Use of Reverse Genetics to Enhance the Oncolytic Properties of Newcastle Disease Virus

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Abstract

Naturally occurring strains of Newcastle disease virus (NDV) have shown oncolytic therapeutic efficacy in preclinical studies and are currently in clinical trials. Here, we have evaluated the possibility to enhance the cancer therapeutic potential of NDV by means of reverse genetics. Mice bearing s.c. implanted CT26 tumors were treated with intratumoral (i.t.) injections of a recombinant NDV modified to contain a highly fusogenic F protein. These treated mice exhibited significant reduction in tumor development compared with mice treated with the unmodified virus. Furthermore, mice in a CT26 metastatic tumor model treated with an i.v. injection of the genetically engineered NDV exhibited prolonged survival compared with wild-type control virus. In addition, we examined whether the oncolytic properties of NDV could be improved by expression of immunostimulatory molecules. In this regard, we engineered several NDVs to express granulocyte macrophage colony-stimulating factor, IFN-γ, interleukin 2 (IL-2), or tumor necrosis factor α, and evaluated their therapeutic potential in an immunocompetent colon carcinoma tumor model. Mice bearing s.c. CT26 tumors treated with i.t. injections of recombinant NDV expressing IL-2 showed dramatic reductions in tumor growth, with a majority of the mice undergoing complete and long-lasting remission. Our data show the use of reverse genetics to develop enhanced recombinant NDV vectors as effective therapeutic agents for cancer treatment. [Cancer Res 2007;67(17):8285–92]

Introduction

Newcastle disease virus (NDV) is a nonsegmented, negative-sense, single-stranded RNA virus of the *Paramyxoviridae* family with a natural avian host range. Several clinical trials have reported NDV as a safe and effective therapeutic agent for cancer treatment in patients with no reports of pathologic affects in humans other than mild flu-like symptoms and conjunctivitis (1). NDV is reported to selectively replicate in and destroy tumor cells while sparing normal cells, and therefore it has been sought for use as a clinical oncolytic agent (2–6). These inherent oncolytic properties, shared by several viruses (including NDV), are believed to derive, at least in part, from defective IFN signaling pathways in tumor cells (7, 8). Normal cells, with an effective antiviral response, inhibit viral replication before significant damage is instigated, thereby providing a mechanism for using NDV as a safe and effective cancer therapeutic agent. The known sensitivity of NDV to IFN and its inability to replicate in normal mammalian cells are likely to contribute to its shown safety in humans (9). In addition, NDV is known to enter cells by binding to sialic acid residues present on a wide distribution of human and murine cancer cell lines (2, 10, 11), making it suitable for use in a broad range of cancer cell types.

Reports from phase I and II clinical trials using naturally occurring strains of NDV as oncolytic agents show promise and warrant investigation into methods of improving the therapeutic potential of these viruses (12, 13). Although NDV has been in use in clinical trials longer than most other oncolytic agents, genetic development of an improved viral vector for the treatment of tumors has not been reported. The ability to modify NDV through reverse genetics affords the opportunity to develop improved therapeutic vectors engineered for increased antitumor efficacy. Recent reports have shown that expression of the highly fusogenic F protein of NDV in transfected cancer cells can efficiently kill tumor cells through the formation of large multinucleated cells, called syncytia (14). Therefore, we first investigated whether the inclusion of a highly fusogenic F protein into a replicating nonpathogenic chicken vaccine strain of NDV, Hitchner B1 (NDV/B1), will enhance its antitumor properties. For this purpose, the F protein of NDV/B1 was genetically modified to contain a multibasic cleavage and activation site. NDV expressing this modified F protein (rNDV/F3aa) is highly fusogenic and able to form syncytia (15). In addition, vesicular stomatitis virus (VSV) engineered to express this highly fusogenic F protein has shown increased oncolytic potential in treating hepatocellular carcinoma in the livers of immunocompetent rats (16). In this report, we show an improved therapeutic response in immunocompetent tumor-bearing mice treated with rNDV/F3aa compared with rNDV/B1.

Second, we evaluated the potential to genetically engineer rNDV as a gene therapy vector to enhance its therapeutic efficacy. The expression of immunomodulatory molecules by oncolytic vectors [such as granulocyte macrophage colony-stimulating factor (GM-CSF; ref. 17) and interleukin 2 (IL-2; ref. 18) expressed from vaccinia virus or GM-CSF (19) expressed from herpes simplex virus] has shown great promise in clinical trials. In addition, preclinical reports of other viruses modified to express cytokines were able to confer an increase in their therapeutic efficacy in several tumor models (20–27). In this regard, we developed several NDV vectors carrying GM-CSF, tumor necrosis factor (TNF)-α, IFN-γ, or IL-2 and tested them for increased therapeutic efficacy. Although the recombinant expression of these cytokines has been described in other viruses, we sought to understand the effect they would have in the context of NDV treatment of a mouse tumor model. Our results describe the first reported genetic modifications of NDV for increased cancer therapeutic efficacy.

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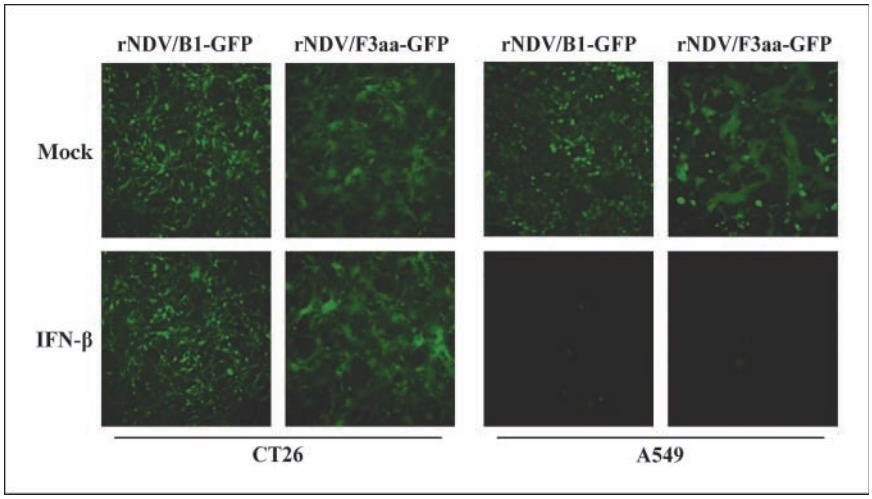

Figure 1. rNDV infection of CT26 colon carcinoma and A549 human lung cells. Cells were infected at an MOI of 2 and visualized 24 h postinfection for green fluorescence. Cells were pretreated with 2,000 units/mL of universal IFN-β or mock treated 24 h before infection, as indicated. Loss of GFP expression is only observed in the IFN-responsive A549 cells and not in the CT26 cell line that remains permissive to NDV infection even in the presence of IFN-β. Both A549 and CT26 cells infected with rNDV/F3aa form large multinucleated cells or syncytia.

Materials and Methods

Cell lines. CT26 is an *N*-nitroso-*N*-methylurethane–induced BALB/c colon carcinoma cell line generously provided by Dr. Nicholas P. Restifo (National Cancer Institute, NIH, Bethesda, MD) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 100 μg/mL streptomycin, 100 μg/mL penicillin, and 0.03% L-glutamine. A549 cells were obtained from American Type Culture Collection and were maintained in DMEM with 10% FBS.

Viruses. The NDV cDNA sequence was derived from the Hitchner B1 lentogenic strain, which is commonly used as a live attenuated vaccine in chickens. Viruses are plaque purified and grown in 10-day-old pathogen-free chicken eggs (Charles River Laboratories, SPAFAS). Virus stock preparations are tested for contamination by streaking them on sheep blood agar plates and incubating them at 37°C overnight (data not shown). HI titers were determined as previously described (28).

Generation of recombinant viruses. The NDV mutant viruses engineered to express the modified F cleavage site were generated as previously described (29). The *luciferase* and green fluorescent protein (*GFP*) genes were subcloned as an extra transcriptional unit from p55C1B and phrGFP plasmids, respectively, into the *Xba*I site created between the *P* and *M* genes of pT7NDV/B1 and pT7NDV/F3aa. The cytokines were cloned from mouse splenocytes and subcloned into pT7NDV/B1 and pT7NDV/F3aa vectors in the same manner as luciferase and GFP. Viruses were rescued from cDNA using methods previously described (30) and sequenced by reverse transcription-PCR for insert fidelity.

Animal studies. All procedures involving animals followed NIH protocols and were approved by and done according to guidelines of the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine. Six-week-old female BALB/c mice were purchased from Taconic Farms and housed in a pathogen-free environment. Tumor volume was monitored every other day using digital calipers in two dimensions. Tumor volume was calculated using the following formula: tumor volume $(V) = 4/3 \times \pi \times S^2/2 \times L/2$, where S is the smallest measured diameter and L is the larger diameter. Animals were culled when tumor size reached 18 mm in any dimension or at defined experimental time points.

To assess the systemic toxicity after i.v. inoculation of rNDV/B1 or rNDV/F3aa, we determined the kinetic profiles of blood chemistries at days 1 and 3 postinjection. I.v. injection of 5×10^7, 1×10^7, or 0.2×10^7 plaque-forming units (pfu) of rNDV/B1 or rNDV/F3aa caused a similar and very mild transient induction of circulating IL-6, alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine 24 h postinjection, well below concentrations associated with systemic toxicity in animals and in human clinical trials. Specifically, day 1 postinjection, serum levels were <100 pg/mL, 139 units/L, 25 mg/dL, and 0.2 mg/dL (IL-6, ALT, BUN, and creatinine, respectively) for the recombinant viruses, and <100 pg/mL, 56 units/L, 22 mg/dL, and 0.2 mg/dL, respectively, for mock-treated mice. By day 3 postinjection, no increases were detected. Toxic thresholds in clinical trials for these cytokines are reported as 40,000 pg/mL, 2,000 units/L, 100 mg/dL, and 1.5 mg/dL for IL-6, ALT, BUN, and creatinine, respectively (31).

Luciferase imaging. *In vivo* luciferase imaging was done using an IVIS Imaging System Series 200 (Xenogen Corp.) with Living Image acquisition and analysis software (version 2.11, Xenogen). Mice were anesthetized with isofluran-mixed oxygen. Each mouse is then injected i.p. with 150 mg luciferin/kg body weight (D-luciferin Firefly Potassium Salt, Xenogen). The imaging system first takes a photographic image in the chamber under dim illumination; this is followed by luminescent image acquisition. The overlay of the pseudocolor images represents the spatial distribution of photon counts produced by active luciferase. An integration time of 5 min was used for luminescent image acquisition for all mouse tumor models. We use Living Image software to integrate the total bioluminescence signals (in terms of photon counts) obtained from mice.

ELISA. CT26 cells were infected at an multiplicity of infection (MOI) of 10. The supernatant was collected at 24 h postinfection and assayed by ELISA kit (R&D Systems) following the supplier's protocol. NDV- or mock-infected tumors were dissected and homogenized using a tissue grinder. Following centrifugation, supernatants were removed and tested by ELISA kit. Recombinant cytokines were used for a standard curve and samples from mock-infected cells or tumors were used as a control. Tumors were dissected on day 5 and manually dissociated with scissors and forceps in 2 mL of PBS. Cell clumps were dissociated by gently pressing them between the frosted ends of two coverslips in a circular motion. Cell homogenates were centrifuged at $100 \times g$ and 100 μL of supernatants were collected and immediately frozen.

Flow cytometry. The tumors of sacrificed animals were analyzed for the presence of $CD4^+$ and $CD8^+$ cells by fluorescence-activated cell sorting (FACS) analysis. These tumors were dissected and manually dissociated with scissors. Dissociated tissue was then collected and incubated at 37°C in RPMI and 80 μL of Liberase Blendzyme 3 (Roche Diagnostics). After 20 min of incubation, 120 μL of 0.5M EDTA were added to the cell homogenates and mixed for 5 min. Cells were then filtered using a cell strainer and stained with CD3, CD4, and CD8 (17A2, GK1.5, and 53-6.7, respectively; BD PharMingen) and flow cytomety was done in a Cytomics FC500 machine (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

Results

Phenotypic characterization of rNDV/F3aa in CT26 cells *in vitro*. To determine the syncytia-forming ability of rNDV/F3aa in tumor cells, we infected CT26 cells *in vitro* with rNDV/B1 or

rNDV/F3aa expressing GFP (rNDV/B1-GFP and rNDV/F3aa-GFP, respectively). At 24 h postinfection, large multinucleated cells were observed in cells infected with rNDV/F3aa-GFP. In contrast, CT26 cells infected with rNDV/B1-GFP did not exhibit syncytia formation. Syncytia formation by rNDV/F3aa was also observed in two other mouse tumor cell lines tested (B16.F10 melanoma and Renca renal cell carcinoma; data not shown). In addition, CT26 cells pretreated with 2,000 units/mL of universal IFN-β 24 h before NDV infection were not resistant to infection by NDV, whereas A549 cells, which are well characterized for their IFN signaling abilities (32), dramatically inhibited NDV replication, as seen in Fig. 1. The sensitivity of NDV infection to IFN suggests a possible mechanism for its safety and efficacy reported in preclinical and clinical studies. Furthermore, we have previously shown that the V protein of NDV/B1, although specifically inhibiting the IFN system in avian hosts, is nonfunctional in human cells, rendering NDV highly sensitive to the human IFN system (9).

Tumor-bearing mice treated with recombinant NDV containing a modified F protein cleavage sequence exhibited enhanced tumor growth inhibition. To assess the *in vivo* therapeutic efficacy of rNDV/F3aa as an improved oncolytic agent, 5×10^5 CT26 cells were s.c. implanted in the right flank. Tumors were allowed to establish for 2 weeks until a palpable tumor mass developed. When tumors reached ~50 to 100 mm^3 in volume, mice were then intratumorally (i.t.) treated with 1×10^7 pfu every other day for four injections. Treatment with the control wild-type strain, rNDV/B1, produced a significant reduction in tumor growth compared with control mice treated with PBS (Fig. 2*A* and *B*). Treatment with rNDV/F3aa had an improved tumor growth inhibitory effect compared with rNDV/B1, with 2 of 10 of the tumors treated with rNDV/F3aa undergoing complete tumor regression (Fig. 2*C*). To determine if rNDV/F3aa induces a long-lasting protective immune response, mice that underwent complete tumor regression from the experiment in Fig. 2*C* were challenged s.c. with a second dose of 5×10^5 CT26 cells on the contralateral flank 60 days after treatment initiation. In our experience, the average complete tumor regression occurs between the second and third week after treatment initiation. All of these mice (n = 2) were protected from tumor challenge whereas age-matched control mice developed tumors (data not shown).

Prolonged survival of mice systemically implanted with CT26 tumors after i.v. treatment with NDV/F3aa. The therapeutic efficacy of NDV/F3aa was not limited to s.c. implanted CT26 tumor cells. Five mice per group were inoculated i.v. with 5×10^5 CT26 cells and treated 3 days later with one i.v. injection of 1×10^7 pfu/mouse of rNDV/B1, rNDV/F3aa, or PBS. As shown in Fig. 2*D*, mice treated with rNDV/B1 had a prolonged survival compared with PBS-treated mice and a further enhanced prolongation of survival when treated with rNDV/F3aa. However, although the modification of the F protein of NDV resulted in a significantly enhanced tumor growth reduction in our s.c. tumor model, only 20% of mice developed complete tumor regression. Whereas it may be possible that further modifications of the NDV backbone vector could improve the therapeutic response of this virus (perhaps through modification of the HN protein), we wanted to investigate the possibility of further enhancing the therapeutic potential of NDV by genetically engineering the NDV genome to express cytokines.

Construction of recombinant NDVs expressing GM-CSF, IFN-γ, IL-2, or TNF-α. Previous studies have identified numerous cytokines that can play a critical role in the immune response. Some of the well-shown and most effective cytokines for cancer treatment used in both animal tumor models and in cancer patients are GM-CSF, IFN-γ, IL-2, and TNF-α (25). The ability of

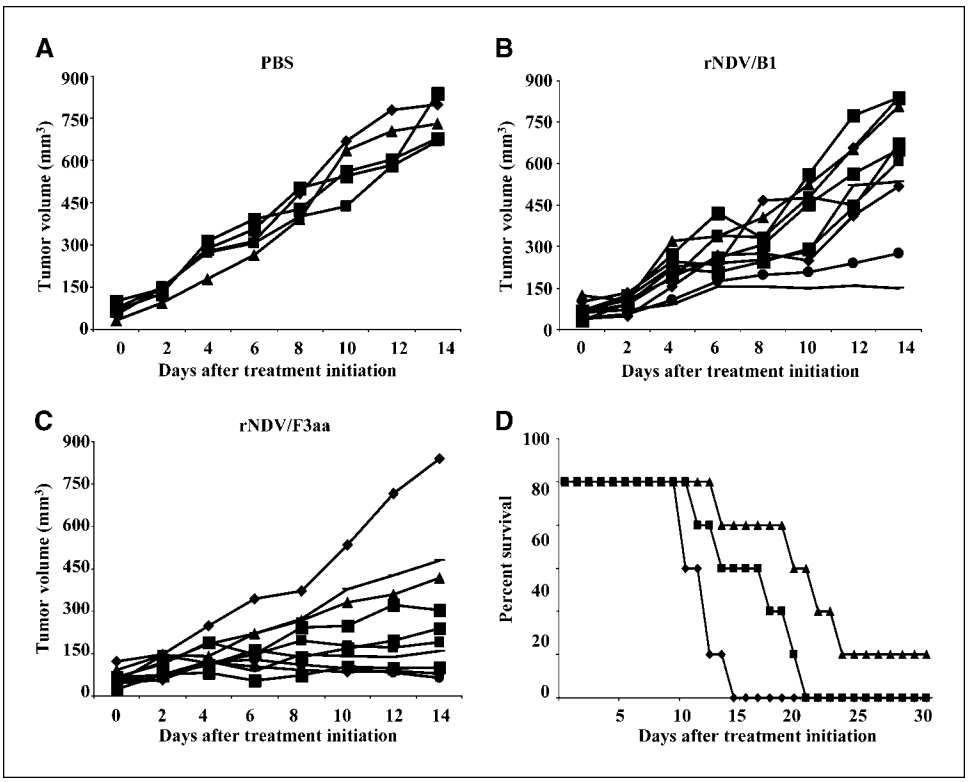

Figure 2. *A* to *C,* comparison of tumor growth inhibition between unmodified rNDV/B1 and the recombinant NDV expressing a modified F protein (rNDV/F3aa). Groups of 5 and 10 mice were s.c. inoculated with 5×10^5 CT26 cells in the right flank (PBS- and NDV-treated groups, respectively). After palpable tumors formed, animals were treated every other day with four i.t. injections of PBS, 1×10^7 pfu of rNDV/B1, or 1×10^7 pfu of rNDV/F3aa. Tumor volumes were monitored every other day for the duration of the experiment and results of individual mice are plotted. Mice treated with rNDV/F3aa had a delayed tumor growth compared with rNDV/B1 (P = 0.0001, between PBS and rNDV/B1; P = 0.01, between rNDV/B1 and rNDV/F3aa) and 2 of the 10 mice treated with rNDV/F3aa underwent complete tumor regression compared with 0 of 10 mice treated with rNDV/B1. *D,* NDV/F3aa mediates extended survival of mice with CT26 tumor metastases. Groups of five mice were inoculated i.v. with 5×10^5 CT26 tumor cells and then treated i.v. 3 d later with 10^7 pfu of rNDV/B1 (■), rNDV/F3aa (▲), or PBS (♦). Animals were monitored daily for survival.

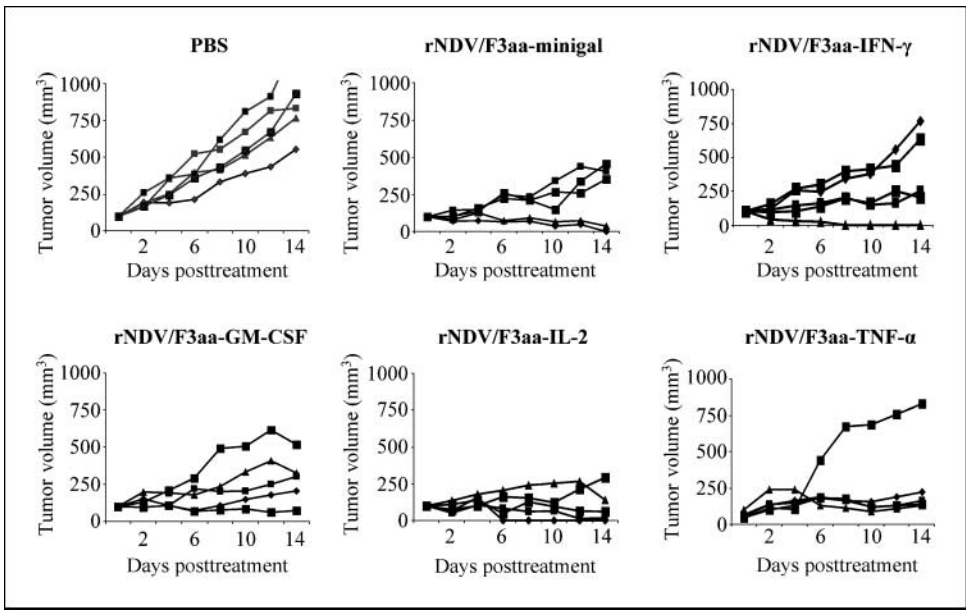

Figure 3. Tumor growth inhibition of tumor-bearing mice treated with recombinant NDV expressing minigal, GM-CSF, IFN-γ, IL-2, TNF-α, or PBS. Five mice per treatment group were evaluated in the same s.c. tumor model as in Fig. 2*A* to *C*, and results of individual mice are plotted.

these molecules to enhance antitumor therapeutic effects has been investigated with several oncolytic viruses, but never with NDV. Considering these reports, we have designed and constructed four viruses expressing murine GM-CSF, IFN-γ, IL-2, or TNF-α and tested them in our tumor model. As a control for potential changes caused by insertion of a foreign gene into NDV genome, we created a virus that expresses a β-galactosidase peptide fragment (rNDV/F3aa-minigal). Cells infected with this virus did not produce detectable levels of any of the immunomodulatory molecules (data not shown). In contrast, supernatant 24 h postinfection from CT26 cells infected with an MOI of 10 resulted in 368 ± 28.8 (SD), 1,354 ± 72.6, 1,950 ± 24, and 255 ± 6.4 ng/mL for rNDV/F3aa-GM-CSF, rNDV/F3aa-IFN-γ, rNDV/F3aa-IL-2, and rNDV/F3aa-TNF-α, respectively.

Treatment of s.c. implanted CT26 tumors with rNDV/F3aa-IL-2 results in enhanced tumor reduction. To assess and compare the therapeutic potential of NDV/F3aa recombinant viruses expressing IFN-γ, GM-CSF, IL-2, and TNF-α, mice were tested in our s.c. tumor model as described earlier. Tumor growth inhibition caused by rNDV/F3aa expressing IFN-γ, GM-CSF, IL-2, or TNF-α was compared with rNDV/F3aa-minigal as a control (results shown in Fig. 3). In this tumor model, mice treated with GM-CSF–, TNF-α–, or IFN-γ–expressing viruses did not exhibit a clear change in the therapeutic efficacy of NDV compared with rNDV/F3aa-minigal. Although mice treated with NDV expressing TNF-α had a noticeably prolonged delay in tumor growth, four of five of the mice eventually succumbed to the tumor (data not shown). Mice treated with NDV expressing GM-CSF and IFN-γ did not exhibit a significant change in tumor growth inhibition compared with rNDV/F3aa-minigal. However, tumor-bearing mice treated with rNDV/F3aa-IL-2 virus had a marked tumor reduction and underwent complete tumor regression in four of five mice, compared with two of five rNDV/F3aa-minigal–treated mice. To further show that rNDV/F3aa-IL-2 is able to significantly enhance the therapeutic efficacy of NDV, we increased the sample size to 10 mice per treatment group and repeated the experiment. Mice treated with rNDV/F3aa-IL-2 had a significant effect on the reduction of tumors with 6 of 10 mice undergoing complete regression compared with 2 of 10 mice treated with the control virus ($P = 0.028$; Fig. 4*A–C*). Furthermore, mice treated with 1 log less of virus per treatment (1×10^6 pfu for four injections) maintained the ability to cause complete regression in 5 of 10 mice compared with 0 of 10 mice in the control virus group (summarized in Fig. 4*D*).

Sixty days after treatment, all of the mice that had undergone complete tumor regression (summarized in Fig. 4*D*) were challenged with a s.c. injection of 5×10^5 cells on the contralateral flank of the mouse. Mice in all treatment groups that underwent complete tumor regression were completely protected from challenge (Fig. 4*C*) whereas naïve age-matched control mice developed tumors (data not shown). The results from these experiments prompted us to investigate the mechanism for the improved therapeutic response from mice treated with rNDV/F3aa-IL-2.

S.c. tumors produce IL-2 only within the tumors of rNDV/F3aa-IL-2–treated mice. Tumors were dissected on day 5 and supernatants of tumor homogenates were tested for the *in situ* presence of IL-2 by ELISA. As seen in Fig. 5*A*, tumor homogenates from rNDV/F3aa-IL-2 and not from control infected tumors had detectable levels of IL-2 within the tumor. This IL-2 was only detected locally within the tumors of rNDV/F3aa-IL-2–infected mice and was not detectable in blood sera (data not shown). Although IL-2 has shown cancer therapeutic potential and is approved by the Food and Drug Administration for cancer treatment, toxicity of systemically administered IL-2 is of concern and limits its widespread use. Mice are able to tolerate 50- to 300-fold higher serum concentrations of IL-2 than humans, and our inability to detect IL-2 within the sera of rNDV/F3aa-IL-2–treated mice suggests that the expression levels of IL-2 from NDV will be nontoxic to humans.

Imaging of the *in vivo* expression of a foreign gene by rNDV/F3aa. To determine *in vivo* distribution of NDV after s.c. treatment, we constructed a recombinant NDV expressing firefly luciferase. This virus can be used to measure the expression of a foreign gene within a mouse tumor and allows us to visualize the distribution of expression *in vivo*. Construction of rNDV/F3aa expressing firefly

luciferase (rNDV/F3aa-Fluc) was generated by insertion of the firefly *luciferase* gene between the *P* and *M* genes as were previous viruses. Mice were s.c. injected with 5×10^5 CT26 cells in the flank and allowed to establish for 2 weeks. Mice then received a single i.t. injection of either 1×10^6 pfu of rNDV/F3aa-FLuc or mock infected. Twenty-four hours after treatment, luciferase expression from rNDV/F3aa-FLuc was visualized *in vivo* by IVIS imaging. As seen in Fig. 5*B*, rNDV is able to express luciferase only within the tumor of the mouse 1 day postinjection and this single i.t. injection is detectable by *in vivo* luciferase imaging for 4 days (data not shown). These results allowed us to conclude that NDV can be used as an effective gene therapy vector to efficiently express a foreign gene within a CT26 s.c. tumor model, and that this expression is limited in distribution to the mouse tumor. In addition, the expression of luciferase seen only within the tumor is consistent with the increased IL-2 produced only within the tumors treated with rNDV/F3aa-IL-2 and not with rNDV/F3aa-minigal. Together, these data show that the expression of IL-2 seen in the mouse tumor treated with rNDV/F3aa-IL-2 can be at least partially attributed to direct expression from the recombinant NDV gene therapy vector.

S.c. implanted tumors treated with rNDV/F3aa-IL-2 have marked T-cell infiltration compared with control virus–treated tumors. Tumors from mice treated with PBS, rNDV/F3aa-minigal, and rNDV/F3aa-IL-2, as previously described, were removed on day 5 after treatment initiation and stained with H&E. The intense H&E staining pattern of the rNDV/F3aa-IL-2–treated animals suggested an increase in tumor-infiltrating lymphocytes compared with rNDV/F3aa-minigal and PBS controls (Fig. 6*A*). To specifically identify cells within the infiltrate as $CD4^+$ or $CD8^+$, tumors were removed and dissociated into single-cell suspension. These cells were then stained with anti-CD3 and costained with either anti-CD4 or anti-CD8 antibody and analyzed by FACS analysis. As shown in Fig. 6*B* and *C*, tumors treated with rNDV/F3aa-minigal and PBS have comparable levels of $CD4^+$ and $CD8^+$ T cells. However, rNDV/F3aa-IL-2–treated tumors showed significant increase in $CD4^+$ and $CD8^+$ cells as compared with those from PBS- and rNDV/F3aa-minigal–treated mice. Tumor-draining lymph node–derived cells (LN) removed on days 5 and 14 were cocultured with irradiated CT26 cells and production of IFN-γ was measured as an indication of T-cell function (Fig. 6*D*). LN cells from PBS-treated mice had undetectable levels of IFN-γ. Moreover, LN cells from rNDV/F3aa-IL-2–treated mice produced higher levels of IFN-γ compared with those from rNDV/F3aa-minigal–treated mice. IFN-γ production was dependent on coculture with CT26 cells, and supernatants of LN cells alone in culture did not produce detectable levels of IFN-γ (data not shown). These data suggest that the increased therapeutic efficacy of rNDV/F3aa-IL-2 is at least associated with a stronger CT26-specific immune response by T cells from rNDV/F3aa-IL-2–treated mice and an increased number of $CD4^+$ and $CD8^+$ T cells within rNDV/F3aa-IL-2 tumors.

Discussion

We have previously reported the construction of a NDV with a modified F protein (F3aa) to be used as a dual vaccine vector against Newcastle disease and avian influenza in chicken (29). This virus was genetically modified to contain the consensus F protein cleavage recognition sequence of virulent strains of NDV (33). Highly virulent strains of NDV in birds are classified as velogenic, intermediate strains as mesogenic, and nonvirulent strains as lentogenic based on the mean death time in chicken eggs (34). The precursor F_0 protein is only fusogenic after it is cleaved into F1 and F2 polypeptides. The different F protein cleavage sequences of NDV

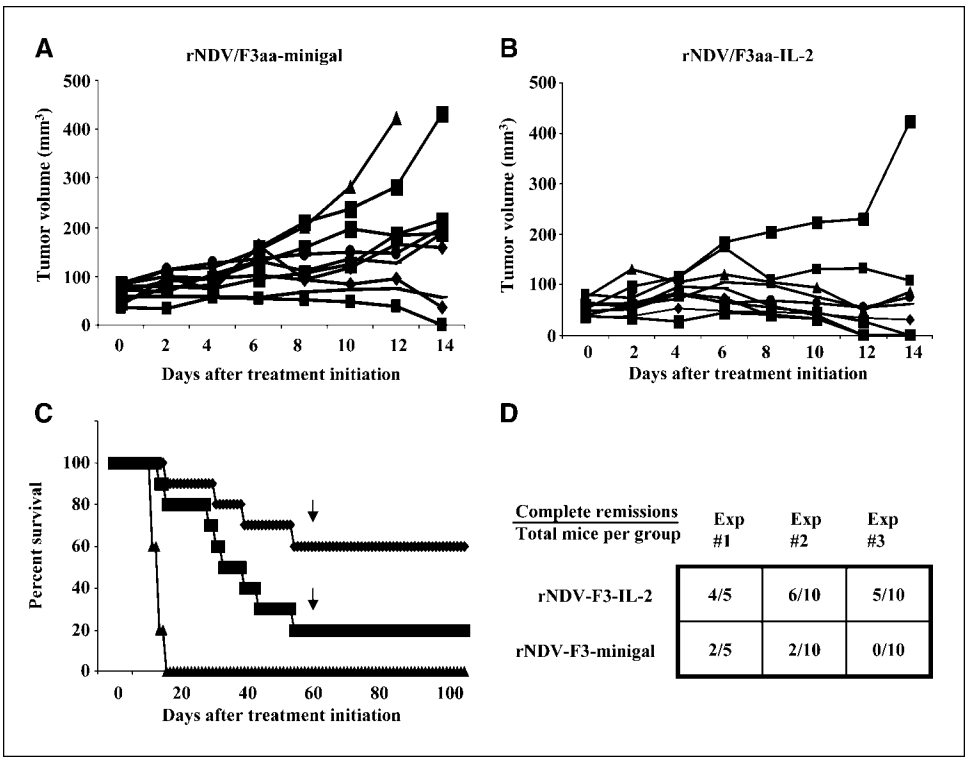

Figure 4. Antitumor effects of rNDV/F3aa-IL-2 virus. *A* to *C*, ten mice per treatment group were tested in the same s.c. CT26 tumor model as in Fig. 3. *A*, tumor volumes in mice treated with rNDV/F3aa-minigal. *B*, tumor volumes in mice treated with rNDV/F3aa-IL-2. *C*, percent survival of tumor-bearing mice treated with PBS (▲), rNDV/F3aa-minigal (■), and rNDV/F3aa-IL-2 (♦). Six of 10 mice treated with rNDV/F3aa-IL-2 underwent complete tumor regression compared with 2 of 10 mice treated with rNDV/F3aa-minigal ($P = 0.02$). Surviving animals were challenged with a new s.c. injection of CT26 cells (*arrows*). *D*, complete remissions of tumors observed in three independent experiments [experiments 1 and 2 correspond to animals in Fig. 3 and in (*C*); experiment 3 was done with 10^6 pfu per injection].

strains are recognized by distinct cellular proteases (35). The F_0 proteins from lentogenic viruses are only cleaved by trypsin-like proteases found in the respiratory and intestinal tracts of birds, whereas the F proteins of velogenic strains can be cleaved by a broad range of proteases found in a wide range of tissue. Furthermore, syncytia formation caused by the F protein requires the HN protein for the fusion process (36). Expression of a highly fusogenic F protein is able to mediate an antitumor response when transfected into cells (14) or when expressed as a transgene from VSV (37). Although the F cleavage site is a major determinant of virulence in NDV (15), modification of the F protein from the attenuated Hitchner B1 strain of NDV resulted in a virus that only exhibits intermediate virulence for birds (29) based on a mean death time in embryonated eggs assay. Other reports show that not only the F protein cleavage site but also regions of the HN protein play a critical role in virulence (38). This would explain why a highly attenuated lentogenic NDV (NDV/B1) modified to contain only the F protein cleavage site sequence of velogenic viruses does not exhibit highly pathologic properties in embryonated eggs.

Replication of NDV, an avian paramyxovirus, seems to be restricted in other species, including human cells. However tumor cells, which are usually deficient in the antiviral IFN-mediated response, are permissive to NDV replication (2). Our recombinant clone of NDV is capable of replicating in numerous tumor cell lines to high titers ($\sim 10^6$ pfu/mL). This parental strain usually requires the addition of exogenous protease to propagate in most tumor cells, whereas rNDV/F3aa (the NDV/B1 clone with a modified F cleavage sequence) can grow to similar titers in the same cells without addition of exogenous protease. In the present study, we constructed a NDV modified to express the highly fusogenic F protein for the treatment of a model colon carcinoma. Expression of this F protein is sufficient to induce syncytia formation in tumor cells and cause what is referred to as a "bystander effect" (14). This effect recruits nontransfected cells into the growing syncytia and has been reported as an effective means for enhancing oncolysis of tumor cells. Here, we show that tumor-bearing mice in the CT26 metastatic tumor model treated with a single i.v. injection of 10^7 pfu of NDV/F3aa displayed a prolonged survival compared with wild-type NDV/B1. These data are consistent with our results from the s.c. cancer model where we observed an enhanced therapeutic efficacy after four i.t. injections of NDV in mice harboring established s.c. tumors. Moreover, complete regression of these s.c. tumors was observed in 2 of 10 of animals treated with the modified virus, compared with 0 of 10 mice undergoing complete regression with the wild-type NDV/B1. This enhanced therapeutic efficacy is consistent with previous reports from transfected and VSV-infected tumor cells also expressing a highly fusogenic F protein and remains to be tested in other tumor models. Although there is a significant improvement in therapeutic efficacy, we wanted to explore additional means of enhancing the therapeutic potential of NDV by reverse genetics. To this end, we explored the use of NDV/F3aa as a vector for gene therapy.

Formation of syncytia in CT26 cells *in vitro* and an enhanced therapeutic response shown in both s.c. and metastatic models by rNDV/F3aa led us to choose this vector as our backbone to develop further reverse genetic modifications of NDV for an enhanced therapeutic potential. Additional modifications to rNDV/F3aa allow for the possibility of generating an even more effective agent compared with rNDV/B1. This second-generation therapeutic agent was designed to evaluate whether genetically engineered NDV carrying an immunomodulatory gene can be created and, secondly, whether such viruses are more efficacious in tumor therapy. Although gene therapy vectors carrying immunomodulatory genes have been tested in a number of virus vectors, the overall therapeutic effect of the immunomodulatory genes varies, depending on the vector used and the cancer model. In this report, we show that NDV can be used as a gene therapy vector and, secondly, that this virus can be modified to express cytokines for enhanced therapeutic efficacy. Specifically, the expression of IL-2 was able to dramatically improve the therapeutic efficacy of rNDV in our colon cancer model, with a majority of the rNDV/F3aa-IL-2–treated mice undergoing complete and long-lasting tumor regression. Furthermore, these mice develop a potent immunologic memory response protecting them from further CT26 tumor challenge. Whether it is be possible that GM-CSF, TNF-α, are IFN-γ are

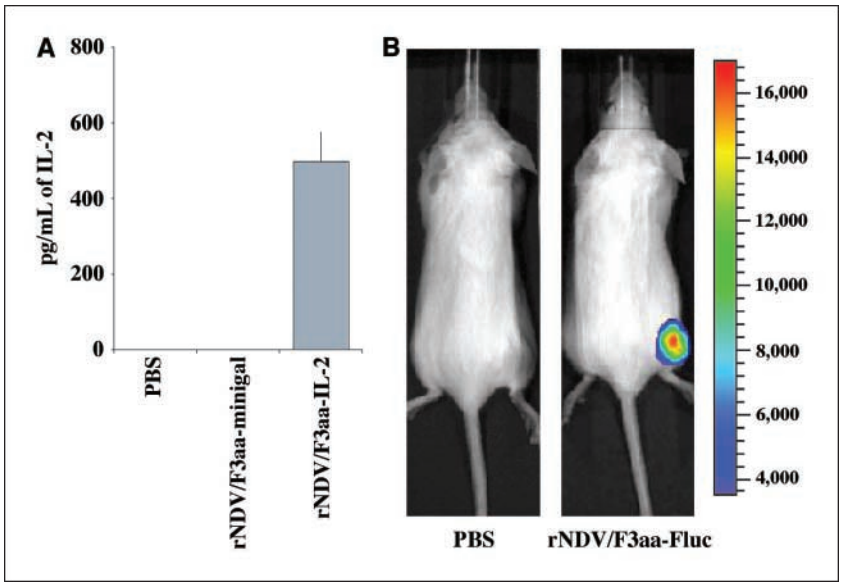

Figure 5. *In vivo* expression of foreign genes by rNDV/F3aa. *A,* tumor homogenates from mice removed on day 5 posttreatment were tested for IL-2 by ELISA. *B,* mice were i.t. injected with 1×10^6 pfu of rNDV/F3aa-Fluc and monitored for luciferase by *in vivo* imaging using the IVIS imaging system. In the representational image, luciferase expression was observed only within the tumors of the rNDV/F3aa-Fluc–treated mice and was not detected in any other locations. Units are in photon counts.

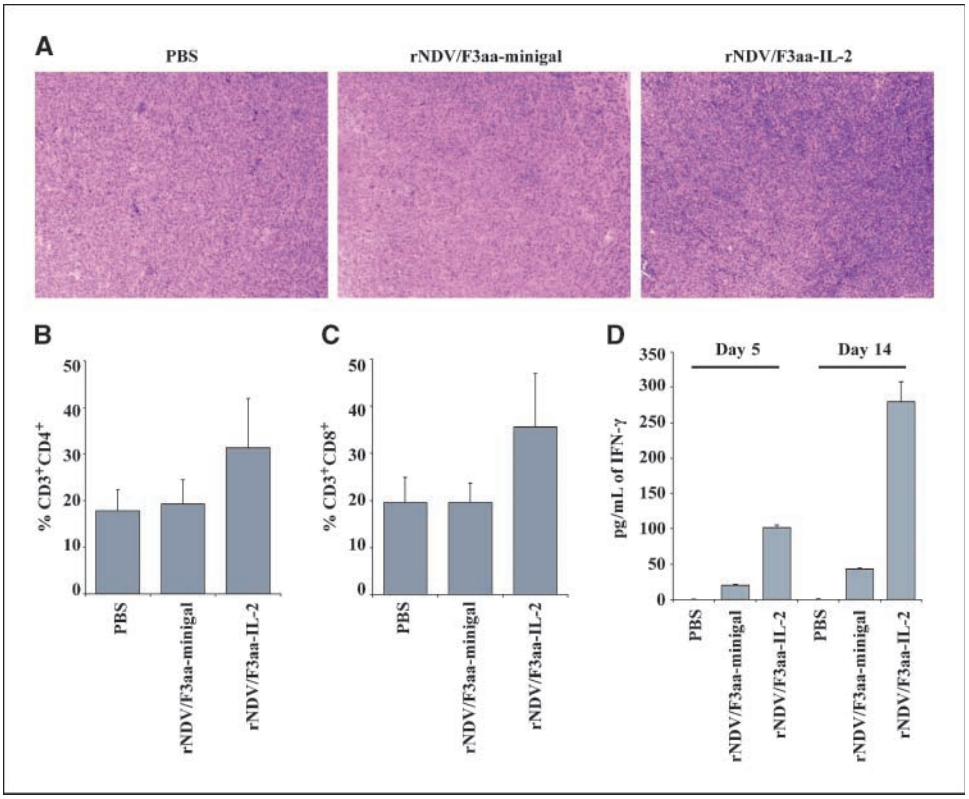

Figure 6. *A,* representative sections of H&E-stained mouse tumors dissected on day 5 after treatment initiation. Intense infiltration was observed in the rNDV/F3aa-IL-2–treated tumors compared with both PBS- and rNDV/F3aa-minigal–treated tumors. *B* and *C,* quantification of $CD4^+$ and $CD8^+$ T cells within the tumors of mice on day 5 posttreatment. Tumors were removed on day 5, dissociated into single-cell suspensions by collagenase treatment, stained with anti-CD3, and costained with either anti-CD4 or anti-CD8 antibody for FACS analysis. Treatment with rNDV/F3aa-IL-2 produced a marked increase in the percentage of $CD4^+$ and $CD8^+$ T cells within the tumors ($n = 6$ mice per group; $P = 0.031$ and $P = 0.010$, between $CD4^+$ and $CD8^+$ cells in tumors from rNDV/F3aa-minigal– and rNDV/F3aa-IL-2–treated mice, respectively). *D,* LN cells were removed from mice on days 5 and 14. LN cells were cocultured with irradiated CT26 cells or plated alone and were measured for IFN-γ production 3 d later.

capable of enhancing the therapeutic efficacy of NDV in other cancer models is yet to be determined.

Although the focus of this work is on using reverse genetics to improve the therapeutic potential of NDV, the dramatically enhanced therapeutic efficacy of NDV expressing IL-2 prompted us to examine the immune response generated from this virus. IL-2 is a well-characterized cytokine with therapeutic anticancer efficacy produced mainly by CD4, but also by CD8, T cells (25). IL-2 is a T-cell growth factor that binds to a tripartite receptor on T cells and exhibits autocrine functions. To this end, we examined the tumors for an increased presence of T cells by FACS analysis of tumor homogenates. Our data show that there is indeed an increase in number of $CD4^+$ and $CD8^+$ T cells within the tumors of mice on day 5 after treatment initiation in tumors treated with rNDV/F3aa-IL-2 but not in control virus– or PBS-treated tumors. Next, we dissected tumors on day 5 posttreatment and tested them for the presence of IL-2. Mice treated with rNDV/F3aa-IL-2 showed the presence of IL-2 within the tumor homogenates by ELISA, whereas IL-2 concentrations from control-infected mouse tumors were undetectable. Local expression of a foreign gene within the tumor of treated mice was confirmed by *in vivo* imaging of rNDV/F3aa-Fluc. By using IVIS imaging of luciferase, we were able to conclude that expression of the gene insert into NDV/F3aa vector was only observed within the tumors of mice and that this expression was detectable within the tumor for 4 days after a single injection of recombinant virus.

Importantly, all of the mice that underwent complete regressions of tumors exhibited a long-lasting protection from tumor challenge regardless of the recombinant virus treatment received, although mice treated with rNDV/F3aa-IL-2 had significantly more mice undergoing complete tumor regression. In addition, LN cells from NDV/F3aa-IL-2–treated mice on days 5 and 14 produced higher levels of IFN-γ only when cocultured with irradiated CT26 cells, compared with rNDV/F3aa-minigal control treated mice. Interestingly, sera taken on day 14 from mice treated with i.t. injections of rNDV/F3aa-IL-2 had higher antibody titers to NDV than the control vector as detected by hemagglutination inhibition assay.[5] Collectively, these facts and the increase in CD4 and CD8 T cells within the tumors of rNDV/F3aa-IL-2–treated mice suggest that both an increased T-cell function and the presence of $CD4^+$ and $CD8^+$ T cells within the tumor mediate an enhanced tumor reduction. Our future plan is to further investigate the precise mechanism of this enhanced therapeutic potential of rNDV/F3aa-IL-2 in CT26 colon carcinoma.

In this report, we have investigated the potential to enhance the inherent therapeutic efficacy of NDV by use of reverse genetics. Clinical therapeutic efficacy using NDV was first noted in 1965 for an advanced cervical cancer patient treated with a direct inoculation of the virus into the tumor (39). Since this initial report, there have been many encouraging reports of cancer therapy treatments using NDV in clinical and preclinical studies with naturally occurring strains of the virus (1). Here, we describe the first genetically engineered NDV designed as an improved cancer therapeutic agent. Specifically, we investigated the potential to engineer the backbone vector of NDV for an enhanced efficacy. In addition, we explored the potential to engineer the virus for use as a gene therapy vector for cancer therapy. The present study focuses on the potential to use reverse genetics to improve the therapeutic efficacy of NDV and allows us to carry out

[5] Unpublished data.

comprehensive mechanistic studies of tumor therapy using genetically engineered NDVs.

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Mount Sinai School of Medicine owns patent positions for reverse genetics of Newcastle disease viruses.

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Use of Reverse Genetics to Enhance the Oncolytic Properties of Newcastle Disease Virus

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