV F3aa-S or NDV F3aa-S-$_{STOP}$V inoculated chickens (**Figure 4**). More importantly, infectious virus was only detected in the conjunctivae and choanae of the contact animals of NDV F0 inoculated chickens, not in any of the samples collected from the contact animals of NDV F3aa-S or F3aa-S-$_{STOP}$V inoculated chickens. Sequence analysis of the V and F regions of viral genomes present in collected samples from chickens inoculated with NDV F0, F3aa, F3aa-S or F3aa-S-$_{STOP}$V, revealed no mutations in these collected virus genomes (data not shown). In addition, seroconversion was only detected in contact animals from the NDV F0 group. Of note, no viral RNA was detected in environmental swabs taken from the bedding or drinking water in any of the isolators.

To assess differences in dissemination of the four NDV strains in the inoculated chickens, the presence of virus was determined for 19 tissues harvested during necropsy. The highest numbers of NDV positive tissues were detected in NDV F0 inoculated chickens, followed by NDV F3aa and F3aa-S inoculated chickens and the

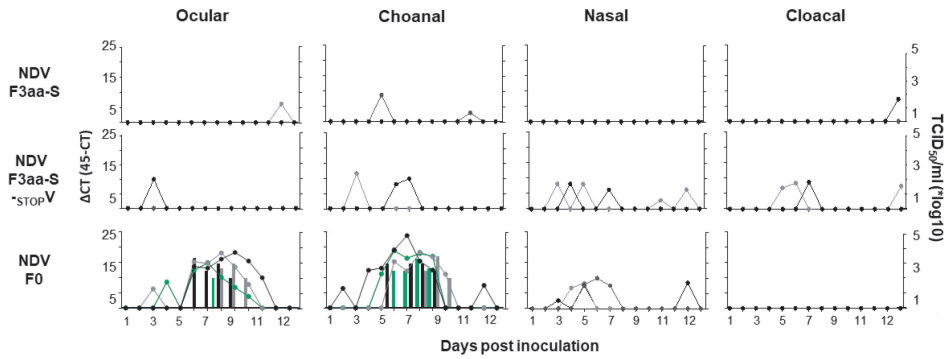

Figure 4. Virus shedding by contact animals of chickens inoculated with NDV F0, NDV F3aa-S and NDV F3aa-S-$_{STOP}$V. Ocular, nasal, choanal and cloacal swabs were collected daily and the presence of NDV genomes was determined by qRT-PCR assays and infectious virus by titrations. Values for detection of viral genomes are shown in ΔCT: higher values for ΔCT indicate the presence of more viral genomes in the samples (connected dots, left Y-axis). Virus titers are given as $TCID_{50}$/ml and depicted in bars (right Y-axis). Each of the three chickens is represented by an individual colour.

lowest numbers were detected in NDV F3aa-S-$_{STOP}$V inoculated chickens (**Figure 5**). Infectious virus was only detected in the nasal conchae of three animals inoculated with NDV F3aa and in that of one animal inoculated with NDV F0. The significant higher amount of shedding and spreading by the NDV F0 inoculated chickens compared to the other inoculated chickens indicate a more efficient replication of NDV F0 in six-week-old chickens compared to NDV F3aa and the variants thereof.

Analysis of the tissues by immunohistochemistry demonstrated the absence of virus infected cells in all tissues collected from NDV F3aa-S-$_{STOP}$V inoculated chickens (data not shown). In samples collected from NDV F0, NDV-F3aa and NDV-F3aa-S inoculated chickens, virus infected cells were not detected in any other tissue than the nasal conchae at day 3 post inoculation and those were no longer observed in the chickens euthanized at day 7 post inoculation (**Figure 6A**). Nasal conchae from NDV F0 infected chickens showed a more pronounced infection in the respiratory epithelial cell layer, with multiple foci of infected cells, some of which had loss of cilia. In contrast, the nasal conchae from those animals which did show infection with NDV F3aa and F3aa-S displayed only a few solitary infected cells without any syncytia formation and associated loss of cilia. In the nasal conchae of NDV F3aa and F3aa-S inoculated animals, infected cells were also detected in the olfactory epithelial layer, which were not observed in those of NDV F0 inoculated animals. No ulcers or erosions were observed in the areas with infected cells in any of the animals (**Figure 6B**). The influx of immune cells was assessed by counting granulocytes in infected areas of the nasal conchae. There did not appear to be differences in the number of granulocytes in birds x than between infected areas of NDV F3aa, F3aa-S and F0 inoculated chickens (**Figure 6C**). Together, these data indicate that NDV F0 replicated more efficiently in the respiratory epithelial cell layer of nasal conchae of chickens than NDV F3aa and F3aa-S, but that only NDV F3aa and NDV F3aa-S infected the olfactory epithelial cells, however without dissemination to the brain as no infected cells were detected in the brain.

These data indicate that infection of six-week-old chickens with NDV F0 resulted in more severe histopathological changes than infection with NDV F3aa, that the replication of NDV F3aa-S-$_{STOP}$V in six-week-old chickens was attenuated compared to that of NDV F3aa-S and NDV F0, and that NDV F0 was transmitted more efficiently by chickens than NDV F3aa and variants thereof.

Virulence determined by ICPI

Previously, the virulence of NDV F0, F3aa, F3aa-S and F3aa-S-$_{STOP}$V was assessed by determination of the virus induced MDT of chicken and duck eggs.[192] Here, the ICPI of the viruses was determined by intracerebral inoculation of one-day old chicks followed by scoring disease and mortality during 8 days and calculation of the ICPI according to World Organisation for Animal Health and EU guidelines.[35] The

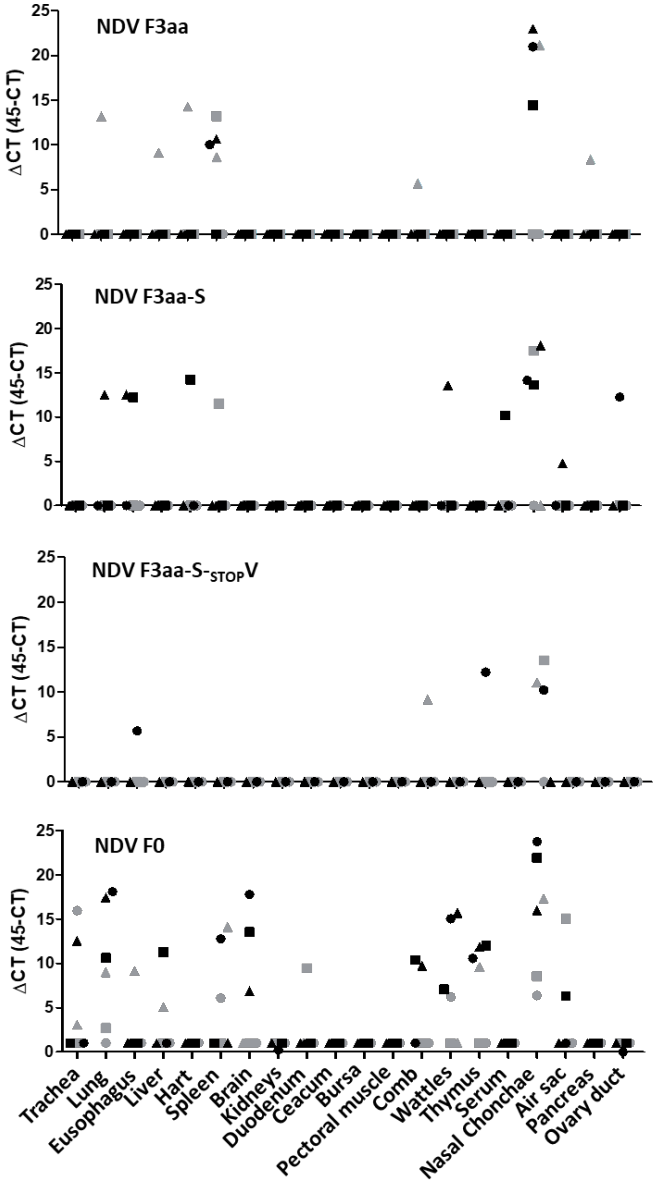

Figure 5. Virus dissemination in six-week-old chickens upon inoculation with NDV F0, NDV F3aa and variants thereof. The presence of viral RNA in tissues of inoculated animals determined by qRT-PCR assays and depicted as ΔCT values for each inoculated chicken individually (indicated by □, ○, or Δ) at day 3 (black) or day 7 (grey). Higher values for ΔCT indicate the presence of more viral genomes in the samples.

recombinant non-virulent LaSota strain (NDV F0) had an ICPI of 0.01 and engineering of an MBCS in this virus (NDV F3aa) resulted in an ICPI of 1.4 for this virus (**Table 2**). This pathogenicity index confirms that NDV F3aa classifies as a virulent virus.[191] The F to S substitution at position 117 of the F protein of NDV F3aa resulted in a decreased ICPI for that virus of 1.3, classifying NDV F3aa-S also as a virulent virus. The disruption of the V protein ORF in F3aa-S-$_{STOP}$V resulted in an ICPI for this virus of 0.03, which was comparable to that of the non-virulent NDV F0 and confirms that disruption of the V protein ORF decreased the virulence of NDV F3aa.

Table 2. Virulence of recombinant NDV strains as assessed by induced mean death time of embryonated chicken eggs and ICPI assays in one-day-old chicks

Virus	ICPI*	MDT**
NDV F0	0.01	107
NDV F3aa	1.42	52
NDV F3aa-S	1.27	58
NDV F3aa-S-$_{STOP}$V	0.03	>160

**Intracerebral pathogenicity index, OIE classifies the NDV strains as virulent based on ICPI values greater than 0.7.*[35]
***MDT, mean death time of chicken eggs, values reported previously.*[192] *NDV strains classify as virulent based on MDT values.*[196]

Together, these data show that NDV F3aa-S-$_{STOP}$V classified as a non-virulent virus based on the ICPI and pathogenicity in chickens upon choanal inoculation. These data also demonstrate that although the ICPI classified the recombinant NDV F0 containing an engineered MBCS (NDV F3aa) as virulent, natural infection of six-week-old chicken with this engineered virus did not result in pathogenicity. In fact, choanal infection with non-virulent NDV F0 resulted in more viral shedding and dissemination than infection with NDV F3aa.

Discussion

Several studies have shown that a non-virulent LaSota strain in which an MBCS at the cleavage site of the F protein was engineered (NDV F3aa) had significant higher efficiency in inducing cell death of tumor cells in *in vitro* assays and in murine tumor models compared to the non-virulent strain NDV F0.[51,52,155] However, NDV strains containing an MBCS are considered virulent for chickens based on their sequence and official standards that are based on the ICPI.[31,191] In order to decrease the virulence of LaSota NDV F3aa for poultry, we generated an NDV strain with an MBCS, but with a disrupted disruption V protein ORF.[26,27,193,194] Infection of tumor cells with NDV with an engineered MBCS (NDV F3aa) will result in increased spread of the virus in the tumor, while disruption of the V protein ORF in this virus would result in activation of the immune system upon infection of chickens and therefore decreased pathogenicity. In addition, the previously described F-117-S mutation in

the F protein was introduced for improve ment of the replication and oncolytic efficacy, resulting in NDV F3aa-S-$_{STOP}$V. Previously, this virus was found to be attenuated in chicken eggs and based on MDT assays the virus classified as non-virulent.[192] The formal standard to determine the virulence of NDV strains is the sequence of the cleavage site or the ICPI in one-day old chickens. Following this standard, NDV F3aa-S-$_{STOP}$V virus classified as non-virulent virus, while both NDV F3aa and F3aa-S classified as virulent, which is in agreement with previously reported MDT and ICPI values for recombinant non-virulent viruses with an engineered MBCS.[25,31,156,191,197]

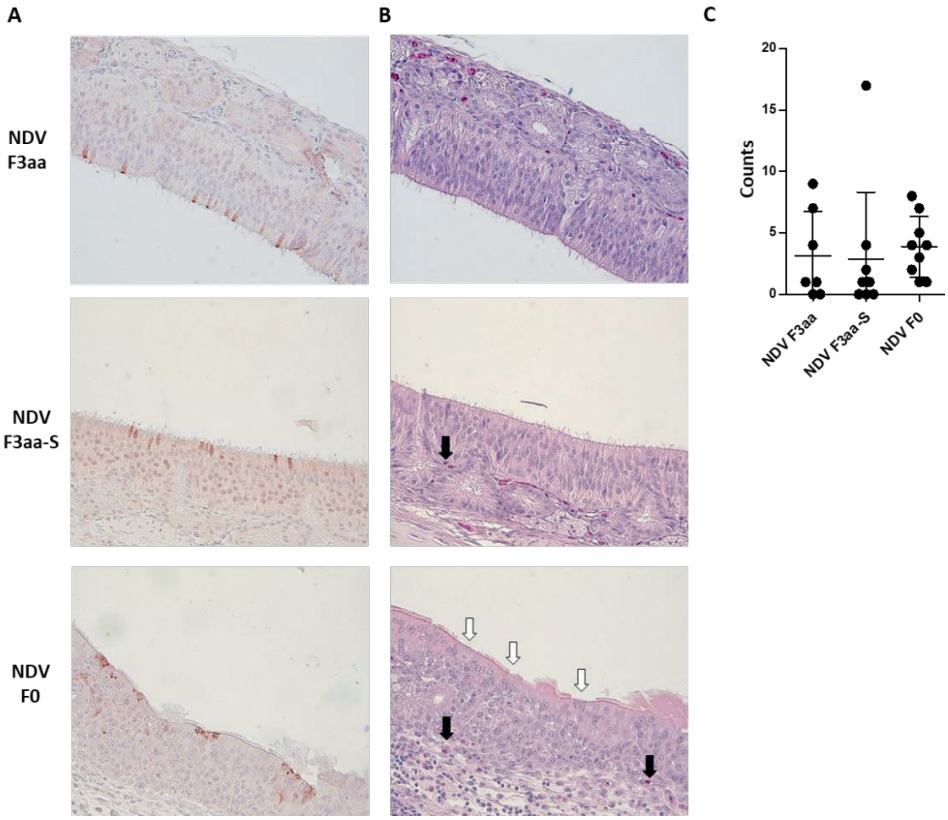

Figure 6. Histopathology and immunohistochemistry of NDV F3aa, NDV F3aa-S and NDV F0 infection in the nasal conchae of chickens. (A) Representative image of NDV infected tissues of the nasal conchae of inoculated chickens (immunoperoxidase staining for NDV, showing virus antigen expression in ciliated respiratory epithelial cells (original magnification 20x) and (B) H&E staining. Black arrows indicate the infiltration of granulocytes. White arrows indicate damaged ciliated cells. (C) Number of granulocytes in infected areas of a 40x magnification field of an H&E image. Mean and standard deviations are depicted.

The higher virulence of recombinant F3aa compared to F0 as determined by the MDT and ICPI assays did not agree with the pathogenicity observed upon inoculation of six-week-old chickens via a natural route of infection, as inoculation of chickens with the recombinant non-virulent NDV F0 led to more virus shedding and dissemination than inoculation with recombinant NDV F3aa(-S). These data indicate that only changing the mono-basic cleavage site to an MBCS in recombinant non-virulent NDV does not necessarily increase the pathogenicity of the virus upon infection of chickens. These observations are in agreement with another study where inoculation of four-week old chickens with recombinant NDV LaSota F3aa and F0 resulted in similar pathogenicity.[191] In these four-week-old chickens an increased immune cell influx to the infection site was observed in NDV F3aa inoculated chickens compared to NDV F0 inoculated chickens. This increased immune response was not seen in the six-week-old chickens in our study, which might be related to the age of the chickens. These observations were also in agreement with other studies reporting that the ICPI of recombinant non-virulent viruses with an engineered MBCS did not correspond with pathogenicity observed after inoculation via a natural route.[32,191] When the cleavage site of the non-virulent LaSota strain was replaced with the MBSC of the virulent, neurotropic, Beaudette C strain the ICPI of this virus was increased from 0 to 1.12.[32] However, upon intranasal inoculation of three-week old chickens no clinical signs or dissemination to the brain was observed. In contrast, inoculation with the original virulent Beaudette C strain did result in clinical signs and dissemination to the brain.[32] Thus, our data, in combination with results from other studies, demonstrate that only substitution of the cleavage site of a non-virulent with a virulent motif did not change a non-virulent into a virulent strain when the virus was inoculated through a natural route of infection.[25,32,191] This suggests that other viral factors are important for pathogenicity or entry into the central nervous system upon infection through the natural route, which is in agreement with other studies.[25]

In addition, our study is in agreement with other reports that question raises concerns on the use of sequencing the cleavage site and determination of the ICPI for virulence determination for viruses used in research, such as viro-immunotherapy studies, as intracerebral inoculation is not the natural way of infection.[191,198] This study shows that the current criteria for defining a virulent NDV by means of sequencing of the cleavage site or by determination of the ICPI are not in agreement with the virulence and pathogenicity induced by NDV in six-week old chickens. Previously, we have shown that intravenous injection of non-human primates with recombinant NDV F3aa did not result in disease and that the very low amounts of virus shedding that were detected did not lead to successful infection of contact animals.[55] This study suggested that patients receiving viro-immunotherapy using NDV F3aa will probably shed very low amounts of virus and that it would be highly unlikely that this shedding will result in productive infection of chickens. The present data obtained with NDV F3aa inoculated chickens also show that in case of virus

shedding by treated patients, the virus would be unlikely to spread further to other chickens or result in pronounced disease.

Altogether, our data indicate that NDV F3aa-S-$_{STOP}$V can be safely used in human viro-immunotherapies without posing a threat to chicken farms and that the use of NDV F3aa in such therapies could also be considered by regulatory authorities. In addition, our data show that official determination of NDV virulence by sequencing the cleavage site in the F protein or by determination of ICPI values do not correlate with induced pathogenesis in chickens upon infection via the natural route, which raises challenges for determination of the virulence of engineered NDVs used for viro-immunotherapies.

Part II

The immune modulating ability and anti-tumor efficacy of recombinant NDV

"I cannot fix on the hour, or the spot, or the look or the words, which laid the foundation. It is too long ago. I was in the middle before I knew that I had begun"
(Jane Austen, *Pride and Prejudice*, 1813)

Chapter 5

Armed oncolytic viruses: a kick-start for anti-tumor immunity

J.F. de Graaf , L. de Vor, R.A.M. Fouchier, B.G. van den Hoogen

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Department of Virology, Erasmus Medical Centrum, Rotterdam, The Netherlands

Abstract

Oncolytic viruses (OVs), viruses that specifically result in killing tumor cells, represent a promising class of cancer therapy. Recently, the focus in the OV therapy field has shifted from their direct oncolytic effect to their immune stimulatory effect. OV therapy can function as a 'kick start' for the antitumor immune response by releasing tumor associated antigens and release of inflammatory signals. Combining OVs with immune modulators could enhance the efficacy of both immune and OV therapies. Additionally, genetic engineering of OVs allows local expression of immune therapeutics, thereby reducing related toxicities. Different options to modify the tumor microenvironment in combination with OV therapy have been explored. The possibilities and obstacles of these combinations will be discussed in this review.

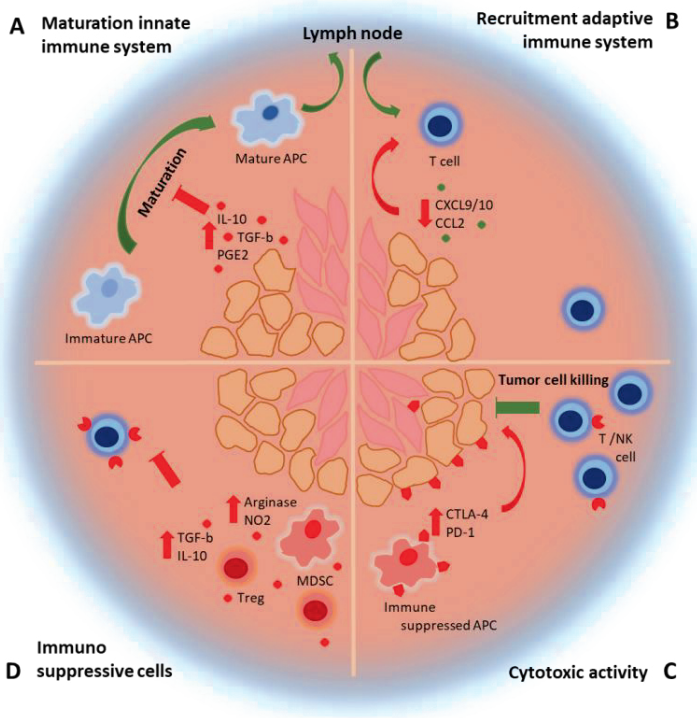


Figure 1. The immunosuppressive tumor micro environment. (A) Tumor cells (orange) and stromal cells (pink) secrete immune suppressive molecules, which inhibit the maturation of APCs. Maturated APCs migrate to the lymph node to activate the adaptive immune system. (B) As a result, activated T cells migrate to the tumor driven by a chemokine gradient. However, the secretion of chemokines is lowered in the tumor resulting in reduced T cell infiltration. (C) T cells that enter the TME to target the tumor cells are inhibited by immune suppressive receptors expressed by the tumor, stromal cell, but also immune suppressed APCs. (D) Tregs and MDSCs are recruited to the TME, which secrete more immune suppressive molecules and inhibit the T cell response even further.

Introduction

The anti-tumor immune response and the immune profile of tumors

The innate and the adaptive immune system work together to detect transformed cells and remove them before they form a tumor.[199] The anti-tumor response starts with the release of tumor associated antigens (TAA) from dying cancer cells and accompanying signal molecules, which attract and activate cells of the innate immune system.[200,201] Whereas NK and γδ-T cells can recognize and kill tumor cells directly, antigen presenting cells (APCs), such as DCs and macrophages, take up TAAs to activate the adaptive immune system.[202,203] The maturation of APCs by the accompanying danger signal molecules determines the skewing to a preferred T helper cell (Th) 1 response. These Th1 signals constitute of pro-inflammatory cytokines, such as interleukin (IL)-12, type I interferons (IFNs) and tumor necrosis factor (TNF), and damage-associated molecular pattern molecules (DAMPs), such as nuclear protein HMGB1, heat-shock proteins and ATP.[201] The Th1 cytokines stimulate the generation of tumor specific cytotoxic CD8+ T cells (CTLs), which are crucial effector cells in the antitumor response [204]. Subsequently, effector T cells, including T helper cells and CTLs, are attracted to the tumor site via a gradient of T cell attracting chemokines, including chemokine (C-C motif) ligand 2 (CCL2), CCL5/RANTES, chemokine (C-X-C motif) ligand 9 (CXCL9), and CXCL10.[8] At the site, CTLs recognize and kill tumor cells mediated by MHCI-T cell receptor interactions. If the immune system succeeds in destruction of the beginning tumor, the host remains free of cancer.

In some cases, tumor cells are reprogrammed to evade the immune system resulting in an equilibrium between dying tumor cells and tumor cells surviving the immune attack. As a consequence, a selection of immunosuppressive or less immunogenic tumor cell variants is introduced, which cannot be eliminated by the immune system.[205] These tumor cells establish a tumor microenvironment (TME), in which the function of anti-tumor immune cells is attenuated (**Figure 1**).[206] First of all, tumor cells and stromal cells (endothelial and epithelial cells and fibroblasts) produce factors such as transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2) and IL-10, that disrupt APC maturation in the TME.[8,201,206] As a result, DCs isolated from the TME often display a partly matured, immune suppressive phenotype and secrete cytokines that induce non-favorable Th2 responses.[8] Secondly, tumors inhibit infiltration of effector T cells by repressing the production of T cell attracting chemokines CXCL9/10 and modification of CCL2.[8,207] Thirdly, effector T cells that can infiltrate the tumor are attenuated by expression of several immunosuppressive molecules and persistent exposure to tumor antigens. As a result, T helper cells and CTLs isolated from the TME often present an exhausted phenotype, characterized by high level expression of immune checkpoint receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1).[207] Ligation of these receptors with their ligands expressed on tumor and

stromal cells, but also immunosuppressed APCs, leads to inhibition of the tumor specific T cell response. Fourthly, regulatory immune cells such as $CD4^+$ regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) are recruited to the tumor site. Similar to tumor cells, Tregs secrete IL-10, Indoleamine 2,3-dioxygenase (IDO) and TGF-β, leading to further attenuation of the T cell response [8,9]. Furthermore, Tregs consume IL-2, which is indispensable for T cell activation [8]. MDSCs contribute to the suppression of effector T cells through production of arginase and nitric oxide, which deprives T cells from amino acids necessary for proliferation.[208]

Despite all these evasion mechanisms, CTLs and Th1 T helper cells are still considered to be the most crucial effector cells in anti-tumor immunity and their infiltration into the TME is associated with good prognosis in various types of cancer [209]. Proper activation of these cells is key for an effective antitumor response and the abundance of mechanisms used by tumors to suppress these cells offers many targets for cancer immunotherapy strategies. At the moment, multiple strategies to target the TME are being explored. Recent successes have led to the FDA approval of checkpoint inhibitors anti-CTLA-4 (clinical responses in 10-15% of treated patients) and anti-PD1 (clinical responses in 30-40% of patients) for treatment of melanoma.[135,210] Clinical trials have shown that dual, synergistic blockage improved antitumor responses against melanoma, indicating that it might take more than one approach to induce powerful and long lasting anti-tumor immunity.[211,212] However, systemic administration of these checkpoint inhibitors, as well as other immunotherapies, often coincides with severe immune-related adverse effects similar to autoimmune diseases.[211,212] A promising treatment option to potentially overcome this obstacle is oncolytic virotherapy.

Oncolytic viral therapy

Oncolytic virotherapy is an approach that uses oncolytic viruses (OVs), either with natural tropism for neoplastic cells or genetically modified to enhance selectivity for tumor cells.[213,214] Tumor cells often lack an adequate antiviral response, making them more susceptible to OV infection than healthy cells. The viral infection leads to tumor regression through two distinct mechanisms: direct killing of tumor cells by replication dependent induced cell death and promotion of an antitumor response towards all tumor cells, including non-infected cells, by inducing immunogenic cell death. Types of immunogenic cell death, such as immunogenic apoptosis, necrosis and autophagic cell death, are characterized by the release of TAAs in combination with DAMPs and viral pathogen associated molecular patterns (PAMPs).[214] Following the secretion of DAMPs and cytokines, more innate immune cells, such as macrophages, DCs, NK cells and neutrophils infiltrate the tumor environment. The immune stimulating cytokine secretion leads to maturation of APCs and hence presentation of TAAs and viral antigens to activate the adaptive immune system in the lymph nodes. Cytotoxic T cells will start infiltrating the tumor again and

specifically eliminate cancer cells. Simultaneously, memory T cells are formed, which improves protection against new tumor challenges in mouse models [215,216]. Therefore, it is evident that OV therapy can function as a 'kick start' for the antitumor immune response by providing TAAs in an immunogenic manner and inducing infiltration of immune cells.

Recently, the focus in the oncolytic virotherapy field has shifted from their oncolytic effect to their immune stimulatory effect. Recombinant OVs armed with immune modulators further enhance the activation of the immune system and overcome the immunosuppressive TME [213]. The first armed OV approved by the FDA is oncolytic Herpes-Simplex-Virus (HSV)-1 expressing GM-CSF showing improvement of melanoma treatment, but no cure yet (T-VEC).[21] Therefore, many more immune modulator armed OV therapies followed and their obstacles and opportunities have come to light. This review will give an overview of the state-of-the-art therapies used in combination with immune modulators to treat cancer patients and give a hint on potential future directions.

Immune therapy

Immune therapies for treatment of cancer aim at overcoming the tumor immune suppressive environment and at increasing antitumor immunity. Most immune therapies target directly or indirectly the inhibitory or stimulatory receptors on immune cells and are often based on monoclonal antibodies. Other therapies intend to restore the intratumoral balance of cytokines and chemokines into a more favorable inflammatory TME to attract and activate immune cells. The most interesting noncellular therapies for combination with OV therapy for solid tumors are described below.

Cytokines

Cytokines are key players in stimulating and regulating antitumor immune responses. For this reason, one of the first immune therapeutic approaches in cancer treatment was the administration of recombinant cytokines. As described before, the most essential cytokines in the anti-tumor response are IL-12, GM-CSF, IL-2 and IL-2-related cytokines IL-15 and IL-21, and they all stimulate different parts of the immune system.[217] GM-CSF recruits APCs to the TME,[212] IL-12 is normally expressed by APCs and stimulates polarization of T helper cells to a Th1 phenotype, IL-2 is a T cell growth factor and improves T cell expansion,[206] and the IL-2-related cytokines promote survival of T cells, but also play an important role in NK cell activation.[217] Despite the their plethora of immune modulatory actions, cytokines have lost their popularity as a monotherapy, because of their low objective response rates and non-negligible side effects upon systemic administration.[218,219]

Inhibitory receptors

One of the most promising immune therapeutics are checkpoint inhibitors. Inhibitory receptors such as CTLA-4 and PD-1 act as checkpoints to avoid over activation and are expressed by T cells and inhibit the T cell activation in the lymph nodes and survival in the TME.[220] Several checkpoint inhibitors have been approved by the FDA of which the first were against CTLA-4 and PD-1. Treatment with these inhibitors results in reactivation of the suppressed immune cells.[135,210] In addition, blocking of CTLA-4 can lead to depletion of Tregs.[219] Combinations of a-CTLA-4 antibody and a-PD-1 antibody have shown to increase responses to treatment of advanced melanoma. However, the frequency of severe immune-related adverse events was also enhanced in clinical studies (16.3 -27.3 % in monotherapy group vs. 55% in combination therapy group).[211] Another disadvantage of these therapies is the fact that tumors can become resistant towards checkpoint inhibitors.[221] As a result, a variety of other co-inhibitory receptors, such as lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin and mucin receptor protein 3 (TIM-3), have recently been identified. LAG-3 is normally activated by its ligand MHCII on APCs and indirectly reduces T cell proliferation.[222] Interaction of TIM-3 with its ligand galectin-9, expressed by Tregs, induces cell death in Th1 cells.[222] Blockage of these inhibitory receptors can unleash potent antitumor CTL responses and are now in clinical or preclinical development.[212,222]

Co-stimulatory receptors

Agonistic antibodies are also in development to activate co-stimulatory receptors expressed by T cells, such as 4-1BB and OX40.[212] Stimulation of these two receptors by their ligands or monoclonal antibodies induces activation, proliferation and survival of T cells. Another interesting target is CD40, which is expressed on DCs. CD40 ligation with CD40L or therapeutic antibody stimulates DC maturation and presentation of antigens, which leads to efficient T cell priming.[222] More stimulatory co-receptors are explored today, such as B7.1 and GITR,[223–225] which might benefit the effect of current therapies.

BiTEs

Another class of therapeutics constitute of dual specificity recombinant antibodies, also called bispecific T cell engagers (BiTEs), which have shown promise as anti-tumor therapeutics. These antibodies simultaneously bind to CTLs via the T cell receptor (TCR) and a tumor antigen expressed on the tumor cell resulting in bypassing MHC dependent antigen presentation.[226] An example of such therapy is Blinatumomab, which engages the CTL to CD19+ tumor cells. Currently, it is approved by the FDA for treating acute lymphoblastic leukemia, which is an hematologic malignancy.[226]

Combination of immune therapy and oncolytic virotherapy

Therapeutic treatment of solid tumors could be enhanced by combination treatment of both immune and OV therapy. By introducing these discussed immune stimulators, checkpoint inhibitors and cytokines as immune modulators in viral vectors, adverse events can be reduced, resistance reverted and treatment responsiveness enhanced.

Combination of OVs with cytokines and chemokines

Several viruses have been engineered to express different cytokines or chemokines. Cytokines and chemokines are attractive transgenes, because they are encoded by small genes and are in general easy to build in a viral genome. Moreover, they often have pleiotropic effects, which means they can target different immune cells simultaneously.[217,226] A complete overview of all cytokines and chemokines used in oncolytic virotherapy is given in **Table 1**.

GM-CSF

The most extensively studied transgene is the cytokine GM-CSF. GM-CSF promotes DC recruitment and maturation. GM-CSF has been successfully used to arm HSV and this armed virus has been approved by the FDA under the name of T-VEC for treatment of metastatic melanoma patients.[21] Besides HSV, other viruses have also been armed with GM-CSF.[227,228] Phase 1 clinical trials in patients with colorectal and hepatocellular carcinoma, neuroblastoma and Ewings sarcoma have proven the efficacy and safety of oncolytic vaccinia virus (VV) expressing GM-CSF (Pexa-Vec).[88,118,140] In addition, two different adenovirus serotypes expressing GM-CSF induced long term survival of patients with, amongst others, ovarian, colon, pancreatic and breast cancer, with no severe side effects.[121,228] Biopsies obtained from these patients with different metastatic tumors showed increased infiltration of T cells and macrophages, which also correlated with patient survival and hence the additive effect of GM-CSF as vectorized immune modulator.[121]

IL-12

IL-12 both activates and promotes survival of NK cells, but also Th1 effector cells.[229,230] Several viruses have been armed with IL-12 and tested in different tumor models (**Table 1**).[231–236] HSV-IL-12 induces tumor infiltration of effector T cells, NK cells and APCs in neuroblastoma and glioma mouse models.[233,235] When comparing HSV-IL-12 with HSV-GM-CSF, HSV-IL-12 was demonstrated most effective in tumor growth inhibition of injected tumors as well as metastases in a squamous cell carcinoma mouse model and a prostate cancer mouse model.[237,238] In addition, mice were better protected against re-challenges with tumor cells in the HSV-IL-12 treated group, indicating the formation of a long term, anti-tumor response.[237] Similarly, adenovirus armed with IL-12 showed improved tumor reduction compared to

adenovirus armed with GM-CSF in a thyroid cancer rat model.[239] In conclusion, multiple studies demonstrate that OVs armed with IL-12 yield better anti-tumor effects than vectorized GM-CSF and can even be further improved by combining different immune modulators.

IL-2 and IL-15

IL-2 and IL-15 both signal via cytokine receptors of the common γ chain family and are important for the stimulation, proliferation and survival of T cells and NK cells.[217,240] Systemic treatment with IL-2 is associated with major adverse side effects in humans [218]. Therefore, local delivery of IL-2 by OVs has been tested by several research groups.[56,241–245] In these murine studies, reduced tumor growth and increased T cell infiltration of the tumors was reported. No distress was observed in mice and IL-2 production was limited to the tumor site, which might indicate less side-effects when administrated to humans.[56,243,244] Also, mice were protected from re-challenge with tumor cells suggesting induction of long term tumor specific immunity and thus show promise as vectorized immune modulator.[56,242–244]

Despite the promising effects of IL-2, IL-15 showed to have several advantages over IL-2. In contrast to IL-2, Il-15 can stimulate only NK and effector T-cells, whereas IL-2 has also the undesirable effect of stimulating Tregs.[240] Even though systemic treatment with IL-15 induces less toxic effects local expression of IL-15 mediated by armed Vescilular Stomatitus virus (VSV) enhanced anti-tumor activity compared to VSV treatment combined with systemic IL-15 in murine models.[246] Vectorization by NDV demonstrated that IL-15 induced more CTL infiltration and increased activation of tumor-specific effector cells resulting in improved survival rates compared to IL-2 in a melanoma mouse model.[247] Altogether, these studies indicate that treatment with OVs expressing IL-15 is more efficient than systemic treatment with IL-15 or treatment with OVs expressing IL-2.

Type I and II interferons

IFN-α/β are important in antiviral responses, but also play a role in anti-cancer immunity by inducing DC maturation and CTL and NK cell activation.[217,226] In addition, they result in upregulation of MHC I expression on tumor cells and can have direct effect on cell proliferation. Only a few studies have investigated OVs armed with type I interferons.[50,248,249] The direct anti-tumor effects of IFN-β expressed by the measles virus (MV) and NDV has been demonstrated in immune deficient mouse models leading to some improvement of the viral therapy. Buijs *et al.* showed that a virus expressing IFN-β interfered with oncolytic NDV replication, whereas Willmon *et al.* and Li *et al.* found that viral IFN-β expression did not interfere with VSV and MV replication.[50,248,249] However, the immune stimulating effect of virus mediated IFN-β expression still needs to be assessed in an immunocompetent model to determine the potency of IFN-β as transgene.

Type II interferon (IFN-γ) is an important Th1 effector cytokine, secreted by activated Th1 cells, CTLs and NK cells.[217] IFN-γ upregulates MHC I expression in tumor cells

and promotes Th1 skewing via an autocrine loop.[217] A recent study describing VSV expressing IFN-γ suggested that this virus induces a stronger immune response by increasing MHC I antigen presentation on tumor cells, enhancing DC maturation and attracting T cells to the tumor site by inducing CCL2 expression in mice.[250] The authors did not observe any difference in viral replication, even though IFN-γ is also known for its antiviral activity. In another murine study, treatment with NDV- IFN-γ did not result in a significant beneficial effect compared to unarmed NDV.[56] In conclusion, arming OVs with IFN-γ has been shown to be effective, but efficacy may depend on the virus used.

Effector cell attracting chemokines

Insufficient infiltration of effector lymphocytes in the tumor often correlates with low efficacy of T cell stimulating immunotherapies.[8,207] Therefore, OVs armed with chemokines, which attract effector cells, may improve antitumor efficacy in addition to the endogenous chemokine release upon viral infection. Important chemokines in the TME are CCL2, CCL5 (RANTES), CXCL9 and CXCL10, which attract Th1 cells and CTLs and CCL22, which attracts undesirable Tregs.[251] Combination of OV therapy with a chemokine modulating cocktail, which induces production of CCL5 and CXCL10 while reducing CCL22, was shown to promote trafficking of T helper cells and CTLs to the TME and resulted in improved survival in a murine colon cancer model.[252] Administration of viruses armed with CCL5 or CCL2 both resulted in increased numbers of infiltrating Th1 cells in colon cancer and neuroblastoma.[253–255] In conclusion, these studies have shown that the use of OVs armed with chemokines efficiently increased the infiltration of T cells into the tumor. However, none of the studies have assessed the infiltration of immune cells in distant tumors and tumor reduction has not been mentioned.

Combination of OVs with blocking of co-inhibitory receptors

The most popular immunotherapeutics are the checkpoint inhibitors, anti-CTLA-4 or anti-PD-1/PD-L1, yielding promising effects but coinciding with major adverse effects, as described above.[256] In addition, some tumors are resistant to these immunotherapies, dependent on the immunogenicity of the tumor and the suppression of the anti-tumor immune responses in the TME.[257] Combination of systemic immunotherapeutics with localized OV therapy may enhance their therapeutic efficacy and may overcome tumor resistance and reduce immune related adverse effects.

CTLA-4

CTLA-4 is a checkpoint inhibitor that inhibits early stages of T cell activation in the lymph nodes, but also stimulates undesirable Treg functions.[222,256] Blockage of CTLA-4 signaling releases a brake on T cell activation and even depletes intratumoral Tregs.[222] Intratumoral administration of NDV combined with systemic

treatment with anti-CTLA-4 (Ipilimumab) showed improved antitumor effect mediated by CTLs and NK cells in a murine melanoma model.[258] Studies using combinations of systemic CTLA-4 blockade with other OVs (VV[259,260] and VSV[215]) showed prolonged survival in renal,[259] lung,[260] and mammary[215] tumor models, long term protection to re-challenge with tumor cells[259,260] and even cured mice.[215] Moreover, T-VEC combined with systemic CTLA-4 blockade has been evaluated for therapy in melanoma patients, which yielded a tumor growth control that was significantly greater than observed after both monotherapies.[114] The incidence of severe adverse effects was similar to Ipilimumab monotherapy.[114,256] Local expression of anti-CTLA-4 induced by administration of adenovirus armed with anti-CTLA-4 resulted in significantly higher concentrations of the antibody in the treated tumor, while plasma levels remained at concentrations indicated as safe in murine models.[261] However, Engeland *et al*. demonstrated that treatment with MV armed with anti-CTLA-4 was less efficient than intratumoral treatment with MV combined with systemic administration of anti-CTLA-4, presumably because the major site of action of CTLA-4 is in the lymph node and not in the periphery.[262] Though local expression may lead to transient expression of the transgene in tumor draining lymph nodes, this study indicated that systemic anti-CTLA-4 therapy may be required for optimal responses.

PD-1 and PD-L1

With a completely different mechanism of action, PD-1 is also a checkpoint inhibitor expressed by effector T cells.[256] While CTLA-4 inhibits T cells in early activation stages in the lymph nodes, PD-1 signaling limits the function of activated T cells at later stages of the immune response taking place in tumors and tissues.[256] Multiple OVs such as MV,[263] reovirus,[264,265] and VSV[266,267] have been combined with systemic PD-1 blockade in treatment of murine glioblastoma,[263] melanoma[264,265] and acute myeloid leukemia [266] tumor models, resulting in enhanced influx of CTLs in the tumors and prolonged survival in mice. Specifically CTLs and NK cells, but not T helper cells, were shown to mediate the beneficial effects [264,266,268]. Moreover, a phase 1b clinical trial with the FDA approved T-VEC combined with PD-1 resulted in good response rates in patients.[113] Arming Myxoma Virus (MYXV) with anti-PD-1 did result in improved survival in mice as well.[268] However, local expression induced by administration of MV or VV armed with anti-PD-1 or anti-PD-L1 demonstrated to be as efficient as therapy with unarmed VV combined with systemic anti-PD-1 / anti-PD-L1 treatment in murine tumor models.[262,269] In conclusion, these studies suggest that OVs combined with checkpoint inhibitors improve treatment, but the benefits of vectorization differ per oncolytic virus.

Combination of OVs with activation of co-stimulatory receptors

B7-1

B7-1 (CD80) is expressed by APCs and a potent co-stimulatory molecule for T cells. B7-1 provides co-stimulation via interaction with CD28 on T cells, but inhibits T cells through interaction with CTLA-4.[222] Nevertheless, several OVs armed with B7-1 have

been tested in (pre)-clinical trials for the treatment of melanoma.[231,270–272] Replication defective HSV expressing soluble B7-1 induced a prolonged tumor specific immune response in mice bearing neuroblastoma tumors.[270] VV expressing B7-1 was eventually evaluated in melanoma patients, where the treatment was well tolerated, but only few patients responded.[272] Overall, OV expression of B7-1 seems to have additional benefits in combination with other transgenes, but not as a single transgene.

4-1BB

4-1BB is a surface protein primarily present on activated T cells and NK cells. 4-1BB signaling promotes Th1 skewing over Th2, protects T cells from activation induced cell death and enhances cytotoxic activity of T cells and NK cells.[222] 4-1BB signaling has been shown to be more potent in T cell activation compared to the CD28 co-stimulation by B7-1.[273] A combination of oncolytic VV with systemic anti-4-1BB administration in a breast cancer model resulted in increased survival and tumor infiltration by CTLs compared to both monotherapies in patients (40% survival vs. 0% survival).[274] Local expression of 4-1BB ligand (4-1BBL) by oncolytic VV in mice has demonstrated to enhance tumor regression and this effect was enhanced even more in combination with lymph node depletion in order to slow down viral clearance [275]. Local injection of the armed virus resulted in an improved CTL/Treg ratio in the TME. In another study, vectorization of both IL-12 and 4-1BBL by adenovirus increased T helper, CTL and DC infiltration, which resulted in improved survival of the mice.[232] In other murine models, DC vaccination combined with the armed adenovirus combined treatment yielded even better results and DCs showed enhanced migration to the tumor draining lymph node, where they activated T cells.[232] These studies have shown that vectorization of this immune modulator in OVs enhances its therapeutic efficacy.

CD40

The maturation and activation status of DCs is often a limiting factor in the induction of antitumor immune responses. Ligation of the CD40 receptor provides a strong activating signal to DCs, resulting in upregulation of MHC II and co-stimulatory molecules and production of IL-12, which is important for skewing of T cells towards a Th1 phenotype.[222] As DC activation takes place in the TME, treatment with OVs armed with a stimulatory CD40 antibody or CD40 ligand (CD40L) may lead to enhanced therapeutic efficacy. Adenoviruses armed with CD40L have been tested in melanoma mouse models and patients with different types of cancer.[276–278] Treatment with a CD40L expressing VV resulted in tumor growth inhibition and increased infiltration of effector T cells, NK cells, DCs in a melanoma mouse model. However, also numbers of Myeloid derived suppressor cells increased of which its effects are unknown.[279] When evaluated in a clinical trial, patients showed disease control for 3-6 months and a systemic tumor specific immune response was induced.[278]

OX40

Similar to checkpoint inhibitors, more and more co-stimulatory targets, such as OX40 and GITR, are being discovered as anti-tumor targets. The OX40 receptor is expressed by activated T cells and induces the production of Th1 and Th2 cytokines upon interaction with OX40 ligand (OX40L).[222,280] OX40 ligation also directly blunts the suppressive effects of Tregs.[222,280] Arming of OV with OX40L has been described once: adenovirus expressing OX40L led to suppression of melanoma, lung and colon tumor growth in mice. This effect was mediated by Th1 cells and CTLs and an increase in Th1 rather than Th2 cytokine expression.[281] A study combining systemic OX40L therapy with systemic 4-1BBL therapy and IL-12 expressing adenovirus, revealed increased expression of Th1 cytokines. thereby the antitumor CTL response were enhanced leading to tumor rejection in a colorectal cancer model involving liver metastases compared to OX40L monotherapy and combined 4-1BBL and IL-12 armed OV treatment.[282] This makes OX40 an interesting transgene, but more research needs to be performed to elucidate its beneficial effects in other tumor models and OVs.

GITR

GITR is a stimulatory receptor expressed on activated T cells, but it is also constitutively expressed on Tregs.[222,280] Activation of the receptor promotes proliferation and cytokine production in T effector cells, whereas it inhibits Tregs.[222,280] Expression of GITR ligand itself by an adenoviral vector resulted in increased infiltration of T helper cells and CTLs and suppression of tumor growth, leading to prolonged survival.[225] Systemic administration of a stimulating GITR antibody together with intratumoral injection of an adenoviral vector armed with IFN-α resulted in enhanced tumor growth inhibition in injected and distant tumors compared to single treatment in colon and pancreatic murine cancer models.[283] This makes GITR ligand attractive as a transgene, but similar to OX40L, more research needs to be performed. More research needs to be done towards these targets, just like for the newly discovered LIGHT and CD27 anti-tumor targets (**Table 1**).[284,285]

Combination of OVs with other immune stimulatory approaches

Prostaglandin E2 blockade

Prostaglandin E2 (PGE2) is a principal mediator of inflammation expressed by tumor cells and immune cells and stimulates accumulation of MDSCs in the TME. Moreover, PGE2 induces IDO and IL-10 expression in DCs, whereas it reduces IL-12 expression, which is necessary to induce a Th1 response.[8,207] A recent study, revealed PGE2 as an important mediator of resistance to OV therapy and other immunotherapies.[286] Treatment of mice with VV expressing a PGE2 inactivating enzyme named hydroxyprostaglandin dehydrogenase (HPGD), resulted in a decreased number of MDSCs in the tumor and yielded a better response than other PGE2 blocking agents.[286] The virus was also shown to sensitize an otherwise resistant renal tumor to anti-PD-1 treatment [286]. Thus, blockade of PGE2 showed

promising results but this approach needs further validation in other tumor models and with other OVs.

Pathogen receptors

Toll like receptors (TLRs) are part of the innate immune system and recognize pathogenic molecules such as bacterial lipids and proteins or viral DNA and RNA.[287] The type of TLR that is activated upon infection is an important determinant for the skewing of the subsequent adaptive immune response.[287] Manipulation of TLR signaling can therefore switch the induced immune response from Th2 to Th1. TRIF mediates TLR3 signaling, whereas other TLRs signal via MyD88.[287] Moreover, this pathway induces the production of Th1 cytokines instead of Th2 cytokines. To manipulate TLR3 signaling, a VV was engineered to express TRIF to increase TLR3 signaling.[288] In addition, the viral particle was deglycosylated, resulting in reduced Th2 responses. Treatment of mice with deglycosylated VV-TRIF resulted in increased production of Th1 but not Th2 cytokines in the TME. Also, the virus induced infiltration of T helper cells and CTLs. These CTLs were both virus and tumor cell specific. VV-TRIF showed improved anti-tumor efficacy in colon and renal tumor models compared to VV-GM-CSF (Pexa-Vec). Similar results were obtained using intracellular pattern recognition receptor DNA-dependent activator of IFN-regulatory factors (DAI) as an vectorized immune modulator.[289] These studies show that the antitumor immune response can be switched from Th2 to Th1 by manipulating intracellular pathogen receptor signaling pathways in tumor cells.

BiTEs

BiTEs (bispecific T cell engagers) are a class of bispecific monoclonal antibodies that have shown promising anti-tumor effects.[290] However, they have a very short half-life and therefore require continuous infusion. Vaccinia virus (VV) armed with a BiTE with specificity for the TCR and a TAA was tested in a xenograft lung cancer mouse model. The study showed increased tumor cell killing through T cell activation and skewing towards Th1 responses.[291] A disadvantage of BiTEs is that they target only one TAA and therefore stimulate the immune response directed to only that TAA, while other immune-modulatory transgenes induce immunity to multiple TAAs that are released by dying tumor cells.

Combining different immune modulators

To further improve the efficacy of OV treatment, different combinations of therapies are being exploited. OVs armed with several cytokine combinations have been tested with promising results. Adenovirus expressing both GM-CSF and IL-12 induced infiltration of effector T cells, NK cells and activated APCs combined with DC vaccination in a melanoma mouse model, as well as long-term protection to re-challenge with tumor cells [292]. Moreover, viral expression of GM-CSF and IL-12 in

the TME shifted the intratumoral cytokine profile from a Th2 to a preferred Th1 response and improved DC migration to the tumor site.[292,293] Similarly, viral co-expression of IL-12 and IL-18 showed enhanced therapeutic efficacy and increased infiltration of DCs and effector T cells compared to mono-expression of IL-12 in murine models.[231,232,294] The combination of IL-12 and IL-18 showed to have synergistic effects on IFN-γ production, an important Th1 effector cytokine, by T cells and NK cells and polarization towards a Th1 response.[222,295] Combining IL-12 expression with CCL2, an important T-cell chemokine, resulted in enhanced neuroblastoma growth inhibition compared to administration of armed HSV alone demonstrating the potential of combining different cytokines and chemokines in mice.[255]

In addition, cytokines have also been combined with immune stimulatory receptor ligands. Treatment with adenovirus armed with GM-CSF and B7-1 in a melanoma mouse model resulted in prolonged survival and resistance to tumor re-challenge compared to treatment with unarmed adenovirus.[271] Similarly, treatment with oncolytic adenovirus armed with IL-12 as well as B7-1 resulted more often in complete regression in a melanoma mouse model compared to adenovirus armed with IL-12 alone.[231] Combination of three armed OVs with IL-12, IL-18, and B7-1 yielded significant better results in inhibition of local and distant tumor growth in a neuroblastoma mouse model, compared to single vector treatment demonstrating the possible synergistic effects between cytokines and co-stimulatory receptors.[224] The combination of checkpoint inhibitors and cytokines work equally well. Co-administration of oncolytic adenovirus expressing GM-CSF and oncolytic adenovirus expressing anti-CTLA-4 has been shown to yield additive antitumor activity in a lung cancer mouse model.[296] Furthermore, T-VEC in combination with systemic treatment with anti-CTLA-4 resulted in significantly improved tumor growth control rates in patients with advanced melanoma compared to both monotherapies alone.[114] Currently T-VEC in combination with anti-PD-1 is being evaluated in clinical trials,[297] but the results are still unknown. In addition, Sorensen *et al*. engineered a replication deficient adenovirus expressing a TAA. By combining administration of this virus with systemic administration of CD40 stimulatory antibodies and CTLA-4 blockage, tumor growth of melanoma was reduced and long term survival was observed in 30-40% of mice in contrast to the monotherapies.[298] Thus, stimulation of both DCs and T cells is necessary for long term anti-tumor immune response. Therefore, these (pre-) clinical trials with combination-therapies demonstrate the potential to improve current therapies.

Taken together, these studies provide a strong rationale for further evaluation of OVs as a local delivery vector for immunotherapies targeting co-stimulatory and co-inhibitory receptors on anti-tumor effector cells or other parts of the TME.

Discussion and future directions

OVs represent a class of promising agents to treat cancer. Besides their direct oncolytic effects, they can function as a 'kick-start' for anti-tumor immunity. Combining OV therapy with existing immune therapies enhances the potential of both therapies by synergizing their effects. Many different combinations have been tested and are summarized in this review (**Table 1**). These studies have broadened our understanding of the strengths of OV immune therapies, but also of its limitations. As a result, these learning lessons enable us to discuss the potential future directions and further considerations in deciding on effective immune modulators and viral combinations and reducing risks.

Effective immune modulators

One of the greatest advantages of OVs armed with immune modulators is that this therapy often induces safer systemic and/or more effective localized concentrations of the modulator than systemic monotherapy.[247,272,278] Nevertheless, vectorization of checkpoint inhibitors can also limit the potency of the immune therapies by incorrect localization and timing and no resilience in case of tumor resistance. With regard to localization, a checkpoint receptor, such as CTLA-4, is mainly functional in the lymph node and requires systemic delivery of the blocking antibodies.[260] For instance, the anti-CTLA-4 armed MV proved to be less effective than monotherapy with CTLA-4 inhibitors.[262] Similar results were found for PD-L1 inhibitors delivered by MV and VV,[262,269] illustrating the second disadvantage of OVs armed with checkpoint inhibitors: the timing of administration of combined therapies. Rojas et al. and Gao et al. demonstrated that systemic administration of immune therapeutics given shortly (1-3 days) after OV therapy resulted in additive efficacy of the combination therapy in contrast to immune therapeutics given prior to OV administration.[259,299] However, timing strategies may differ per virus as was seen with the armed MYXV where vectorization of anti-PD-1 did result in additive effects.[268] In addition to localization and timing, multiple studies have shown the development of resistance towards these checkpoint inhibitors over time in which new inhibitory molecules were upregulated on the cancer cells rendering the therapy ineffective.[221] Systemic administration of checkpoint inhibitors would allow for an easier transition between checkpoint therapies if necessary. Therefore, we think that arming viruses with checkpoint inhibitors might not be the most effective combination strategy.

An alternative approach to consider would be the targeting of APCs, NK cells and CTLs by arming viruses with immune stimulating agonists and cytokines. These immune cells express a known and stable subset of cytokine and co-stimulatory receptors and hence circumvent the treatment resistance as is seen with checkpoint inhibitors. Several studies have shown the additive effects of OVs armed with these agonistic agents compared to OV therapy alone (**Table 1**). In addition, the localized expression of agonistic agents reduces adverse effects in contrast to systemic

administration and still allow combination therapy with checkpoint inhibitors.[247,300,301] Based on the reviewed studies, we reason that both cytokines and agonists targeting co-stimulatory receptors are very promising as immune modulators in the OV treatment against solid tumors.

Viral combinations

Another point of consideration is the combination of the virus and the immune modulator. The virus determines both the 'kick-start' effect, which initiates the inflammation, and the concentration and duration of expression of the vectorized immune modulators. However, viruses differ in the effectiveness in killing the target cell or in expressing the immune modulator. For example, in a study comparing VSV with a replication defective adeno virus, both expressing CD40L, it was shown that the replication defective virus was superior in increasing survival rates over the replicative virus.[302] This is probably because of the low immunogenicity of the replication defective virus resulting in reduced viral clearance by the immune system and hence more immune modulator secretion. However, whether the benefits of a weaker immune response hold true for all therapies remains unknown and probably depends on the virus.

In addition to oncolytic activity, the efficacy of the OV immune therapy is influenced by the efficiency of a virus to express the immune modulators. For instance, for NDV it has been shown that the insertion site in the virus determines the expression levels of the transgene.[245] The effect of the insertion site may also hold true for other OVs, but is often not discussed. In addition, the size of the immune modulators is also of importance. Studies using a complete monoclonal antibody, only the light chain or a single-chain variable fragment showed that smaller proteins were more effectively produced than the larger proteins by cells infected with VV. However, the single chain fragment without an IgG domain was degraded faster in the TME, resulting in similar antitumor activity as the monoclonal antibody.[269] As not all viruses tolerate large insertions, this information should be considered when deciding which modulator to incorporate. However, predicting the efficiency of the modulator expression remains difficult, because differences in expression levels can occur between tumors independent of the virus.[296]

Reducing risks

While moving forward with OV immune therapies to the clinic, safety ought to be considered once more. So far, no adverse effects have been reported on the therapies combining OV with immune therapies in murine models or clinical trials. Nevertheless, adverse effects of the viral infection, such as excessive viral replication or expression of the immune modulators leading to an overreaction of the immune system cannot be excluded yet. A possibility to reduce the risks on adverse effects would be the incorporation of a fail-safe mechanism into the OV therapy to abort viral replication if necessary. Only for HSV, drugs are available to inhibit viral

replication.[303] Alternatively, virus with attenuated virulence or viruses with a different host range may be used. In addition, incorporation of suicide genes, such as thymidine kinase [303], rat cytochrome P450[304] or cytosine deaminase[305], would allow inhibition of viral replication by using drugs as well. Moreover, it could add to the tumor lytic efficacy of the therapy.[306] During OV therapy, the immune modulator is not incorporated into the host genome and therefore expression levels are dependent on viral replication which is usually transient. Thus, controlling the viral replication would reduce the risks on severe adverse effects of the immune modulators.

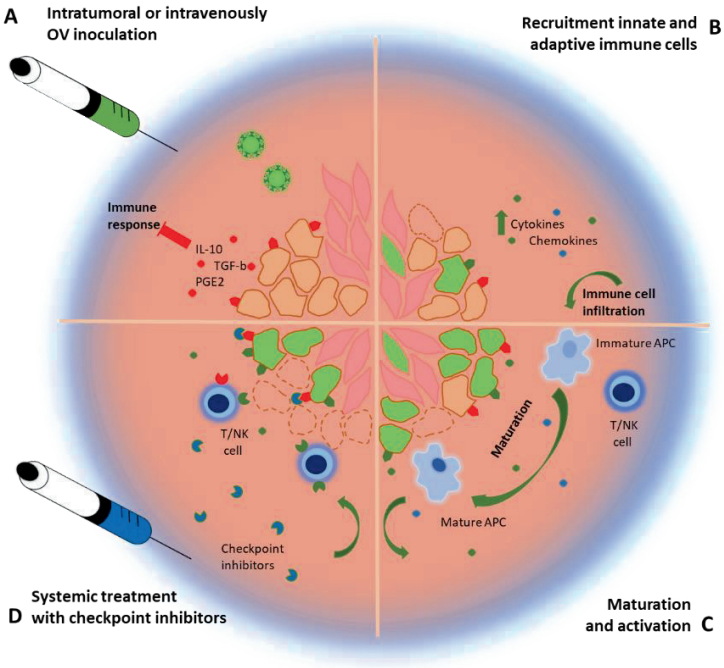


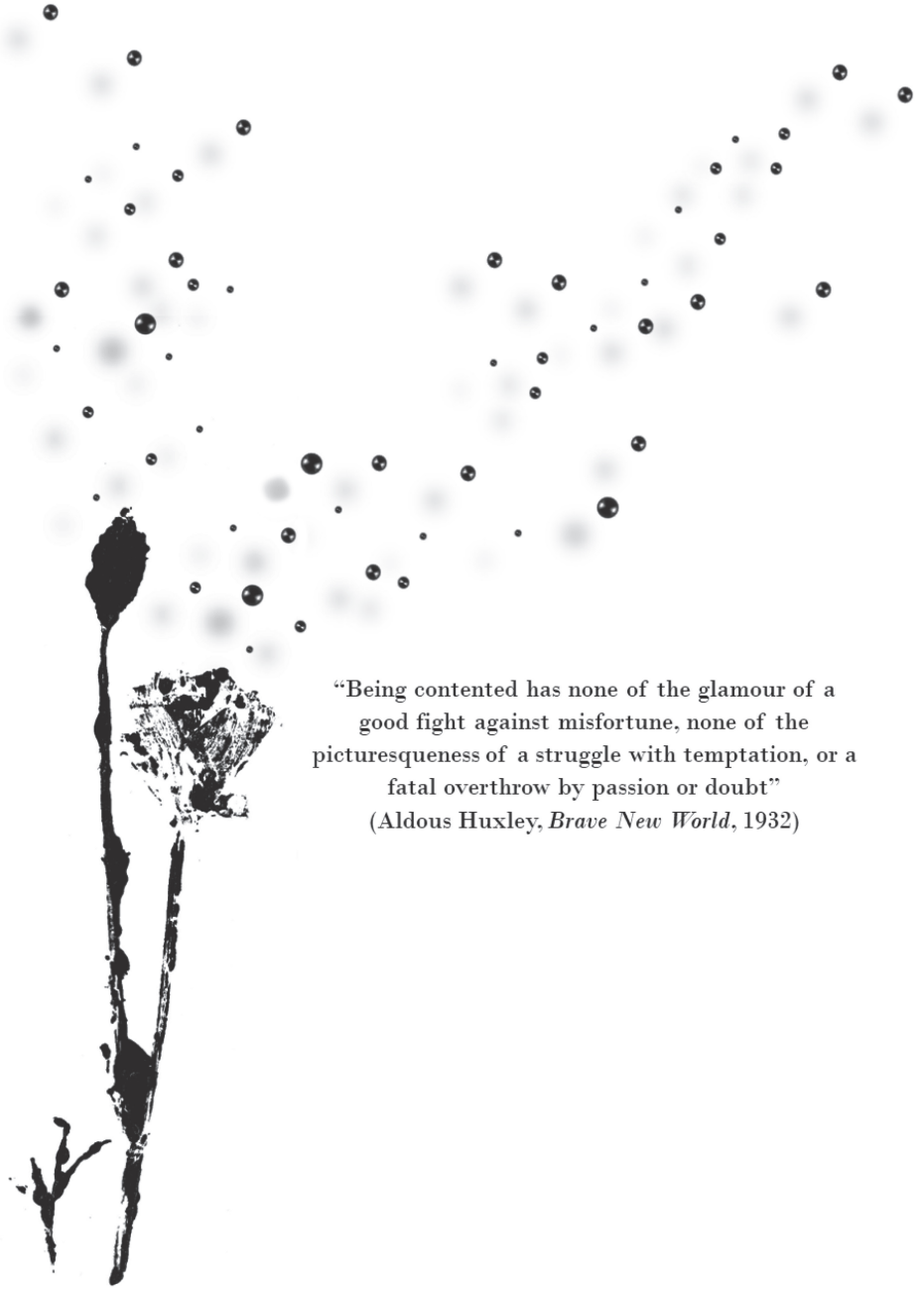
Figure 2. Combining oncolytic viral therapy with immune modulators. (A) Weak immunogenic tumor cells in an immunosuppressive TME are infected by oncolytic viruses (green) armed with immune modulators. (B) Tumor cells start secreting viral induced cytokines and chemokines, but also the immune modulators, which improves the immune activation. As a result, immune cells start to infiltrate the TME. (C) Occasionally, the virus will induce an immunogenic cell death. The TAAs and immune stimulating environment will result in the maturation of APCs. (D) The APCs will activate the adaptive immune system upon which T cells will start infiltrating the tumor attracted by the secreted chemokines. Possible activity of immune suppressive ligands, such as PD-L1, will be prevented by the circulating immune checkpoint inhibitors resulting in clearance of the tumor.

In conclusion, the overall effect of the oncolytic immune therapy depends on the interplay between virus, immune modulator and tumor. Not only tumors develop over time, but the immune system as well. This means that every stage of immune activation should be considered while deciding on the incorporation of an immune modulator (**Figure 2**). Weak immunogenic tumors in an immunosuppressive TME will likely benefit from potent oncolytic viruses, cytokines and innate stimulating agonists to activate the initial innate immune response. If eventually the tumor is inflamed, effector T cells and NK cells responses can be improved by immune activating agonists, such as 4-1BBL, and checkpoint inhibitors. The vectorization of both cytokines and immune activating agonists could reduce possible adverse effects compared to systemic administration, whereas systemic delivery of checkpoint inhibitors improves the timing and localization of the treatment. Future directions will have to explore multiple combinations of dually armed OVs allowing to overcome tumor heterogeneity or at least to use both OVs and immune modulators to their full potential.

Table 1: Combination therapy of armed oncolytic viruses and immune modulators

Transgene	Virus	Tumor	Additive immunologic effects	Toxicity
Cytokines				
GM-CSF	HSV[21,238,307] AdV[228,296,308] MV[227] VSV[309] NDV[310]	Adenocarcinoma[227], Metastasized (phase I)*[21,228], Breast cancer[307], Melanoma (phase I)*[307,308]	Improved peripheral blood mononuclear cell response[310] $CD3^+$ T cell infiltration[227] Long-term immunity against rechallenge with tumor cells [227]	Grade 1 and 2 [21,228]
IL-12	AdV [294] HSV[224,233–235,237,238] VSV[236]	Neuroblastoma[235], Glioma[233], Prostate[238], Squamous Cell Carcinoma[236,237], Melanoma[294]	Infiltration of macrophages, T helper, CTL and NK cells [233,235,237,238] Improved survival [294] and protective against rechallenge [237]	No signs[234,236,237]
IL-2	NDV[56,242–245] HSV[241]	Melanoma[56,244], Hepatoma[242,243], Squamous Cell Carcinoma[241]	Infiltration of T helper and CTL[56,241–245] Immunity against rechallenge with tumor cells [241–244]	No signs[243,244]
IL-15	VSV[246] NDV[247] IAV[311] HSV[312] IAV[313]	Colon carcinoma[246], Melanoma[247,313]	Increase in tumor specific CTLs in the blood[246] Infiltration of T helper and CT[247,312] Immunity against rechallenge with tumor cells[247] Increases survival in mouse model[247,313]	IL-15 only detectable in tumor[247]
IFN-β	MV[249] VV[314] VSV[315] NDV[50]	Non-small cell lung cancer[314,315], Mesothelioma[249], Pancreatic adenocarcinoma[50]	Improved survival mouse model[249]	Not reported
IFN-γ	NDV[56] VSV[250]	Melanoma[56], Mammary and colon carcinoma[250]	Increased cytokine expression and improved DC maturation[250] Increased T cell infiltration[56]	Not Reported
Others: **IL-18,[224,294]** **IL-17,[316]** **TNF,[56]** **MIP1a,[317]** **FLT3L[317]**	NDV[56] VSV[316] AdV[294] HSV[224]		Improved T cell responses	Not reported

Chemokines: CCL5,[253] CCL2,[255] CCL19,[318] CXCL11[252,319]	VV[252,253,318,319] HSV [255]	Colon carcinoma	Improved DC maturation[253] Improved infiltration T helper cells and CTLs[252,253,318,319] Induces a Th2 response, but reverts to a Th1 response in combination with DC vaccination[253]	Not reported
Co-stimulatory ligands				
B7.1/CD80	HSV[270] VV[272]	Neuroblastoma[270] , Melanoma (patients)*[272]	Immunity against rechallenge with tumor cells[270] Response in 3/11 patients[272]	Low grade[272]
4-1BBL/ CD137L	VV[275]	Melanoma	No difference in DC maturation Infiltration of CTL	Not reported
CD40L	AdV[278] AdVdd[276] VV[279] VSV[276]	Melanoma[279], Solid tumor (patients)*[278]	More Th1 cytokines[278] Infiltration of T helper, CTL, NK, DC, MDSC[279] Improved survival, but not with armed VSV[276]	No signs[278]
OX-40L	AdV[281]	Melanoma, lung carcinoma	Infiltration of T helper and CTL	Not reported
GITRL	AdV[225]	Melanoma	Infiltration of T helper and CTL	No signs
LIGHT	AdV[285]	Prostate	Recruitment of effector T cells Reduced Treg suppression	Not reported
CD70	VV[284]	Colon adenocarcinoma	Reduced tumor growth	Not reported
Checkpoint inhibitors				
anti-CTLA4	MV[262] AdV[261,296]	Melanoma	Infiltration of T helper and CTL [262,296] Decreased infiltration of Tregs [262]	No signs [262,296]
anti-PD1 or PD-L1	MV[262] VV[269] MYXV[268]	Melanoma	Infiltration of T helper and CTL [262,269] Decreased infiltration of Tregs [262,269] Improved survival [268]	No signs [262]
Combinations				
GM-CSF + IL-12	AdV[292,293]	Melanoma	Secreted cytokine profile shifted from Th2 to Th2 response[293] Infiltration of T helper, CTL, NK and DC[292,293] Immunity against rechallenge with tumor cells[293]	Not reported
IL-12 + IL-18	AdV[294]	Melanoma	Infiltration of T helper, CTL, NK	Not reported
IL-12 + CCL2	HSV[255]	Neuroblastoma	Reduced tumor growth	Not reported
B7.1 + IL-12	AdV[231]	Melanoma	Infiltration of T helper, CTL and DC	Not reported
B7.1 + IL-18	HSV[320]	Neuroblastoma, Prostate	Reduced tumor growth No significant difference in survival	Not reported
B7.1 + GM-CSF	AdV[271]	Melanoma	Infiltration of T helper, CTL and DC Immunity against rechallenge with tumor cell	Not reported
4-1BBL + IL-12	AdV[232]	Melanoma	Infiltration DC, T helper and CTL	No signs
Others				
HPGD	VV[286]	Several tumor models	Expression of Th1 cytokines, CXCL10/11, CCL5 Decreased infiltration MDSC More DCs secreting IL-12 in LN	Not reported
TRIF	VV[286]	Renal cell carcinoma	Increased immune stimulatory cytokine response Improved survival	Not reported
DAI	VV[289]	Melanoma	Improved $CD8^+$ cell infiltration Reduced tumor growth	Not reported



"Being contented has none of the glamour of a good fight against misfortune, none of the picturesqueness of a struggle with temptation, or a fatal overthrow by passion or doubt"
(Aldous Huxley, *Brave New World*, 1932)

Chapter 7

Summarizing discussion

Summarizing discussion

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with few to no effective treatment modalities.[346] Viro-immunotherapy has shown promise as a treatment strategy for a variety of tumors, including PDAC.[347] This therapy uses oncolytic viruses (OVs) to infect and kill tumor cells.[12,13] Application of viro-immunotherapy results in tumor specific cell death by direct oncolysis as well as by activation of the anti-tumor immune response.[348] Newcastle disease virus (NDV) is such an OV, which has shown potential as an oncolytic agent in several clinical trials.[43,95,96]

NDV, a replication competent virus, belongs to the family *Paramyxoviridae* and has an avian host range under normal conditions. NDV strains can be categorized into three main pathotypes: lentogenic (non-virulent), and mesogenic and velogenic (both virulent), depending on the severity of the disease they induce in chickens. The cleavage site in the fusion protein (F) of NDV has been shown to be a major determinant for virulence.[25,31] NDV is classified as virulent (mesogenic/velogenic) if the F protein contains an multi-basic cleavage site (MBCS) motif or if the virus has an intracerebral pathogenicity index (ICPI) value of 0.7 or higher.[25] Several studies have demonstrated a correlation between ICPI values, the induced mean death time (MDT) of virus inoculated chicken eggs and the virulence of NDV.[25,349,350]

NDV does not cause substantial disease in humans. Only a few, mostly mild, cases have been reported and these infections were generally observed during the mass vaccination campaigns of poultry, when veterinarians apply large amounts of nebulized spray containing live attenuated NDV strains to poultry flocks.[38,351–353] Such infections with NDV may result in a mild clinical picture of acute conjunctivitis and laryngitis in humans, which clears up rapidly and spontaneously. There is no evidence that infections with mesogenic and velogenic viruses result in disease in humans and there are no reports of human-to-human transmission of NDV.

The potential of NDV as OV has been demonstrated in multiple studies using *in vitro* and *in vivo* tumor models, including those for PDAC.[50–52,189,247,354] However, several studies showed that the efficacy of the treatment could be improved using lentogenic viruses with an engineered MBCS in the F protein. These viruses had significant higher efficiency in killing tumor cells in *in vitro* assays and in murine tumor models compared to the lentogenic NDV F0 strain.[50,56] Another approach to increase the efficacy of viro-immunotherapy is the use of OVs armed with immune modulatory proteins. The incorporation of such transgenes is aimed to overcome the immune suppressive tumor micro environment (TME) upon treatment with these viruses.[50,56,355,356]

The studies described in this thesis focus on two topics: (1) environmental safety and toxicity of modified recombinant NDV LaSota strains containing an engineered MBCS and (2) the immune modulating ability and the anti-tumor efficacy of recombinant NDV LaSota strains containing an engineered MBCS or expressing an immune modulatory protein.

Improving environmental safety and efficacy of recombinant NDV

In the first part of this thesis, the most optimal route of administration of NDV as an oncolytic virus was investigated. The intratumoral route is often used in clinical trials, because this route allows the exact administration and local dosing of the OV to the tumor in contrast to intravenous administration (reviewed in **Chapter 1**). Therefore, intratumoral administration strategy was suggested to be safer and more effective in anti-tumor therapy. However, intravenous administration results in in targeting of metastatic lesions by systemic delivery of OV therapy. In **chapter 2**, we have shown that injection with recombinant NDV LaSota containing an engineered MBCS (NDV F3aa) was safe when administered intratumorally or intravenously to immune deficient tumor bearing mice. In addition, both intratumoral and intravenous injections with NDV F3aa resulted in decreased tumor burden, which confirmed the anti-tumor efficacy observed in our previous study, as well as other studies using NDV with an MBCS.[50–54,56,357]

As NDV F3aa might pose a treat for the environmental safety, we aimed to generate mutant viruses with expected lower virulence for poultry but with equal oncolytic efficacy. Mutants of NDV F3aa were generated with abrogated expression of the avian specific interferon (IFN) antagonist, the V protein, but also of the W protein (function unknown), resulting in NDV F3aa-$_{STOP}$V. Our study described in **chapter 3,** showed that, although NDV F3aa-$_{STOP}$V was indeed less virulent in chicken cells than NDV F3aa, the NDV variant without V protein expression unfortunately also had decreased oncolytic efficacy compared to NDV F3aa.

Subsequent assessment of the genetic stability upon passaging of the virus in different cell lines demonstrated that the mutations introduced in the open reading frame (ORF) of the V protein gene, to generate NDV F3aa-$_{STOP}$V, were stable. However, in the genomes of these passaged viruses a substitution of phenylalanine to serine (F-117-S) directly after the MBCS of the F protein was observed (**Chapter 3**). Subsequently, mutants of NDV F3aa and NDV F3aa-$_{STOP}$V were generated that contained this F-117-S substitution (NDV F3aa-S). In **Box 1,** additional data is presented of preliminary results obtained in this project, but not reported on in the thesis, on the comparison between NDV F0, NDV F3aa, NDV F3aa-S and NDV F3aa-S-$_{STOP}$V for induced oncolytic efficacy in a xenograft murine model for PDAC. These preliminary data indicated that NDV F3aa and NDV F3aa-S had both higher oncolytic effectivity than NDV F0. And although NDV F3aa-S-$_{STOP}$V still displayed oncolytic efficacy, this was lower than that induced by NDV F3aa. Additional data obtained in **chapter 3** revealed that NDV F3aa with the F-117-S substitution replicated to higher end point titers than NDV F3aa in Vero cells, which is beneficial for production of clinical grade virus batches to be used in clinical trials.

In **chapter 4**, we describe a study where the virulence of the different NDV variants was determined upon challenging 6-week-old chickens via the natural route and by

Box 1: Assessment of the oncolytic efficacy of NDV F0, NDV F3aa, NDV F3aa-S and NDV F3aa-S-$_{STOP}$V upon intratumoral treatment in a xenograft model for PDAC

In this preliminary study, immune deficient mice were subcutaneously engrafted with human BxPC3 PDAC cells and subsequently three times intratumorally injected with NDV F0, NDV F3aa, NDV F3aa-S, NDV F3aa-S-$_{STOP}$V and (**Figure 1A**). The absence of weight loss indicated that the health of the animals was not severely affected by intravenous injections with NDV (**Figure 1B**). Tumor growth was most profoundly reduced in mice treated with NDV F3aa and NDV F3aa-S compared to mock treated mice. Treatment with NDV F3aa-S-$_{STOP}$V did reduce the tumor growth in this model compared to mock treatment, but not as much as treatment with NDV F3aa-S (**Figure 1C**).

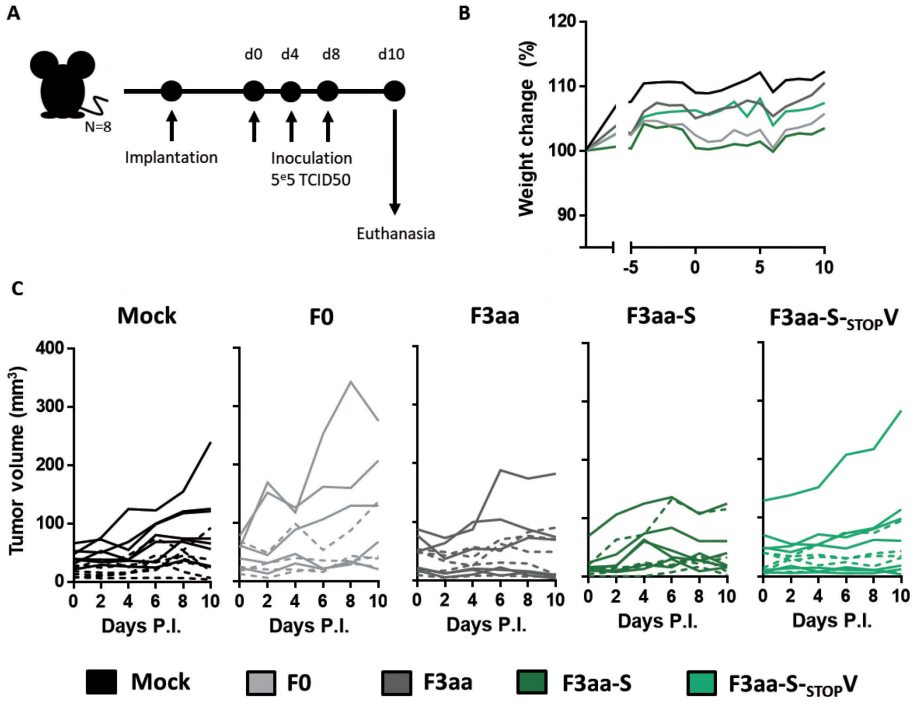


Figure 1. Comparison between oncolytic efficacy upon intratumoral injection with NDV F0, F3aa, F3aa-S and F3aa-S-$_{STOP}$V. (A) The experimental setup of the BxPC3 xenograft model including tumor cell inoculation, injections with NDV and euthanisation (d=day). (B) Relative change in average weight per animal group in the time period after tumor inoculation. Arrows indicate days of virus injection. (C) Tumor volumes per individual mice during 10 days after three intratumoral injection (P.I.= post first injection).

determination of the ICPI. This study showed that incorporation of the F-117-S substitution in the backbone of F3aa or F3aa-$_{STOP}$V did not increase the virulence compared to the parental viruses. This in contrast to another study that reported that the F-117-S substitution could increase the virulence of the virus.[180] In our study, both NDV F0 and F3aa-S-$_{STOP}$V classified as non-virulent based on the ICPI values and both viruses were not pathogenic for 6-week-old chicken. This was in agreement with other studies that observed decreased replication of NDV without V expression in both avian and mammalian cells.[26,27,194] Surprisingly, our study also showed that, although NDV F3aa classified as a virulent virus based on the ICPI, the virus was less pathogenic in six-week-old chickens than lentogenic NDV F0 upon inoculation through the natural route of infection. Thus, the results obtained in this thesis demonstrated that the MDT or ICPI values for viruses with an engineered MBCS did not correspond with the induced pathogenesis in six-week-old chickens.

These results were in agreement with results from other studies reporting no virulence for originally non-virulent strains with an engineered MBCS in the F protein.[25,32,191] Results from these studies and ours indicate that other viral factors than the cleavage site of the fusion protein are important for the virulence, such as the polymerase protein or Hemagglutinin-neuraminidase (HN) protein. [25,32,191] Based on these results, the NDV LaSota strain with an engineered MBCS (NDV F3aa) could be considered safe to the environment based on the absence of pathogenesis in choanal inoculated chickens, although officially still virulent according to the reports published for determination of virulence.[25] Taking in consideration that patients treated with oncolytic NDV will probably be in isolation until negative for virus-shedding, the change of exposure of poultry to the virus is small. Moreover, such exposure would be via a natural route. Altogether, these results indicate that the virulence of engineered NDV strains for use in viro-immunotherapy should be evaluated with different assays or standards than natural strains to determine environmental safety. As NDV F3aa(-S)-$_{STOP}$V viruses were considered too attenuated, future focus was put on NDV F3aa and NDV F0 armed with immune modulators.

Arming oncolytic viruses to enhance anti-tumor responses

Oncolytic viro-immunotherapy, such as with NDV, has the potential to revert the immune suppressive TME to an immune stimulating environment. This immune stimulating environment could be further increased by combining OV therapy with different immunotherapies separately or encoded by the virus, such as immune stimulating cytokines, checkpoint inhibitors, co-stimulatory activating antibodies or depletion of antibodies, such anti-PD-1 or anti-CTLA4. In this study, the application of NDV encoding immunomodulatory transgenes was investigated. To select the most optimal immunomodulatory transgene for NDV, we conducted a review study in **chapter 5**. Based on this review, we chose to arm NDV F0 with several immune modulators including Granulocyte-macrophage colony-stimulating factor (GM-CSF),

Box 2: Characterization of armed NDV F0 mutants

Several mutants of NDV were generated that were armed with immunomodulators, based on an in-depth literature study as described in **Chapter 5** (summarized in **Figure 2A-B**). *In vitro* assays demonstrated that upon inoculation of Vero cells the transgenes were subsequently expressed (**Figure 2C**) and that incorporation of these transgenes in the viral genome did not attenuate the virus (**Figure 2D**). These viruses need further assessment for oncolytic efficacy in an immune competent murine model for PDAC.

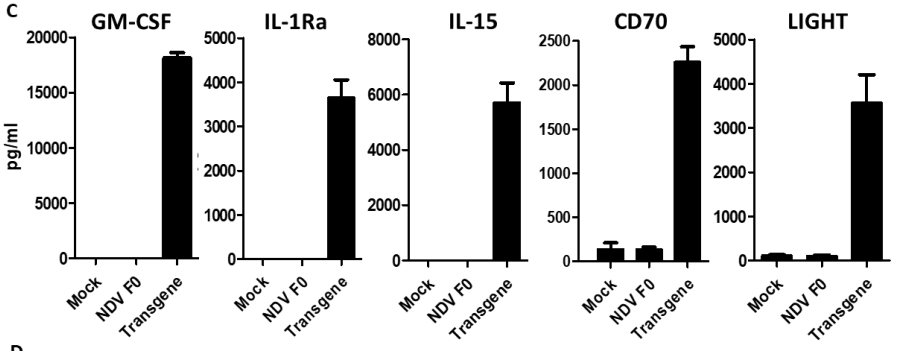
A

B

	Target	Function	Innate	T cells	NK cells	T regs
GM-CSF	-	Tissue infiltration and maturation of monocytes	↑	(↑)	?	?
IL-12	-	Th1 differentiation, increased CTL activity in NK and T cells, anti-angiogenic	(↑)	↑	↑	?
IL-15	-	NK proliferation, NK and T cell activation	(↑)	↑	↑	?
CD70	CD27	Survival T and NK cells	-	↑	↑	?
LIGHT	LT-betaR/ HVEM	Cell Death/co-stimulatory receptor naive T, B cells and immature APCs	↑	↑	↑	(↓)
Il-1Ra	IL-1R	Blockade suppresses tumor growth and is associated with improved beneficial TME	?	?	?	?

↑, stimulating; ↓, repressing; (x), not described as function, but was reported; -, no effect; ?, unknown

C



D

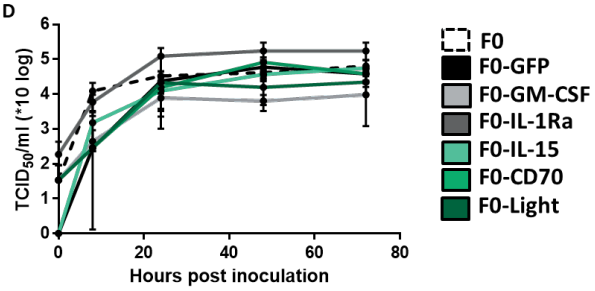


Figure 2. Characterizations of NDV F0 mutants with immune modulators. (A) Schematic representation of the genomes or recombinant NDV armed with an immune modulator. (B) Summary of the function of the incorporated immune modulators. (C) Expression levels of murine immune modulator proteins upon inoculation of Vero cells with the armed viruses at 24 hours after inoculation, as measured by ELISA. GM-CSF, IL-1Ra and IL-15: in cytoplasm; CD70 and LIGHT: in cells. Bars and whiskers represent the mean and standard deviation. (D) Replication kinetics of armed NDV F0 in Vero Cells. Cells were inoculated at an MOI of 0.05 in triplo. At the indicated time points samples were taken and titrated in Vero cells. The experiment was conducted two times in triplo. Means and standard deviations of triplicates of a representative experiment are plotted.

CD70, soluble CD70, tumor necrosis factor superfamily member 14 (TNFSF14, also known as LIGHT), and IL-1 receptor antagonist (IL-1Ra), to increase the anti-tumor immune response induced by armed NDV. In **box 2,** a schematic representation is given for the armed viruses created in this project, with both the murine and human variants of the transgene. Preliminary data show that subsequent infection of Vero cells with these viruses resulted in effective expression of these immune modulators without substantial attenuation of viral replication (**Box 2, Figure 2C-D**). For one of these viruses, NDV F0 armed with CD70, the oncolytic efficacy and safety was assessed in an immune competent murine model (**chapter 6**). The other generated armed NDV strains still need to be evaluated in immune competent models for toxicity and anti-tumor efficacy.

Chapter 6 describes a study where the safety and anti-tumor efficacy of NDV armed with murine CD70 was assessed. CD70 is a ligand of the TNF family, which is involved in activating the adaptive immune responses by ligation of the CD27 receptor. It hence improves T cell maturation and increases cytotoxic activity of T cells characterized by elevated CD8 and PD-1 levels.[358–362] Intravenous injection of immune competent tumor bearing mice with NDV F0-mCD70 resulted in increased percentages of tumor infiltrating T cells. However, the induced oncolytic effect of NDV F0-mCD70 was not increased compared to that induced by NDV F0. **Chapter 6** also reports, for the first time, on the side-by-side comparison between NDV F0 and NDV F3aa for induced immune responses upon intravenous injection in an immune competent tumor model for PDAC. This study showed no differences between NDV F3aa or NDV F0 for induced anti-tumor efficacy or induced immune activation, at least during 10 days after treatment. In addition, minimal differences in viral dissemination were observed between animals injected with NDV F0 or NDV F3aa. These results are somewhat unexpected, because in *in vitro* assays F3aa replicated better in tumor cells than NDV F0. These data suggest that, even though NDV F0 seemed less effective than NDV F3aa in killing tumor cells in *in vitro* assay*s*, treatment with NDV F0 could induce an immune response strong enough to activate an anti-tumor response and create a favorable TME in which the immune system is able to clear the tumor by itself.

Overall, our studies have demonstrated the possibilities of arming NDV F0 with immune modulators to enhance oncolytic efficacy upon intravenous injection. More recent studies have shown the beneficial effects of intratumoral injection of NDV armed with transgenes, such as IL-12, IL-15, IFN-λ, TNF-related apoptosis-inducing ligand (TRAIL), macrophage inflammatory protein-3 alpha (MIP-3α), OX40 ligand (OX40L) or CD137 ligand (CD137L).[242,355,363–370] However, intratumoral or intravenous injections with armed NDV could lead to different preclinical and clinical results as these immunomodulatory proteins function differently at systemic level or in the tumor micro environment.

Future perspectives of Newcastle Disease Virus in oncolytic viro-immunotherapy

Translation from preclinical to clinical studies

The translation of preclinical study results with OVs to the clinic appears to be difficult as multiple (armed) OVs have been evaluated in preclinical trials, but only few were successful as treatment modality in oncolytic viro-immunotherapy.[24] The most successful armed oncolytic virus so far is T-VEC, an Herpes Simplex Virus (HSV) expressing the transgene GM-CSF, which is the first Food and Drug Authorisation (FDA) approved virus for use in oncolytic viro-immunotherapy.[371,372] Studies with T-VEC demonstrated that oncolytic viruses armed with immune modulatory proteins are able to induce an anti-tumor response. However, the immune responses in these clinical studies were less robust than observed in preclinical studies as is more often the case for studies using recombinant GM-CSF as treatment modality[373,374], demonstrating the limitations of translating preclinical studies in mice to the clinic.

Translating preclinical results towards the clinic starts with choosing the appropriate preclinical model. In this thesis, we have used immune deficient and immune competent murine tumor models to assess the efficacy and safety of viro-immunotherapy with oncolytic NDV. It is well known that the use of different murine models, with different genetic backgrounds, can result in different experimental outcomes.[122] For example, high increases in IFN-γ responses have been observed after NDV treatment in C57/BL6 mice, but not after treatment of BALB/c or Swiss mice.[375,376] Other murine models for pancreatic cancer include BALB/c nude mice, the K-ras$^{LSL.G12D/+}$; p53$^{R172H/+}$; PdxCre (KPC) model, a well-defined PDAC model from the C57/BL6 lineage[332], or an immune competent syngeneic murine model with a mixed genetic background of 129S4, B.6129 and 129 Black Swiss mice.[327] In this latter model, also presented in this thesis, no increased interferon responses, nor increased levels of other cytokines, were observed upon inoculation with NDV at 10 days after the first injection. These data suggest that NDV treatment of tumor-bearing mice, might result in different anti-tumor responses in different mouse strains, due to the differences in immune responses. Moreover, the type of murine model used, subcutaneous or orthotopic engraftment, can largely affect the outcome of the results[377], such as previously shown in a study in which mice were treated with FOLFIRINOX chemotherapy.[329] Here, it was demonstrated that, even though orthotopic tumors grew faster, they were more sensitive to systemically administered chemotherapy compared to subcutaneous tumors.

Likewise, our used murine immune competent immune model demonstrated large differences in histology between tumors derived from subcutaneous and intraperitoneal injected tumor cells (**Chapter 6**). Thus, the chosen model determines the efficacy of the anti-tumor therapy as much as the experimental setup, such as the route of administration as shown in **Chapter 2**. In addition, differences between

the human and murine immune systems are even larger than those between murine strains, which signifies the difficulties in extrapolating preclinical data of mice to humans. At last, preliminary data from *in vitro* studies in our research group have demonstrated that murine PDAC cells were less susceptible to NDV infection and virus induced cell death than human PDAC cells (data not shown). This data indicates that the efficacy of an avian virus in a murine model, cannot be directly translated to that in humans due to major host-range differences.

To improve translation of viro-immunotherapy with oncolytic NDV to the clinic, patient derived organoids, small spherical organs made from resected tumor tissues, would be more informative then human 2D cell lines, which are in general lab adapted. An important part of viro-immunotherapy is the stimulation of an anti-tumor response, and, as described before, many studies aim to improve this response by arming the oncolytic viruses with immune modulators. As the murine immune system does not always reflect the human immune response, study results obtained in mice with these armed viruses can often not be directly translated to humans. By co-culturing patient derived organoids with autologous peripheral blood mononuclear cells (PBMCs) of the patients, the participation of the human immune response to the anti-tumor response can be mimicked. Although these techniques have already been established for other tumor models[378,379], these co-culture techniques have yet to be established for PDAC organoids. However, studies using direct inoculation of human immune-cell populations have shown the immune stimulatory effect of oncolytic NDV[363,380], and these studies in combination with results obtained on cytokine release by inoculated organoids will add important information on the immune stimulating capacity of -for instance- armed oncolytic viruses. This, and future research on PDAC patient derived organoids in combination with autologous immune cells, will hopefully result in a better translation of the effectiveness of preclinical studies to clinical trials eventually.

Toxicity studies

In addition to efficacy studies, toxicity studies are necessary to identify and characterize potential toxicities upon treatment with OVs before clinical trials can be initiated. These studies are preferably performed in several animal models, including inbred or outbred mouse strains, or non-human primates. In the murine studies described in this thesis, no toxicities were observed in treated immune deficient and immune competent mice, demonstrating that intravenous injections with recombinant NDV did not induce adverse effects, at least in murine models. In addition, a previous study in cynomolgus macaques demonstrated that intravenous injections with NDV F0 or F3aa did not lead to clinical symptoms, abnormalities in haematological parameters and basic serum chemistry profiles or pathological abnormalities.[55] More importantly, several clinical trials have been reported using intravenous inoculation with intermediate virulent (mesogenic) NDV strains and none

of the treated patients developed severe side effects directly related to viral pathogenesis.[43,95,96]

The safety of NDV as a vector is already generally appreciated in the field of virology and is increasingly used as a vaccine modality for various infectious diseases.[381–383] Most of the studies involved immunogenicity studies in mice to determine the efficacy of vaccination for Severe Acute Respiratory Syndrome (SARS)-2, Respiratory Syncytial Virus (RSV) and Influenza virus.[382,384,385] But also more elaborate studies have been conducted in non-human primates to evaluate the protective immunity of NDV based vaccines against ebolavirus or highly pathogenic influenza virus.[385,386] These studies have demonstrated that the use of armed NDV is safe and that the large insertions, such as immune modulatory change genes, are genetically stable with minimal chance on recombination.[383,385]

Overall, these studies indicate that OV therapy based on NDV F0 and F3aa can be safely applied to patients, although the first clinical trials with NDV F0 to be conducted at ErasmusMC have to provide the ultimate proof for this.

Good manufacturing practices

In addition to environmental safety, anti-tumor efficacy and toxicity studies, good manufacturing practices (GMP) grade virus batches need to be generated for use in clinical trials. GMP grade viruses are required by regulatory instances to bring a licensed product to the clinic. The proceedings to generate these viruses cover all manufacturing processes, including production, storage and quality control of the product. During the last years, these aspects have been investigated for NDV F0 and NDV F3aa at Erasmus MC. The production of both viruses was optimized by testing different cell lines and by evaluating different concentration and purification methods. These methods are in line with what has been reported before by several groups on the production of NDV batches with high titers.[387–390] However, most of these reported studies have used NDV generated in embryonated chicken eggs. It has been reported that egg-generated NDV, in contrast to viruses generated in mammalian cells, are sensitive to neutralisation by the mammalian complement system.[55,166] We have generated a mutant virus based on NDV F0 for which virus stocks can be generated in mammalian cells. It is expected that this virus will have higher oncolytic efficacy upon human use, as the virus is less efficiently neutralised by the mammalian complement system.

Quality control assays for purity, stability and efficacy of NDV batches have been developed and consist of assays as described in this thesis. These data and protocols will together form the framework for an advanced therapy medicinal product (ATMP) and together with the realisation of a new ATMP facility at ErasmusMC, allows us to produce clinical grade oncolytic virus batches to treat patients at ErasmusMC.

Concluding remarks

Overall, the research described in this thesis has demonstrated that the use of NDV as an oncolytic virus in viro-immunotherapy is a promising treatment for patients with PDAC. The obtained results in *in vitro* and *in vivo* assays showed oncolytic efficacy of the therapy and minimal risks for environmental safety. In regard to future perspectives of this therapy, more research is necessary on the promising efficacy of NDV F3aa and on NDV F0 armed with immune modulators. Future studies using subcutaneous and orthotopic murine models, but even better in patient derived materials as well as in patients, are needed to provide proof on efficacy and safety. The first planned clinical trials will provide proof of principle for safety and efficacy with NDV F0 and, if the therapy is proven to be safe, these first studies should be followed up with improved (and armed) NDVs as generated in this project.

"All we can know is that we know nothing. And
that's the height of human wisdom."
(Leo Tolstoy, *War and Peace*, 1869)

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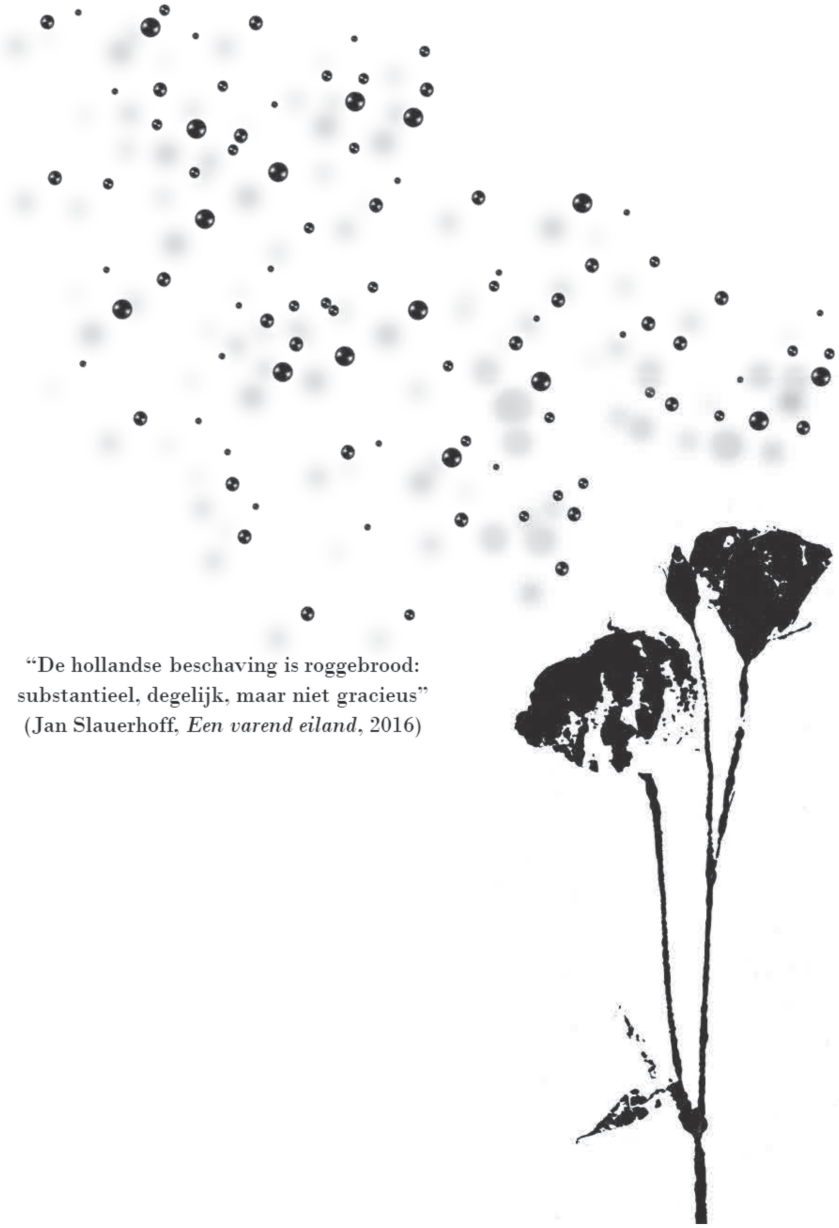

"De hollandse beschaving is roggebrood:
substantieel, degelijk, maar niet gracieus"
(Jan Slauerhoff, *Een varend eiland*, 2016)

Nederlandse samenvatting

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Jaarlijks worden er in Nederland gemiddeld drieduizend patiënten gediagnosticeerd met alvleesklierkanker en die hebben elk een vijfjarige overlevingskans van ongeveer zes procent. Deze kwaadaardige kankersoort wordt vaak te laat ontdekt door het langdurig uitblijven van symptomen. Wanneer deze symptomen wel ontstaan, uiten die zich meestal in zeurende buik- of rugpijn, gewichtsverlies en/of geelzucht. Vaak is op dat moment de tumor al uitgegroeid tot een ver gevorderd stadium van kanker en hebben therapieën zelden effect. De behandelingsmogelijkheden van patiënten met alvleesklierkanker zijn dan ook beperkt. Slechts vijf procent van de patiënten komt in aanmerking voor een operatie, terwijl chemotherapie meestal alleen wordt toegepast als palliatieve therapie.

Verschillende therapieën, waaronder immuuntherapie met ‘checkpoint inhibitors’, zijn veelvuldig getest als behandeling van alvleesklierkanker. Echter, waar deze therapieën goede resultaten geven in bijvoorbeeld patiënten met longkanker of huidkanker, is deze therapie minder succesvol bij patiënten met alvleesklierkanker. Een mogelijke oorzaak hiervoor is dat de tumor het anti-tumor immuunsysteem onderdrukt. Oncolytische viro-immunotherapie, gebaseerd op het gebruik van oncolytische virussen, is een vorm van immuuntherapie die in principe het anti-tumor immuunsysteem kan reactiveren. Oncolytische virussen zijn virussen die specifiek kankercellen kunnen infecteren en lyseren, maar gezonde cellen met rust laten. Wanneer dit oncolytische virus zich begint te vermenigvuldigen in de cel, sterft de kankercel langzaam af en worden nieuwe virussen vrij gelaten die daarna nieuwe kankercellen infecteren. Tegelijkertijd zorgen vrij gekomen tumor eiwitten voor activatie van het anti-tumor immuunsysteem.

Newcastle disease virus (NDV) is één van de veelbelovende oncolytische virussen dat gebruikt wordt in viro-immunotherapie. Dit is een RNA virus dat na infectie ziekte kan veroorzaken in pluimvee, maar niet tot ernstige ziekte leidt in gezonde mensen. Natuurlijk voorkomende NDV varianten worden geclassificeerd op basis van hun vermogen om ziekte te veroorzaken in kippen. Niet virulente, of te wel lentogene, virussen veroorzaken bijna geen ziekte. Wanneer een dier, zoals de legkip, dan besmet raakt met dit virus zal het in eerste instantie een verminderde eierproductie geven. Als het virus een virulente variant is, bijvoorbeeld een mesogene of velogene variant, kan dat leiden tot ernstige ziekte en uiteindelijk sterfte. Mesogene en velogene virussen worden gekenmerkt door de aanwezigheid van meerdere basische aminozuren op de klievingsplaats in hun fusie eiwit, terwijl lentogene virussen een enkelvoudig basische klievingsplaats in hun fusie eiwit hebben.

Tussen 1960 en 2000 zijn verschillende klinische studies met oncolytisch NDV in kankerpatiënten uitgevoerd. Hierbij werden met name lentogene varianten van het virus gebruikt. Deze studies toonden aan dat er wel een anti-kanker effect was, maar dat een verbetering van dit effect wenselijk was. De komst van recombinant

technologieën heeft ervoor gezorgd dat oncolytische virussen zodanig aangepast kunnen worden dat de therapie effectiever wordt. Dit kan bijvoorbeeld door het virus immuunstimulerende eiwitten tot expressie te laten brengen wat kan leiden tot een verhoogde anti-tumor immuunreactie. De effectiviteit van NDV kan verhoogd worden door de virulentie van het virus te verhogen. Dit kan door verandering van de monobasische klievingsplaats in het fusie eiwit van NDV F0 naar een meervoudig basische klievingsplaats zoals in NDV F3aa. Wij, en anderen, hebben eerder aangetoond dat NDV F3aa een betere oncolytische werking heeft dan NDV F0 in immuun gecompromitteerde tumor dragende muizen. Echter, NDV F3aa zou theoretisch een risico voor het milieu kunnen vormen, aangezien virulent NDV zoals NDV F3aa ernstige ziekte kan veroorzaken in pluimvee. Dit proefschrift beschrijft studies naar de milieuveiligheid in pluimvee en anti-tumor effectiviteit van NDV F0, NDV F3aa en genetische gemodificeerde varianten hiervan.

Viro-immunotherapie kan worden toegepast door het geven van een intraveneuze of intratumorale injectie (hoofdstuk 1). Intratumorale injecties kunnen lokaal tot hoge anti-kanker effectiviteit leiden, terwijl intraveneuze injecties juist meer effect kunnen hebben op uitzaaiingen (metastases) van kanker door het lichaam. Echter, intraveneuze toediening kan mogelijk ook meer ongewenste bijwerkingen geven. Om de meest optimale toedieningsroute te bepalen werden beide routes bestudeerd in immuun gecompromitteerde tumor dragende muizen (hoofdstuk 2). Hieruit bleek dat intratumorale injecties met NDV F3aa niet leidde tot ziekteverschijnselen en dat het virus zich niet of nauwelijks naar andere organen verspreidde na injectie. Wanneer dieren intraveneus geïnjecteerd werden met NDV F3aa werden er opnieuw geen ziekteverschijnselen waargenomen, terwijl viraal RNA werd terug gevonden in de longen, milt, lever en soms in de tumor. Beide behandelingen leidden tot een verminderde tumorgroei. De resultaten uit deze studie laten zien dat NDV F3aa een bruikbaar en effectief oncolytisch virus is voor intraveneuze toediening in een immuun gecompromitteerd muismodel van alvleesklierkanker. Echter, NDV F3aa kan nog steeds een risico vormen voor pluimvee.

In de studies beschreven in hoofdstuk 3 is getracht een virus te genereren waarvan de virulentie van NDV F3aa voor kippen werd verlaagd, maar dat nog wel een goede oncolytische werking had. Hiervoor werd een virus gemaakt dat het V eiwit, een virulentie factor specifiek voor kippen, niet meer tot expressie kon brengen (NDV F3aa-$_{STOP}$V). De beschreven studies met dit virus toonde aan dat NDV F3aa-$_{STOP}$V inderdaad verzwakte groei vertoonden in pluimvee cellijnen, kippeneieren en eendeneieren en niet in zoogdiercellen. Onderzoek naar de stabiliteit van dit virus toonde aan dat door het passeren van het virus op zoogdiercellen een mutatie in het genoom van het virus ontstond. Deze mutatie betrof een fenylalanine (F) naar serine (S) aminozuur substitutie in het fusie eiwit, op een positie direct na het meervoudige basische klievingsplaats. Het aanbrengen van deze substitutie in het originele genoom van NDV F3aa (NDV F3aa-S), als ook in dat van NDV F3aa-$_{STOP}$V (F3aa-

S-$_{STOP}$V), verbeterde de groei van beide virussen in zoogdiercellen. Vervolgonderzoek naar het oncolytisch potentieel van NDV F3aa-$_{STOP}$V en NDV F3aa-S-$_{STOP}$V in humane alvleesklierkankercellen liet vervolgens zien dat infecties met deze virussen tot minder oncolytisch effect leidden dan infectie met NDV F3aa en NDV F3aa-S, maar dat dit vergelijkbaar was met dat na infectie met NDV F0. In een poging om de oncolytische effectiviteit te verhogen is vervolgens geprobeerd om de expressie van de fusie en hemagglutinine-neuraminidase eiwitten van het virus te verhogen door een aantal mutaties aan te brengen. Allereerst werd een mutatie in het fusie eiwit, waarvan gerapporteerd was dat het de groei van NDV zou verbeteren. Daarnaast zijn de startmotieven voor eiwittranslatie van het fusie en hemagglutinine-neuraminidase eiwit aangepast naar een optimaal motief voor eiwitproductie in zoogdiercellen. Onze studies in kippen- en eendeneieren lieten zien dat de NDV F3aa-$_{STOP}$V virus varianten met deze aanpassingen minder ziekteverwekkend zouden zijn in pluimvee dan NDV F3aa. Helaas was het oncolytisch potentieel in humane alvleesklierkankercellen maar minimaal verbeterd (hoofdstuk 3).

Deze eerste preklinische studies toonden aan dat het NDV F3aa-S-$_{STOP}$V virus mogelijk geen ziekte zou veroorzaken in pluimvee. Om dit te bevestigen is onderzochte of zes-weken-oude kippen, zonder vaccinatie achtergrond, ziek werden na infectie via de natuurlijke route met NDV F0, NDV F3aa, NDV F3aa-S en NDV F3aa-S-$_{STOP}$V. Daarnaast werd ook de mogelijke transmissie van het virus tussen de kippen onderzocht (Hoofdstuk 4). Geen van de virussen veroorzaakte ziekte in de kippen, en in geen van de kippen werd virus in de hersenen aangetoond. Tegen de verwachting in werd in NDV F0 geïnfecteerde dieren meer uitscheiding en verspreiding van het virus vastgesteld dan in dieren geïnfecteerd met NDV F3aa, NDV F3aa-S en NDV F3aa-S-$_{STOP}$V. Ook werd er meer transmissie tussen de kippen voor NDV F0 gedetecteerd dan voor de andere virussen. Deze data tezamen laten zien dat infectie via een natuurlijke route, in dit geval de choana, van zowel NDV F0 als NDV F3aa, NDV F3aa-S en NDV-F3aa-S-$_{STOP}$V, niet tot ziekte leidt in kippen.

De virulentie van NDV wordt normaal gesproken bepaald op basis van een intracerebrale pathogeniteit index (ICPI) test. In deze test worden 1-dags kuikens in het hersenvocht (intracerebraal) geïnjecteerd met virus en vervolgens worden de dieren acht dagen lang geobserveerd. Een positieve score wordt toegekend voor ieder dier dat ziek wordt of overlijdt ten gevolge van de virus infectie. Uiteindelijk wordt een ICPI score lager dan 0.7 beschouwd als een virus dat veilig is voor pluimvee. In hoofdstuk 4 is de ICPI voor onze NDV varianten bepaald. De ICPI voor NDV F0 en F3aa-S-$_{STOP}$V waren lager dan 0.7 en kunnen daardoor als lentogeen beschouwd worden, terwijl de ICPI voor NDV F3aa NDV F3aa-S, beide hoger dan 0.7, de virussen als virulent classificeren volgens de officiële en mogelijk een risico vormen voor het milieu. Echter, infectie met dit virus via een natuurlijke infectie route veroorzaakte geen ziekte in 6-weken oude kippen. Deze resultaten geven aan dat

de officiële richtlijnen voor bepaling van virulentie niet altijd opgaan voor virussen waarbij alleen de klievingsplaats van het fusie eiwit is veranderd middels recombinante DNA-technologieën, zoals NDV F3aa. Dit is ook door andere onderzoeksgroepen gerapporteerd. Om deze reden zou NDV F3aa waarschijnlijk in klinische trials gebruikt kunnen worden zonder dat er een risico is voor pluimvee.

Een belangrijke component van viro-immunotherapie is de bijdrage aan de (her-)activatie van het anti-tumor immuunsysteem. Dit moet onderzocht worden in immuun competente tumor modellen. Verschillende studies hebben aangetoond dat de effectiviteit van viro-immunotherapie verhoogd kan worden door in het virus genoom genen in te bouwen die immuunstimulerende eiwitten tot expressie brengen. Wanneer het virus zich vervolgens vermenigvuldigd in de kankercellen zal dit eiwit tot expressie komen en zijn functie uitoefenen. Hierbij kan men denken aan immuunstimulerende eiwitten die zowel het aangeboren als het verworven immuunsysteem stimuleren. Uit verschillende preklinische studies bleek dat het toevoegen van immuunstimulerende genen aan een viraal genoom dan ook een positieve werking had op de virus-geïnduceerde vermindering in tumorgroei zoals samengevat in hoofdstuk 5. In onze studie hebben wij gekozen om het immuunstimulerende eiwit CD70 in te bouwen in het genoom van NDV F0 (Hoofdstuk 6). Dit ligand is specifiek betrokken bij de ontwikkeling en activatie van T cellen. Onze studie toonde aan dat het inbouwen van dit eiwit in NDV F0 (NDV F0-mCD70) geen effect had op de groei van het virus. Tien dagen na injectie van de muizen met NDV F0-mCD70 werd er geen expressie van CD70 gezien in de tumor, maar wel in de milt. Echter werd er na deze injectie geen verminderde tumorgroei of veranderde immuunreactie gezien ten opzichte van injectie met NDV F0. Er is meer onderzoek nodig om te bepalen of NDV F0-mCD70 of NDV F0 op latere tijdspunten na behandeling een effect geven en of andere immuun stimulerende eiwitten het effect van NDV F0 kunnen verhogen.

Daarnaast beschrijft Hoofdstuk 6 een studie waarin wij het oncolytisch effect van NDV F0 en NDV F3aa hebben onderzocht in immuun competente tumor dragende muizen waarbij de virussen zijn toegediend via een intraveneuze injectie. Dit onderzoek focuste vooral op het effect tijdens de eerste 10 dagen na injectie om zodanig vroege immunologische reacties te onderzoeken en om eventueel virus in de tumoren te kunnen detecteren. Deze studie liet zien dat intraveneuze injecties met NDV F0 en NDV F3aa beide tot tumor reductie leidden. Tijdens de 10 dagen na injectie werd geen ongewenste overactiviteit van het immuunsysteem gezien, wel werden significante verschillen in immuunreacties waargenomen tussen onbehandelde en virus behandelde dieren. Deze studie toonde aan dat intraveneuze injectie van NDV F0 en NDV F3aa vergelijkbare immuunreacties en anti-tumor effectiviteit gaven in immuun competente tumor dragende muizen, in de eerste 10 dagen na behandeling.

Concluderend, de studies in dit proefschrift laten zien dat NDV F3aa als oncolytisch virus veilig is te gebruiken in immuun competente en immuun gecompromitteerde muizen zonder risico's voor pluimvee. Dit is onder andere aangetoond door onze studie in zes-weken-oude kippen die via een natuurlijke route waren geïnjecteerd met NDV F3aa, waarbij geen ziekte verschijnselen optraden. Onze data tonen ook aan dat NDV anti-tumor effect geeft bij zowel intraveneuze als intratumorale toediening. De effectiviteit van viro-immunotherapie met NDV F3aa of NDV F0 kan waarschijnlijk verder worden verbeterd door virussen immuun stimulerende eiwitten tot expressie te laten brengen. Verder onderzoek moet uitwijzen wat hiervoor de beste kandidaat genen zijn. In de tussentijd is in het Erasmus MC een laboratorium gerealiseerd voor productie van geneesmiddelen voor geavanceerde therapieën (zgn. ATMP laboratorium). Hier zullen op korte termijn klinische batches van NDV F0 worden geproduceerd voor gebruik in de eerste klinische studies in patiënten met alvleesklierkanker. Deze klinische studies zullen de veiligheid en toepasbaarheid van NDV als oncolytisch virus in viro-immunotherapie tegen alvleesklierkanker bevestigen en aanleiding geven tot verdere studies met NDV F3aa met of zonder additionele immuuntherapie.

"If you are under the impression you have already perfected yourself, you will never rise to the heights you are no doubt capable of"
(Kazuo Ishiguro, *The Remains of the Day*, 1989)

About the author

Curriculum vitae

Joanna Fréderique de Graaf was born on the 19th of August 1993, in Arnhem. She obtained her VWO diploma at gymnasium Camphusianum, Gorinchem before she started her academic studies on the University of Utrecht, the Netherlands, in 2011. On the faculty of Medicine, she enrolled the bachelor study 'Biomedische Wetenschappen' and joined their honours program during which she wrote her honours program thesis 'Suppression of the innate immune system by IAV NS1 in humans and chickens at the laboratory of Prof.dr. van Kuppeveld, University of Utrecht. In 2014, Fréderique started her master Infection and Immunity at the university of Utrecht, The Netherlands, during which she obtained her first research training at the laboratory of Prof.dr. Meyaard at the department of Translational Immunology of Utrecht Medical Institute, The Netherlands aiming to unravel the undefined CD200R inhibitory receptor pathway. After her first internship, she moved to Canada for her second internship at the laboratory of Prof.dr. Pezacki at the Biochemistry Department of the University of Ottawa, Canada. Here, she designed and optimized fluorescent labelling of the viral RNA suppressor p19 and evaluating the FRET-based detection of small RNA binding. Fréderique graduated (cum laude) after finishing her master thesis at the laboratory of Prof. Kornmann at ETH Zurich, Switzerland. In 2016, she started her PhD studies on avian paramyxoviruses and their application in oncolytic viro-immunotherapy for treatment of pancreatic cancer patients at the laboratory of Dr. Bernadette van den Hoogen, Prof.dr. Casper van Eijck and Prof.dr. Ron Fouchier, resulting in this thesis. Since 2021, she started working as scientist at Immunetune under supervision of Dr. Jeroen van Bergen, and in close collaboration with the group of Prof.dr. Ossendorp, on synthetic linear DNA as a novel platform for the development of personalized neoantigen vaccines.

List of publications

de Graaf JF, Laksono BM, Vietsch EE, Groeneveld D, van Nieuwkoop S, van Eijck CHJ, Fouchier RAM, van den Hoogen BG,
Virus dissemination and immune responses upon oncolytic viro-immunotherapy with Newcastle Disease Virus with or without multi-basic cleavage site in a syngeneic murine model for pancreatic cancer at early time points after treatment.
In preparation

de Graaf JF, Huberts M, Groeneveld D, van Nieuwkoop S, van Eijck CHJ, Fouchier RAM, van den HoogenBG,
Comparison between intratumoral and intravenously administered oncolytic virus therapy with Newcastle disease virus in a xenograft murine model for pancreatic adenocarcinoma.
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Armed oncolytic viruses: A kick-start for anti-tumor immunity.
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Portfolio

Courses

2019	Organoids, stem cells and regenerative medicine	EMC
2018	The Course in Virology	EMC
2018	Scientific Integrity Course	EMC
2018	The annual course on Molecular Medicine	EMC
2018	PhD Course Infection, Immunity and Tolerance	LUMC
2017	OIC Course Functional Imaging and Super Resolution	EMC
2017	Translational imaging workshop by AMIE: mouse to man	EMC
2017	Patient orientated research: design, conduct and analysis	EMC
2017	The survival analysis course	EMC

Teaching

2019	Lecture I&I oncolytic viruses	EMC
2019	Supervision bachelor thesis	EMC
2018	Supervision master thesis	EMC
2017	Supervision high school thesis	EMC
2016	Supervision master thesis	EMC

Symposia/conferences

2019	Immunotherapy for Cancer and Beyond (poster)	Qindao
2019	European congress of Virology (poster)	Rotterdam
2019	Scientific Spring Meeting KNVM & NVMM (oral)	Papendal
2019	Molmed Day (poster)	Rotterdam
2019	NVGCT Spring Symposium (oral)	Lunteren
2018	PhD retreat	EMC
2018	Conference on Oncolytic Virus Therapeutics	Oxford
2018	Symposium: Improving flu vaccines	EMC
2018	NVGCT Spring Symposium(oral)	Lunteren
2018	Dutch Annual Virology Symposium	Amsterdam
2017	Armed Oncolytic Immunotherapies Summit	Frankfurt
2017	Molmed Day	Rotterdam
2017	NVGCT Spring Symposium	Lunteren

Other activities

2018	Organization of PhD retreat	EMC
2018-2022	Organization of monthly journal club	EMC

"I'd rather take coffee than compliments now"
(Louisa May Alcott, *Little Women*, 1868)