CLINICAL INVESTIGATION

Cancer therapy with Newcastle disease virus: rationale for new immunotherapeutic combinations

Cancer immunotherapy with Newcastle disease virus (NDV) dates back to 1960s, when intratumoral treatment of a cervical cancer patient was noted to result in an abscopal effect with distant tumor regression. Since then, multiple preclinical and clinical studies have demonstrated the inherent ability of NDV to increase immunogenicity of tumor cells. Recently, these properties were explored in animal models within the context of novel immunotherapies such as antibodies targeting immune checkpoints, with demonstration of induction of systemic antitumor immunity and regression of tumors not directly affected by the virus. These studies highlight the immunotherapeutic potential of NDV and provide a strong rationale for exploration of NDV and perhaps other oncolytic viruses in combination with immunomodulatory antibodies in the clinic.

Keywords: CTLA-4 • PD-1 • cancer immunotherapy • Newcastle disease virus • oncolytic

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References to a relationship between natural viral infections and tumor regressions can be found in case reports dating back to mid-1800s [1–8]. In 1904, an American pathologist and clinician, George Dock described a patient with chronic myelogenous leukemia who went into a short-lived remission after an influenza infection [2–4]. Further cases were observed in the mid-1900s of patients with acute appendicitis-induced and measles-induced remissions of leukemia. More recently, Zygiert and Taqi associated regression of Hodgkin's disease to measles virus vaccination [1,4]. These observations laid the foundation for human trials in which oncolytic viruses (OVs) were exploited for therapeutic use. In the years to come, Alice Moore and Chester Southam would pioneer the testing of OVs in animal models and clinical trials [9–12].

Over time, in an effort to force the evolution of viruses with greater tumor specificity and circumvent pathogenicity, clinicians tried to isolate a nonhuman animal virus that retained oncolytic activity even in a host that was not traditionally susceptible to the virus. After numerous attempts, promising results were reported using Newcastle disease virus (NDV). In 1965, Cassel and Garrett inoculated NDV intraperitoneally into mice with Ehrlich ascites and upon re-challenge, showed that this treatment was curative with no adverse side effects [6,7]. Moreover, the first documented case of NDV in a clinical setting was reported by Wheelock and Dingle, where a patient with leukemia responded to intravenously administered NDV [8].

Over the next 50 years, NDV would be evaluated as an anticancer agent in multiple studies in animals and humans, alongside several other viruses, with elucidation of mechanisms of virus-mediated oncolysis and specificity for cancer cells. However, while promising activity has been demonstrated in a variety of animal models, which primarily evaluated the viruses administered intratumorally, the clinical results with systemically-administered OVs have not been as impressive. The major limitation of most OVs has been their poor delivery to metastatic cancer sites and rapid development of neutralizing antibodies, which limits the

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utility of further systemic administration [13]. However, in the few patients who did achieve response to oncolytic virotherapy, the clinical benefit was often durable even after completion of therapy, which strongly implicates the role of antitumor immune response in the observed efficacy [14]. This concept has been explored in greater depth in more recent studies, where OVs have been evaluated as immunotherapeutic rather than lytic agents, with promising activity recently reported in a Phase III study in advanced melanoma with talimogene laherparepvec, an oncolytic herpesvirus expressing granulocyte/macrophage colony-stimulating factor (GM-CSF) [15]. Evidence from many research groups points to the strong immunotherapeutic potential of OVs and provides for rationale for incorporation of OVs into treatment regimens with other novel immune therapeutic modalities [16]. In the current review, we will summarize the evidence behind the immunotherapeutic potential of NDV and will discuss the implications for further studies, integrating NDV into treatment regimens with novel immune therapeutic agents.

NDV as an OV & completed clinical trials

NDV is a negative-strand RNA virus that belongs to the Paramyxoviridae family and is responsible for severe respiratory and CNS diseases in avian species [17]. NDV strains have been categorized as velogenic, mesogenic or lentogenic based on their virulence and severity of disease in birds. Anticancer effect of NDV was first noted in 1950s, when studies by Moore *et al.* and Prince *et al.* demonstrated selective ability of the virus to lyse Ehrlich ascites carcinoma cells [18–22], later confirmed by Eaton *et al.* in murine models of lymphoma and leukemia [23,24]. Research into the development of anticancer agents using NDV as an OV became an increasingly active area, with an optimism for unraveling the exact mechanisms of oncolysis and oncolytic specificity for cancer cells. Depending on their ability to successfully replicate and lyse human cancer cells, NDV pathotypes have been further classified as lytic (velogenic and mesogenic) and nonlytic (lentogenic). The most commonly studied strains MTH-68/H, PV701, 73T, Italien and AF2240 are grouped as lytic strains, while HUJ, Ulster, LaSota and V40-UPM are nonlytic strains. Different NDV strains have been evaluated in xenograft models of human fibrosarcoma, neuroblastoma and carcinoma and immunocompetent models of murine cancers including lymphoma, leukemia, colon carcinoma, head and neck carcinoma, mesothelioma, gastric carcinoma, rat pheochromocytoma, hepatocellular carcinoma and glioma [25–29]. In cell culture, and xenograft tumor models, lytic NDV strains in general have been most effective OVs, due to their superior inherent ability to lyse cancer cells. The studies in immunocompetent tumor models, however, have not been as conclusive, with some studies indicating that nonlytic strains may be just as efficient or even superior to the lytic ones [30]. At present there is, thus, no sufficient evidence to mark some NDV strains as being superior to others, as most of them were never compared head to head and the comparisons of the viruses across the studies are flawed by the differences in experimental models that have been used.

Several studies demonstrated that specificity of NDV for tumor cells is dependent on the cancer-specific defects in the IFN pathways that allow cancer cells to evade IFN-induced inhibitory signals [25,31,32]. For instance, Krishnamurthy *et al.* demonstrated that NDV was able to replicate and spread significantly more in human fibrosarcoma cells than in human fibroblasts [31,33]. He attributed this effect to a poor response of the human fibrosarcoma cell line to IFN-β, which led to a reduced activation of IFN-regulated genes and ultimately to an enhanced replication of NDV.

These notable findings became somewhat contentious when Mansour *et al.* demonstrated effective oncolytic activity in cancer cells with normal IFN responses [34]. The authors demonstrated that NDV selectivity for the non-small-cell lung cancer cell line A549 overexpressing the antiapoptotic protein Bcl-xl was dependent on tumor cell resistance to apoptosis. The rationale was that a delay in apoptosis provided enhancement of early viral replication in these chemotherapy-resistant cells, which then resulted into a higher number of cells being infected and ultimately destroyed. These findings were supported by another recent study demonstrating oncolytic activity of NDV in pancreatic cancer cell lines independent of the defects in IFN signaling pathways [35].

The studies elucidating the mechanisms of NDV specificity for tumor cells were accompanied by promising reports of efficacy with NDV in clinical trials. For instance, clinical evidence for NDV antineoplastic activity was mentioned in 1965 by Cassel and Garrett, where a patient with metastatic cervical carcinoma was treated with injection of NDV directly into the cervical tumor mass [36]. Interestingly, while the woman had marked regression of the pelvic tumor, she also had shrinkage of distant disease in a supraclavicular lymph node [6]. Meanwhile, the first intensive experiments of Csatary *et al.* using an NDV strain MTH-68 on advanced cancer patients via inhalation, spawned attempts to administer NDV systemically [37]. This novel virus strain, which stood for 'More Than Hope 1968,' was used in a randomized, placebo-controlled Phase II trial in advanced solid tumors. Overall 1-year survival was 55% (18 patients) compared with 8% (two patients) in the placebo group [37,38]. The authors

attributed the antitumor activity of MHT-68 to immunostimulation and to the release of cytokines. Indeed, this attenuated virus strain was shown to increase the production of nitric oxide by enhancing the expression of nitric oxide synthase II enzyme and increasing the macrophage population in the peritoneal cavity of rats [39]. Furthermore, Csatary went on to show long-term benefit of intravenously injected MTH-68 in glioblastoma patients as a sole form of therapy [37].

Another lytic NDV virus strain that made it to the clinical trials was PV701 strain, isolated by investigators at ProVirus, Inc. Pecora *et al.* reported on a Phase I clinical trial in which PV701 was administered intravenously to 79 advanced solid cancer patients who were previously unresponsive to standard therapy [40]. The principal objective of this study was to define a maximum tolerated dose for systemic administration of PV701 as a single agent in cancer therapy. In this study, 14 patients demonstrated progression-free survival ranging from 4 to 31 months. In hopes of improving the tolerability and response rates, Hotte *et al.* introduced slower infusion rates followed by safely escalated subsequent infusions of higher doses [41]. In 18 patients, four major and two minor responses were observed. Interestingly, in several patients, the responses occurred long after initiation of therapy and after establishment of neutralizing antibody titers. The responses tended to be durable and in some patients persisted despite therapy discontinuation [41].

Despite the significant clinical promise of the PV701 and MTH-68 NDV strains, unfortunately, the progress into both clinical and laboratory investigation of lytic NDV strains as anticancer agents has been severely hampered by the classification of these viruses as select agents by the US and European regulatory agencies in early 2000s. Due to these restrictions, the majority of the research effort over the past few years has been concentrated on exploration of the immune-activating rather than direct lytic properties of the virus. Indeed, the patterns of response seen in clinical studies, including abscopal effects, delayed responses and durability are highly reminiscent of the responses seen with modern immunotherapeutic agents [42] and present a tantalizing possibility that the therapeutic effect seen in these trials was strongly driven by the immune response to the tumor. These speculations are supported by the findings in animal models. The oncolytic potency of the lytic 73-T NDV strain has been demonstrated successfully in a number of human tumor xenografts grown in athymic mice [43,44]. However, while in tissue culture and in xenograft models, lytic viruses were more effective than the nonlytic strains, the difference was less pronounced in syngeneic tumor models with intact immune system [45]. For instance, as discussed above, Schirrmacher *et al.* showed the lytic strain 73T to be inferior to the nonlytic strain Ulster in immunocompetent mouse model of colon cancer [30]. These findings suggest that therapeutic efficacy of NDV in immunocompetent hosts might not be entirely dependent on the direct lytic ability of the virus and strongly implicate the role of the immune system in the observed antitumor activity.

To formally evaluate the immunotherapeutic potential of NDV in patients, several studies utilized NDV-infected autologous or allogeneic tumor cells to immunize patients with advanced metastatic disease [6,46–50]. Most remarkable results have been reported by Cassel *et al.* and Murray *et al.*, where NDV oncolysate was used as an immunotherapeutic agent in postsurgical management of resected melanoma [47,48]. In their initial study, six out of 13 malignant melanoma patients showed a decrease in the size of skin nodules and lymph node lesions following administration of viral oncolysates [48]. Moreover, a long-term follow-up on 83 stage II melanoma patients in which NDV oncolysate was used as an immunotherapeutic agent in postsurgical management, reported over 60% 10-year overall survival and 55% 15-year survival and freedom from disease recurrence [46,51].

Overall, these findings highlighted that the immune system may play an indispensable role in NDV-mediated therapeutic effect and provided rationale for multiple studies focusing on elucidation of mechanisms of NDV-induced innate immune responses and exploration of strategies to harness these mechanisms to drive stronger adaptive antitumor immunity.

Activation of innate immune response by NDV

The strong link between NDV and the innate immune responses has been proposed for many years. In early 1990s, Zorn *et al.* demonstrated that pretreatment of peripheral blood mononuclear cells (PBMCs) with NDV significantly enhanced their cytolytic activity against tumor cells [52]. This enhanced target cell lysis was attributed to the increased levels of IFN-α, a central mediator of NK cell cytotoxicity. Depleting NK (CD56+) cells from PBMC culture yielded significant decrease in killing of target cells, further validating the major role of NK cells in NDV-mediated cell lysis [52]. The realization that NDV could stimulate the innate immune system brought up the possibility that NDV could have direct immunostimulatory effects on cancer cells as well, which is supported by the earlier studies with NDV oncolysates. In 1998, Haas *et al.* published the findings on the changes in tumor cell surface adhesiveness induced by NDV infection [53]. Introduction of the viral receptor-binding glycoprotein

hemagglutinin-neuraminidase (HN) into the plasma membrane of the tumor cells increased their cell surface adhesiveness for lymphocytes, presumably mediated by interaction between the HN protein and the lymphocyte membrane. The authors in addition went on to show that irradiated tumor cells infected with NDV induced upregulation of activation markers, such as CD69 and CD25, on co-cultured T cells [53]. This effect was dependent on recognition of virus-infected tumor cells by T cells, underscoring the importance of having tumor-associated virus rather than free virus for activation of the effector cells.

In addition to directly binding lymphocytes through expression of the HN glycoprotein, Washburn and Schirrmacher demonstrated that NDV infection results in upregulation of the adhesion molecules ICAM-I and LFA-3, which further aid the recruitment of lymphocytes [54].

Furthermore, NDV-infected tumor cells were found to upregulate HLA molecules, co-stimulatory molecules and the chemokines RANTES and IP-10, thus recruiting T lymphocytes and monocytes to the site of infection [25,54].

Finally, and perhaps most importantly, in multiple studies NDV infection was demonstrated to induce type I IFN, a central event in establishing an antiviral immune response. In fibroblasts and dendritic cells (DCs), NDV-mediated induction of type I IFN was shown to be dependent on activation of RNA helicase RIG-I [55], while absence of RIG-I in tumor cell lines was demonstrated to be a determinant of sensitivity to NDV infection [56]. With evolving understanding of the role of type I IFN pathway in tumor antigen cross-presentation and induction of antitumor immunity, this aspect of NDV becomes particularly important when designing combination immunotherapeutic approaches targeting the adaptive immunity, as discussed below [57,58].

In summary, NDV-mediated activation of the innate immune response involves several different mechanisms, which could all act in concert to promote generation of antitumor immunity. An oversimplistic interpretation of this relationship would be: activation of danger signals required for antigen presenting cell activation such as type I IFN, damage-associated molecular patterns and pathogen-associated molecular patterns, as well as release of TAAs via tumor lysis; recruitment of immune cells via upregulation of chemokines, adhesion molecules and expression of viral HN adhesion glycoprotein on the surface of infected cells; and, promotion of inherent immunogenicity of tumor cells via NDV upregulation of MHC and co-stimulatory molecules (Figure 1). This leads to effective DC maturation and recruitment of additional effectors such as monocytes and NK cells, providing a bridge to initiation of adaptive immune response and improved recognition of tumor cells by the adaptive immune system [59–62].

Using NDV to activate adaptive antitumor immune response

The adaptive immune response plays a major role in recognition, control and elimination of early tumor growth and thus many cancer immunotherapies have focused on the ways to enhance tumor recognition by the T cells. In NDV research, this became possible with advent of genetic engineering, allowing to genetically modify NDV to express cytokines, immunostimulatory ligands and tumor antigens [63,64]. To promote activation and recruitment of antigen presenting cells, Janke *et al.* engineered recombinant NDV (rNDV) expressing GM-CSF [65]. The insertion of GM-CSF gene did not affect the replication or the tumor selectivity of the recombinant virus. Meanwhile, tumor vaccine cells infected by this rNDV were shown to stimulate PBMCs to exert antitumor bystander effects *in vitro* [65]. This phenomenon was attributed to the increased production of IFN-α by monocytes and plasmacytoid DCs. While this vector was not evaluated *in vivo* in animal tumor models, these findings nevertheless suggested that in addition to direct tumor lysis, expression of GM-CSF from NDV could aid antigen presentation and activation of immune effectors.

Several other studies focused on expression of cytokines that could directly augment activation of T cells. Vigil *et al.* generated a panel of NDV vectors expressing murine IL-2, GM-CSF or IFN-γ and evaluated them for intratumoral therapy in CT26 tumor-bearing mice [45]. Interestingly, out of all viruses, only rNDV vector expressing IL-2 led to significant increase in overall survival when compared with mice treated with parental virus. The treated mice were further protected from a second tumor challenge. This long-lasting protective immune response was credited to increased levels of CD4+ and CD8+ T cells in tumors of mice treated with rNDV [45]. Similar findings with the same virus were later demonstrated in the B16-F10 melanoma model [66]. NDV vectors expressing human IL-2 were also developed and studied *in vitro* and in animal models, with promising immune-activating properties seen in all studies [63,67,68]. Most recently, Bai *et al.* published their findings on the anticancer effects of rNDV expressing human IL-2 along with TNF-related apoptosis-inducing ligand TRAIL [63]. The synergistic partnership of IL-2 and TRAIL was rationalized by IL-2 stimulation of T-cell proliferation and upregulation of apoptotic genes (Bax, FasL, caspase-8, caspase-9, caspase-3) in tumor cells via TRAIL. Melanoma

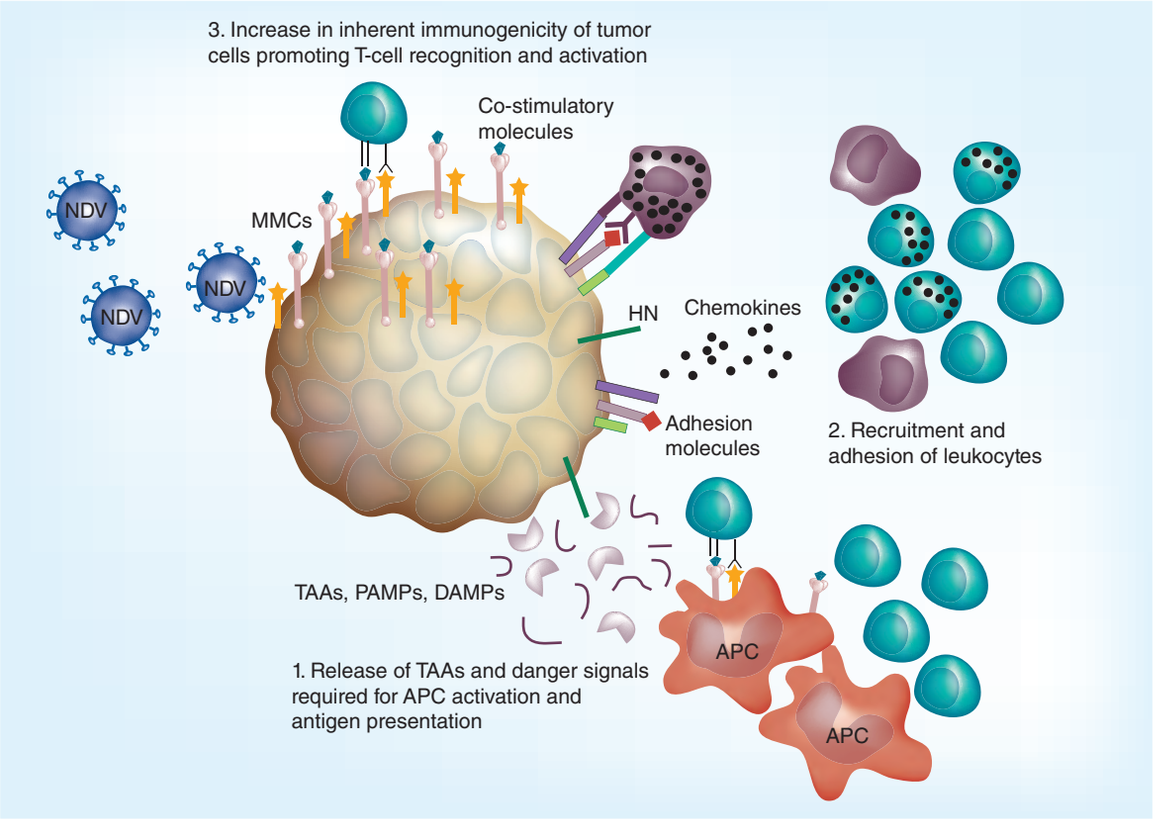

Figure 1. Newcastle disease virus-mediated activation of the immune system. Upon NDV infection, tumor cells undergo apoptosis releasing TAAs as well as 'danger signals' such as PAMPs and DAMPs, leading to activation of APCs, such as macrophages and DCs. In addition, NDV-induced expression of the viral HN glycoprotein on the surface of infected cells, upregulation of cell adhesion molecules, and secretion of pro-inflammatory chemokines lead to recruitment of both innate and adaptive immune cells, such as NK cells, T cells, and DCs. Finally, upregulation of MHC and co-stimulatory molecules on tumor cells makes them function as APCs and facilitates their recognition by the adaptive immune system.
APC: Antigen presenting cell; DAMP: Damage-associated molecular pattern; DCs: Dendritic cells; HN: Hemagglutinin-neuraminidase; NDV: Newcastle disease virus; PAMP: Pathogen-associated molecular pattern; rNDV: Recombinant NDV; TAA: Tumor-associated antigen; TCR: T-cell receptor.

tumor-bearing mice treated with rNDV-IL-2-TRAIL displayed a significant tumor regression and prolonged overall survival when compared with control mice treated with phosphate buffered saline (PBS) [63]. The same group generated an additional rNDV expressing IL-15, demonstrating significant enhancement of antitumor activity over the parental virus with direct intratumoral administration [69].

To formally investigate whether rNDV could be used to stimulate tumor-specific immunity, Vigil *et al.* generated rNDV expressing a model antigen, β-galactosidase MHC I epitope [70,71]. Intratumoral therapy of animals bearing CT26 tumors expressing β-galactosidase with rNDV-β-gal induced a TAA-specific immune response that led to a complete tumor regression in five out of 10 mice, compared with two out of 10 mice treated with control virus. Moreover, this response was significantly boosted by co-administration of rNDV expressing IL-2, with 90% tumor regression seen.

Recognizing the potential of NDV as a vaccine vector, Maamary *et al.* explored the possibility of enhancing the immunogenicity of the rNDV vaccine by targeting the rNDV-encoded HIV antigens directly to DCs [72]. In order to accomplish this goal, Maamary and colleagues generated rNDV expressing a fusion protein of HIV Gag and a single-chain antibody specific for the DC-restricted antigen uptake receptor DEC205. Mice treated with this vaccine were found to have significantly increased levels of Gag-specific CD8+ T cells and CD4+ T cells in the spleen and were better protected from lethal challenge with recombinant vaccinia virus expressing HIV Gag protein, when compared with those treated with rNDV coding for a nontargeted Gag antigen. These findings provide a compelling argument to explore the same strategy for targeting of tumor-associated antigens [72].

To further improve upon the immunostimulatory activity of NDV, Haas and colleagues explored

bispecific adapter proteins as a strategy to enhance the adaptive immune response to tumor cells infected with NDV [73]. In their studies, Haas *et al.* developed recombinant bispecific single-chain antibodies with one arm specific for the HN molecules of NDV and the other arm specific for the CD3 or the CD28 antigen on human T cells. By cross-linking the target cells with effector cells, the researchers observed a polyclonal T-cell response with cytotoxicity of the infected tumor cells at nanomolar concentrations. Maximal antitumor activity in human lymphocytes was further revealed when PBMCs or purified T cells were co-incubated with this modified tumor vaccine for 3 days and then serially transferred to new tumor cell monolayers [74]. The antitumor cytotoxic activity of these effector cells lasted for 10 days. To further augment the immunostimulatory properties of the NDV cellular vaccine, Fournier *et al.* constructed adapter proteins consisting of a single-chain antibody directed against the fusion protein (F) of NDV linked to GM-CSF and a single-chain antibody against HN of NDV linked to IL-2 and GM-CSF [75]. Infection of tumor cells with NDV in combination with either of these bi-specific adapter proteins resulted in stronger activation of PBMCs, providing a rationale for exploration of this strategy *in vivo.* Using a similar strategy Bian *et al.* demonstrated that NDV could be re-targeted to tumor cells expressing specific receptors [76]. In order to re-direct the virus to tumor cells, Bian *et al.* created an adaptor molecule, scHN-IL-2, containing human IL-2 and a single-chain antibody against HN molecule [76]. Selective virus entry into IL-2-receptor-positive human leukemia cells was demonstrated *in vitro* and *in vivo*, and was greatly reduced in the liver, spleen, kidney, lung and thymus in tumor-bearing mice [76,77].

Oncolytic NDV as a potentiator of immunomodulatory antibodies targeting immune checkpoints

In parallel to development of OVs for cancer therapy over the past 50 years, the advances in immunology spearheaded the development of various other immunotherapeutic approaches. Most notably, the identification of the immune receptors regulating T-cell activation, such as the co-stimulatory receptors CD28, GITR, OX40 and 4–1BB, and the co-inhibitory receptors, such as CTLA-4 and PD-1, led to development of agents targeting these proteins in an effort to enhance T-cell activation and to overcome tumor-induced immunosuppression [78,79]. The initial studies with anti-CTLA-4 antibody demonstrated significant activity of this agent in various animal models, which led to eventual development of two clinical agents, ipilimumab and tremelimumab [80–84]. In a pivotal Phase III study in patients with advanced melanoma, ipilimumab demonstrated improvement in overall survival with unprecedented durable disease control and even cures in a subset of patients, which eventually led to its approval by the US FDA in 2011 [84]. Several therapeutic antibodies targeting PD-1 and its ligand PD-L1 have also entered clinical testing over the past few years, with promising activity seen in patients with metastatic melanoma, lung cancer, and renal cell carcinoma, as well as some other cancers, including hematologic malignancies [85–89].

Despite the clinical success of these agents, therapeutic efficacy in patients has not been universal and has not been observed in all cancer types, providing rationale for development of combination therapies in hopes of benefiting a larger patient population. Data from the clinical trials identified a subset of patients who were more likely to respond to immunotherapy with immunomodulatory antibodies. Specifically, patients with evidence of pre-existing antitumor immune response, as suggested by the presence of tumor-infiltrating lymphocytes (TILs), were more likely to benefit [90–92]. Interestingly, an increase in TILs was strongly associated with upregulation of an inflammatory transcriptional signature, which included innate genes involved in type I IFN pathway [58]. Supporting the importance of the type I IFN pathway in tumor immune recognition, type I IFN signaling was demonstrated to be essential in CD8α+ DC-mediated antigen cross-presentation and priming of tumor specific CD8+ T cells [57,58]. These discoveries have provided rationale for evaluation of agents targeting type I IFN pathway as a means to improve sensitivity to immunotherapy with antibodies targeting co-stimulatory and co-inhibitory T-cell receptors.

Given the known potent ability of NDV to induce a type I IFN response, we hypothesized that the immunostimulatory properties of this virus could be employed to drive tumor immune infiltration necessary for the optimal response to immunomodulatory antibody therapy [93]. Specifically, we explored whether local inflammatory responses generated by NDV could be harnessed to generate antitumor responses that would be active on systemic level, thus obviating the need for virus delivery to all tumor sites. To achieve this, we used bilateral flank tumor models with NDV administered to a single flank tumor. Notably, while we were unable to detect viral spread to the distant tumor sites, the microenvironment of those tumors exhibited favorable changes, characterized by brisk infiltration with activated CD8 and CD4 effector T cells, but not regulatory T cells. Consistent with these findings, when intratumoral NDV therapy was combined with adoptive transfer of transgenic

tumor-specific CD4 or CD8 lymphocytes, both NDV-injected and distant tumors exhibited brisk infiltration with the transferred tumor-specific T cells, an effect that was not observed in the absence of NDV therapy. These findings suggested that therapy with NDV was able to diminish the inhibitory effects of the tumor microenvironment, allowing for tumor entry and recognition by the tumor-reactive T cells. Despite these findings, in the challenging B16-F10 model, these effects were not sufficient to induce complete tumor rejection in the majority of animals, suggesting that additional immune inhibitory mechanisms must be suppressing establishment of effective antitumor immunity. Remarkably, when intratumoral therapy with NDV was combined with a systemic antibody targeting CTLA-4, we observed regression of majority of virus-injected and distant tumors, an effect that was not observed with either therapeutic agent alone. This effect was associated with significant increase in TILs, specifically CD8+ and conventional T cells, but not regulatory T cells [93]. Further characterization of TILs revealed an upregulation of ICOS, granzyme B and Ki-67 markers on CD4+ and CD8+ T cells. Therapeutic success was highly dependent on CD8 cells, NK cells and IFN-γ and was completely abrogated in type I IFN receptor-knockout mice. Long-term survivors further displayed protective antitumor memory response when tumor challenged the second time without any further therapy [93]. The combination treatment was also applied in TRAMP C2 prostate adenocarcinoma model and CT26 colon carcinoma model, resulting in similar therapeutic effects, suggesting that this approach could be potentially effective in different tumor types [93].

Conclusion & future perspective

The emerging advances in understanding of pathways driving pathogenesis of cancer provide for new opportunities for the development of novel therapeutics targeting not only tumor-specific defects such as oncogenes, but also the factors driving the suppressive tumor immune microenvironment, such as immune regulatory receptors. Beyond the traditional approaches of surgery, radiation and chemotherapy, immunologic treatments are quickly becoming a part of every oncologist's armamentarium. While treatment modalities using IL-2, IFN-α and BCG have been a routine part of therapy for some cancers over the past 20 years, the major breakthroughs in understanding of mechanisms of T-cell activation and inhibition allowed for development of novel therapeutics with unprecedented durable responses and even cures observed in advanced malignancies. Along these lines, the last 50 years have witnessed an emergence of experimental support for the use of OVs as a form of immunotherapy. While initially deemed unsuccessful due to toxicities or poor systemic delivery, viral therapies recently began to resurface as a form of immunotherapy. The most advanced clinical example of this is talimogene laherparepvec (T-VEC), an oncolytic herpes simplex virus encoding GM-CSF. Intratumoral injection of the virus for advanced melanoma led to tumor immune infiltration and regression not only of the injected lesions, but also at distant sites [15,94]. Analogously, intralesional injection of another OV, coxsackievirus A21, in patients with melanoma similarly led to regression not only of the injected, but also of distant tumors [95].

Since most OVs have not been compared head to head, it is difficult to make any conclusions regarding superiority of one virus over another, especially in clinical setting. With our evolving understanding of mechanisms of viral-mediated oncolysis and antitumor immune response, NDV exhibits several characteristics making it an attractive agent for exploration as a cancer immune therapeutic. First of all, prior clinical experience with rather large doses of systemically administered virus certainly attests to the safety of this agent [14,38]. Second, the ubiquitous nature of the NDV receptor (sialic acid) makes it a potentially useful therapeutic for multiple cancer types. Third, in multiple studies the virus has been demonstrated to be a strong inducer of type I IFN, which has recently re-emerged as a factor playing an essential role in establishment of antitumor immunity. Fourth, the ability to genetically engineer the virus allows it to be used as a vaccine vector or as a vector for introduction of immunostimulatory ligands directly into the tumor microenvironment. Fifth, to attest to the immunotherapeutic potential of NDV in human malignancies, a large body of experimental evidence from clinical trials using NDV-modified tumor cell vaccines suggests that infection of tumor cells with the virus could induce antitumor immune responses. Lastly, recent data from preclinical models indicate that this immune response could be further amplified and lead to improved therapeutic responses through the use of genetically-engineered viruses expressing immunostimulatory ligands/cytokines and combinatorial therapies with systemic immunomodulatory antibodies [93]. It is likely that other OVs may possess similar properties and the development of all viruses as immunotherapeutic agents may proceed in parallel. In fact, OVs are more diverse than some of the other immunotherapeutic agents currently in development (e.g., different anti-PD-1 or anti-PD-L1 antibodies). Thus, it is likely that multiple OVs may end up being approved for different indications, administration routes or combinations with other agents. Taken together, these findings provide a strong rationale to clinically explore

rNDV in combination with systemic therapies targeting the immune co-stimulatory and/or co-inhibitory receptors, whereby local antitumor responses activated by NDV in the tumor microenvironment could be harnessed to be active on the systemic level (Figure 2). To date, NDV has been active in multiple preclinical cancer models, though it is not indicative that those would necessarily be the cancers for which the virus would be effective in clinic. It would be compelling, however, to further study this virus in the context of tumors that have previously been poorly responsive to checkpoint blockade such as prostate cancer and gastrointestinal malignancies.

In addition to providing a rationale for evaluation of NDV in clinical trials, the findings above also open up multiple opportunities for further preclinical exploration to address some additional questions. For example, at present the precise mechanism of action for NDV-induced immune activation is not fully understood, and understanding the balance between the lysis and the ability to induce antitumor immune response would be crucial in the decision on which strain to use, as the viruses with a better replicative potential may preferentially skew the immune response to the virus rather than tumor. Along the same lines, while engineering of rNDV vectors expressing therapeutic antibodies and immunostimulatory cytokines and ligands has been demonstrated to improve the immunotherapeutic efficacy of the virus [93,96], at present it is unknown which ligands would result in establishment of strongest antitumor response without skewing the immune system to predominantly antiviral response, as was observed in the case of recombinant VSV expressing CD40L [97]. Furthermore, each selected transgene might not be universally effective in all OVs. In fact, despite the noted success of oncolytic vaccinia, herpes and adenoviruses expressing GM-CSF in clinical and preclinical studies, previous data from Vigil *et al.* did not reveal the same degree of enhancement of therapeutic efficacy when GM-CSF was expressed by NDV [98]. This could potentially be due to an already known strong ability of NDV to induce DC maturation, whereby further stimula-

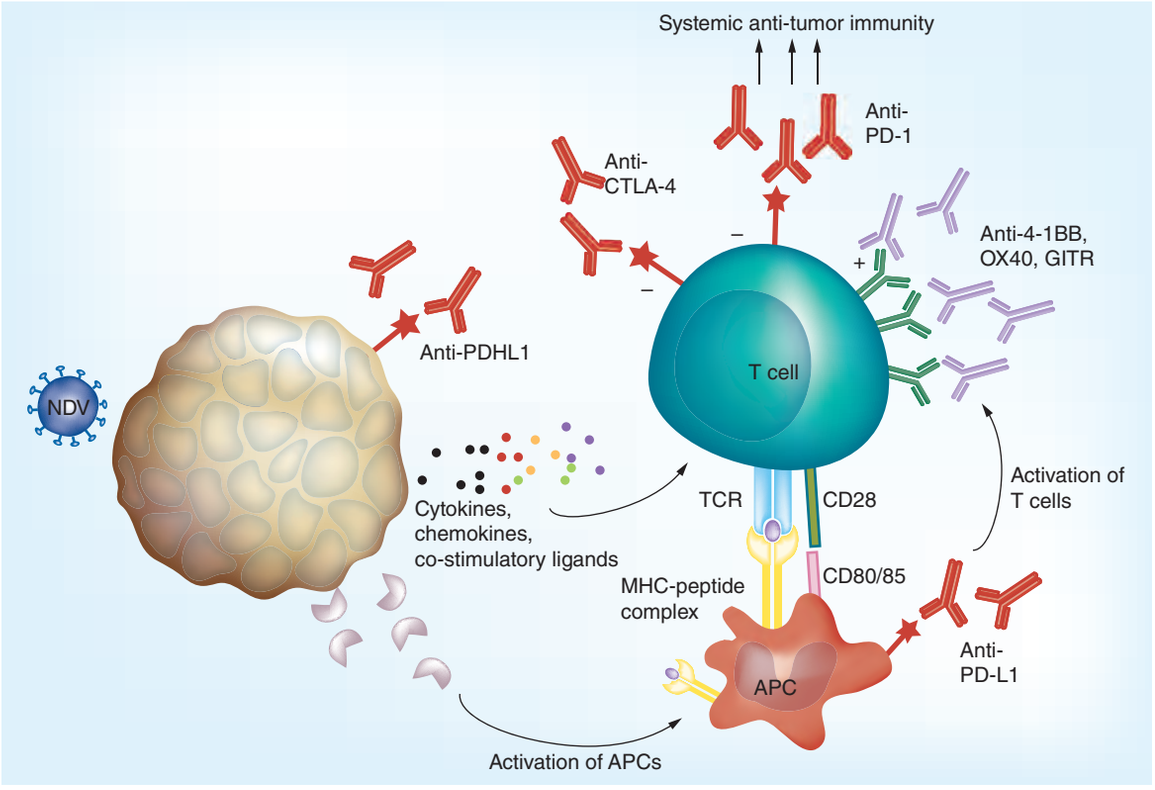

Figure 2. Combination strategies using recombinant Newcastle disease virus and immunomodulatory antibodies for effective systemic cancer immunotherapy. NDV infection of tumor cells results in immunogenic cell death and an inflammatory response with activation of APCs. Expression of cytokines, chemokines or other immunostimulatory ligands encoded by rNDV can further aid the recruitment and activation of tumor-specific T cells. This local inflammatory response can be harnessed for systemic antitumor immunity by using immunomodulatory antibodies targeting co-stimulatory (4 1BB, OX40, GITR) and/or co-inhibitory (CTLA-4, PD-1) receptors and their ligands (PD-L1).
APC: Antigen presenting cell; NDV: Newcastle disease virus; rNDV: Recombinant NDV; TCR: T-cell receptor.

tion with GM-CSF might not provide additional benefit. On the other hand, nonpathogenic NDV strains expressing cytokines and/or ligands that target adaptive immunity, instead of innate immune system, may be more beneficial, as previously demonstrated [66,98]. The ability of NDV to induce immune infiltration in distant tumor sites is certainly also an interesting finding, but the exact mechanism of action is also currently not well understood. Further experiments should focus on delineation of these mechanisms, perhaps through characterization of the effects of NDV on the local production of cytokines and chemokines, its effects on tumor vasculature and tumor-infiltrating innate immune cells, such as DCs and NK cells. In addition, systemic delivery of the virus to distant tumor sites remains a major clinical challenge, as viral sequestration in different organs as well as neutralizing antiviral immune responses, inadequate virus extravasation and spread through the tumors hinder virus delivery to tumors [99]. To address these limitations, multiple groups have developed protocols involving carrier cell systems to better deliver the virus to the tumor site [99–101]. For example, Mader *et al.* described the use of immune and cancer cells to act as carrier of OVs, since these cells possess tumor-homing characteristics [101]. Qiao *et al.* explored loading VSV onto antigen-specific T cells for efficient intratumoral delivery via adoptive transfer in mice bearing melanoma expressing the target antigen [102]. Finally, Ilett *et al.* have recently demonstrated that systemically-administered reovirus associates with PBMCs, granu-

Executive summary

Newcastle disease virus as an oncolytic agent

- Newcastle disease virus (NDV) is an avian paramyxovirus with selective ability to replicate and lyse human cancer cells, an effect that is mediated by predilection for cells with defective antiviral and apoptotic signaling pathways.
- Clinical trials with systemically-administered NDV in cancer patients demonstrated evidence of safety at high doses.

NDV activates innate antitumor immunity and renders tumor cells immunogenic

- In addition to direct lysis, infection of tumors with NDV induces a plethora of immunostimulatory innate effects, which include: release of tumor-associated antigens and danger signals required for antigen presenting cell activation such as type I IFN, damage-associated molecular patterns and pathogen-associated molecular patterns; facilitation of immune cell recruitment and adhesion by upregulation of chemokines, adhesion molecules, and expression of viral HN adhesion glycoprotein on the surface of infected cells; and promotion of inherent immunogenicity of tumor cells by upregulation of MHC and co-stimulatory molecules.
- Clinical trials using vaccination with NDV-modified autologous tumor cells or systemically-administered NDV demonstrated evidence of durable clinical benefit, reminiscent of responses seen with novel immunotherapeutic agents.

Genetically-engineered NDV for enhancement of adaptive antitumor immune response

- Genetic engineering of NDV allows for expression of immunostimulatory molecules such as cytokines directly within the tumor microenvironment, which in many preclinical studies was demonstrated to enhance therapeutic potential of the virus.

Oncolytic NDV as a potentiator of immunomodulatory antibodies targeting immune checkpoints

- Immunomodulatory antibodies demonstrated significant promise in clinical trials, though responses have not been universal and have not been observed in all cancer types.
- Patients with evidence of pre-existing tumor immune infiltration are more likely to respond to immunomodulatory antibody therapy.
- In animal models, intratumorally-administered NDV results in systemic antitumor immune response, with increased lymphocytic infiltration in distant tumors.
- Combination therapy of intratumoral NDV with systemic antibody blocking CTLA-4 led to rejection of distant tumors in different tumor models and long-lasting antitumor immunity.

Future perspective

- Several aspects of NDV, including its immunostimulatory properties, strong safety record and the potential for genetic modification make it an attractive vector for further development as an immunotherapeutic agent.
- Further studies need to define the immunostimulatory ligands/cytokines that can be expressed within the tumor microenvironment and result in most efficient enhancement of the antitumor immunity.
- Preliminary clinical data with other oncolytic viruses administered intralesionally demonstrate evidence of distant tumor regressions, highlighting the role of the immune system rather than direct oncolysis in the observed therapeutic efficacy of these agents.
- Overall, these findings provide a strong rationale for evaluation of combination immunotherapies using locoregionally-administered NDV and other oncolytic viruses with systemic immunomodulatory antibodies.

locytes, and platelets, highlighting that these cells could also potentially be used for systemic delivery [99]. Thus, stimulation of PBMCs with GM-CSF led to an expansion of intratumoral monocyte and macrophage populations and reovirus titers.

Recent data from preclinical models and clinical trials using intralesionally-administered OVs indicate that perhaps systemic delivery of the viruses is not essential to all tumor sites, as a significant part of therapeutic response is driven by the immune system [15,93,95,103]. Indeed, OVs have been demonstrated to have the potential to break immunologic tumor tolerance and lead to an antitumor response. These findings would argue that having an accessible tumor lesion for an '*in situ* vaccination' with an OV should be sufficient to spark an antitumor immune response, which could perhaps be further driven through the use of combinatorial strategies with other systemic immunotherapeutic agents such as immunomodulatory antibodies. This may in addition help to mitigate some of the untoward toxicities seen with systemic OV administration. An early clinical validation of this strategy was recently demonstrated in a Phase I study using combination of T-vec administered intratumorally into accessible lesions with systemic ipilimumab in advanced melanoma patients [103]. In this preliminary report of 18 patients, an objective response rate of 41% including 24% complete response rate was observed, which compares favorably to the historical responses with ipilimumab alone, which nears 10% [84]. With advances in delivery of therapeutic agents, this strategy can be extended to other tumor types, as viral delivery to the 'accessible' tumor lesions could be accomplished via minimally-invasive procedures, such as intraperitoneal or intrapleural infusions, inhalation or hepatic arterial infusion [104].

In summary, recent advances in immunotherapy and OV therapy provided important insights into how these strategies could be integrated to result in effective antitumor immunity. Evidence has emerged indicating that these therapeutic approaches could indeed result in enhanced therapeutic benefit in the instances where either therapy is ineffective alone. With evolving understanding of immunostimulatory properties of OV therapy, rNDV emerges as an attractive agent for further clinical exploration alone and in combination with other immune therapeutics.

Financial & competing interests disclosure

D.Z. is the Bart A. Kamen Fellow of the Damon Runyon Cancer Research Foundation and a recipient of the Young Investigator Awards from the ASCO Conquer Cancer Foundation and the Bladder Cancer Awareness Network. D.Z. is a co-inventor of a pending patent for the use of NDV for cancer immunotherapy. The authors have no other relevant affiliations or financial involvement with organizations or entities with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

No writing assistance was utilized in the production of this manuscript.

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