Systemic Oncolytic Herpes Virus Therapy of Poorly Immunogenic Prostate Cancer Metastatic to Lung

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Abstract

Purpose: Our goal was to evaluate whether systemic administration of NV1042, an interleukin-12 (IL-12) – expressing oncolytic herpes simplex virus, and its noncytokine parental vector NV1023 were effective against preexisting metastatic prostate cancer in an immunocompetent mouse model.

Experimental Design: Metastatic TRAMP-C2 lung tumors established in C57Bl/6 or nude mice were treated on day 21 with four i.v. administrations of NV1042 or NV1023 and sacrificed on day 42 to assess virus efficacy and the potential mechanism of efficacy.

Results: NV1042 or NV1023 treatment was similarly effective in eliminating extrapleural and hemorrhagic tumors present in mock-treated mice. However, NV1042 was further effective compared with NV1023 in controlling the growth of lung tumors (as determined by mean surface tumor nodule number, lung weights, and surface tumor burden) and in extending survival. NV1042-treated mice exhibited a transient increase of serum IL-12 1 day posttreatment, whereas IL-12 levels in tumor bearing lungs persisted a further 2 days at least. Only splenocytes from NV1042-treated mice secreted IFN-γ in response to TRAMP-C2 stimulation and displayed natural killer activity. The IL-12-mediated enhancement observed with NV1042 in the syngeneic model was abrogated in athymic mice treated in a similar manner, thus indicating a role for T cells in the augmented efficacy of NV1042 virus.

Conclusions: Systemic administration of the IL-12-expressing NV1042 virus is more effective than its noncytokine parent, NV1023, against preestablished metastatic lung tumors. Given the clinical safety profile of NV1020, the parental vector of NV1023, and NV1042’s enhanced efficacy and ability to activate the host immune system, NV1042 merits clinical consideration for treating metastatic prostate cancers.

Prostate cancer is the most commonly diagnosed cancer in men in the United States, accounting for 33% of all cancers and claiming the life of one in eight men with this diagnosis (1). Early diagnosis facilitated by screening high-risk populations using prostate-specific antigen and imaging techniques have aided in lowering the mortality rates of patients with localized, early-stage cancer. However, about one third will bear regional or metastatic tumors at the time of diagnosis with another 25% progressively developing metastatic disease during the course of the disease (2). Bone is the primary site of metastasis in 85% of cases followed by lymph nodes, lung, liver, and adrenals, in ~45% of cases.

Currently, there are no curative treatment options available for patients with advanced prostate cancer. Although organ-confined cancer responds well to surgery and radiation therapy, with 5-year survival rates for such patients being 89%, the survival rates for patients with metastatic cancer decreases to 31% (2). Hormonal therapy (androgen ablation) has been the primary treatment of choice since the 1960s for patients with advanced disease. Unfortunately, the effectiveness of hormonal therapy is limited by the inevitable development and outgrowth of hormone-resistant cells either within the prostate or in metastatic deposits. Using prostate-specific antigen levels as an indicator of response, hormonal therapy of metastatic cancer has a median duration of response of only a year (3). Chemotherapy has not yet proved to be a viable option in curing or improving survival of patients with advanced prostate cancer and is mostly given as a palliative measure to improve the physical quality of life (4). Thus, there exists a paucity of novel treatment options for advanced forms of prostate cancer.

In evaluating novel therapies for metastatic prostate cancers, the features that are highly desirable include: (a) the ability of the agent to destroy the overt tumor and (b) its ability to activate the host immune system so as to seek and kill both overt and covert tumors. Replication-competent oncolytic herpes simplex viruses (HSV) designed to differentially target
tumor cells while sparing normal tissues seem to fulfill these criteria in that they can destroy a variety of tumors through their direct lytic activity and also trigger a tumor-specific immune response (5-10). Successful phase I clinical trials of three oncolytic HSVs (G207, 1716, and NV1020) attest to their safety in humans (11-13). G207 and 1716 were inoculated i.t. in glioma and melanoma patients, whereas NV1020 was given intrahepatically in metastatic liver cancer patients. G207 is an HSV-1 with deletions of both copies of γ34.5 and a LacZ insertion inactivating the ICP6 gene (14), whereas NV1020 is an HSV-1/HSV-2 hybrid virus with deletion of one copy of γ34.5 and the internal repeat, UL24 and UL56 genes, and addition of gl, g0, US2, and US3 from HSV-2 (15). Preclinical studies with these viruses have also confirmed their safety and efficacy for prostate cancer. G207 inoculated intraprostastically into BALB/c mice and Aotus monkeys showed safety (16). G207 and NV1020 given intraneoplastically or systemically were effective against human prostate cancer xenografts established in nude mice (17, 18).

To examine the contributory role of immune activation to the cumulative antitumoral efficacy of oncolytic HSVs, a competent immune system is essential; therefore, our current studies use immunocompetent syngeneic prostate cancer mouse models. Mouse cells are more resistant to HSV infection and various strains of mice differ in their LD50 to HSV-1. One of the few immunocompetent mouse models available for prostate cancer is TRAMP (19), derived from C57Bl/6 mice, a strain highly resistant to HSV-1 with an LD50 of 1 × 108 plaque-forming units (pfu; ref. 20). These transgenic mice express SV40 T antigen under the control of rat probasin promoter, thus restricting the expression of T antigen to epithelial cells of the prostate. TRAMP-C2 is a clonal prostate cancer cell line established from a spontaneously arising prostate adenocarcinoma in TRAMP mice and expresses very low levels of MHC class I (21, 22). TRAMP-C2 cells are negative for SV40 T antigen expression (21) and form tumors when implanted into C57Bl/6 mice. Therefore, this syngeneic model can be used to evaluate the efficacy of various oncolytic HSVs.

In a direct head-to-head comparison of various first- and second-generation oncolytic HSVs (G207, G47Δ, and NV1023) in their efficacy for prostate cancer, we identified NV1023 to be most effective against TRAMP-C2 prostate tumors established in syngeneic mice (23). G47Δ is a second-generation mutant of G207 with additional deletions of the ICP47 gene and US11 promoter (24). NV023 is a second-generation mutant of NV1020 with an insertion of the LacZ gene and additional deletions of the ICP47 gene and US11 promoter (25). The ICP47 deletions in G47Δ and NV1023 overcomes the down-regulation of MHC class I expression normally observed in HSV-infected cells. We further showed that an interleukin-12 (IL-12)-expressing virus NV1042 was superior to the granulocyte macrophage colony-stimulating factor–expressing virus NV1034 or their noncytokine parental vector NV1023 (23). Importantly, by using two mouse prostate tumor models that varied greatly in their levels of MHC class I expression (2% versus 91%), NV1042 was observed to inhibit the growth of not only an inoculated tumor but also a distant noninoculated tumor (23). Thus, efficacy of NV1042 was shown to be independent of the level of MHC class I expression of the tumors and to be mediated via the immune and antiangiogenic effects of IL-12.

The goal of this study was to evaluate the use of systemically given NV1023 and NV1042 towards the application for advanced prostate cancer. We hypothesized that besides its direct cytocytotoxicity, IL-12 expression from NV1042 would augment the host immune system to seek and destroy metastatic cancers. Therefore, the low MHC class I expressing TRAMP-C2 cells established as metastatic lung tumors were treated with i.v. (tail vein) delivered IL-12 expressing NV1042 virus and its noncytokine parental virus, NV1023. The results show that the NV1042 virus was significantly more effective than NV1023 in controlling the growth of established lung tumors. The efficacy of NV1042 was mediated by its ability to activate and direct immune cells to seek out tumors, which potentially consists of natural killer (NK) and T cells.

### Materials and Methods

**Cells.** TRAMP-C2 cells were kindly provided by Dr. Norman Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA). The cells were cultured in DMEM with glucose (4.5 g/L; Mediatech, Inc., Herndon, VA) supplemented with 5% inactivated FCS (HyClone Laboratories, Logan, UT), 5% Nu-Serum IV (Becton Dickinson, Bedford, MA), 10−6 mol/L dihydrotestosterone (Sigma-Aldrich, St Louis, MO), 5 μg/mL insulin (Sigma-Aldrich), and 25 μg/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA). Vero (African green monkey kidney) cells (American Type Culture Collection, Manassas, VA) and B16-F10 melanoma cells (American Type Culture Collection) were cultured in DMEM with glucose (4.5 g/L) supplemented with 10% calf serum. YAC-1 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 with 2 mM/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM/L HEPES, 1.0 mM/L sodium pyruvate, and 10% fetal bovine serum.

**Viruses.** Purified virus stocks of NV1023 and NV1042 were obtained from MediGene, Inc. (San Diego, CA). Construction of NV1023 and NV1042 has been described previously (25). The viruses were individually titered on Vero (African green monkey kidney) cells by plaque assay.

**Mice.** Six-week-old male C57Bl/6 mice or athymic nude mice were purchased from Harlan (Indianapolis, IN) or National Cancer Institute (Frederick, MD), respectively. Animal procedures were conducted with approval from the Massachusetts General Hospital Subcommittee on Research Animal Care. All animal studies were blinded.

**Metastatic lung tumor treatment.** TRAMP-C2 lung tumors were established as previously described (26). TRAMP-C2 cells (5 × 106) were implanted i.v. via tail vein into male C57Bl/6 mice (syngeneic model) or into male nude mice. Preliminary studies showed that multiple microscopic tumor nodules and up to 16 surface tumor nodules were present in the lungs of mice by day 21, and that by day 49, most of the mice became severely morbid. Therefore, groups of 8 to 10 mice were treated i.v. (tail vein) with 2 × 107 pfu of NV1023, NV1042, or virus buffer (mock), on days 21, 24, 28, and 31. Treated mice were sacrificed on day 42. The lungs were excised, weighed, and stained with India ink to count surface tumor nodules, which appeared as white spots against the black background. The lungs were photographed, and the diameter of each nodule in the syngeneic model was measured using a vernier caliper.

**Survival studies in the syngeneic (C57Bl/6) mouse model.** Metastatic lung tumors were established and treated as described above in C57Bl/6 mice. Because our institutional animal protocol is not approved to use death as an end point, we chose to assess quality of life (moribidity) as the survival criteria. Mice were monitored for morbidity and sacrificed when observed to have a rough hair coat, hunched posture, lethargy, limited ambulatory movements in response to stimuli, or recumbent posture.

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Cancer Therapy: Preclinical
Serum and lung IL-12 ELISA. C57Bl/6 mice harboring TRAMP-C2 lung tumors were treated with $2 \times 10^7$ pfu of NV1023, NV1042, or virus buffer (mock), on days 21, 24, 28, and 31. Three mice from each treatment group were sacrificed on days 32, 34, and 38. Blood was obtained by cardiac puncture and allowed to clot for 2 hours at room temperature and then centrifuged at 16,000 $\times$ g for 15 minutes to collect serum. Lungs were excised and resuspended in 2 volumes of cold PBS and then homogenized thoroughly to obtain cellular lysate. IL-12 was assayed from sera and lung lysates by ELISA, using Quantikine M kits (R&D Systems, Minneapolis, MN).

Immune activation studies. C57Bl/6 mice harboring TRAMP-C2 lung tumors were treated with $2 \times 10^7$ pfu of NV1023, NV1042, or virus buffer (mock), on days 21, 24, 28, and 31. Splenocytes isolated from three mice from each treatment group on days 32, 34, and 38 were pooled and assayed for immune function.

IFN-γ release assay. TRAMP-C2 or B16-F10 cells were cultured in the presence of recombinant murine IFN-γ (Peprotech, Inc., Rocky Hill, NJ) at 100 µg/mL for 72 hours to up-regulate MHC class I expression. Single-cell suspensions of MHC class I induced TRAMP-C2 cells or B16-F10 cells were then incubated with 50 µg/mL of mitomycin C (Sigma-Aldrich) at 37°C for 20 minutes to induce cell cycle arrest. Splenocytes (1 $\times$ 10⁷) were stimulated in vitro with 1 $\times$ 10⁴ mitomycin C–treated TRAMP-C2 cells in the presence of 50 µg/mL IL-2 (Peprotech). Supernatant was collected 48 hours later and assayed for IFN-γ by ELISA using Quantikine M kits (R&D Systems).

NK cell assay. Single-cell suspensions of YAC-1 cells were labeled with 100 µCi of $^{35}$Cr for 1 hour at 37°C. After thorough washing, the cells were plated at 1 $\times$ 10⁵ per well of a 96-well plate, and 5 $\times$ 10⁶ splenocytes were added to a final effector/target ratio of 50:1. Plates were incubated at 37°C for 4 hours, and the supernatant was collected and counted using a Packard Cobra Gamma counter.

Virus biodistribution studies. TRAMP-C2 lung tumor-bearing C57Bl/6 mice were treated with $2 \times 10^7$ pfu of NV1042 on days 21, 24, 28, and 31. Three mice were sacrificed on days 32, 34, and 38 and perfused with Zamboni's fixative (1.8% paraformaldehyde, 7.5% picric acid, 31. Three mice were sacrificed on days 32, 34, and 38. Blood was obtained by cardiac puncture and allowed to clot for 2 hours at room temperature and then centrifuged at 16,000 $\times$ g for 15 minutes to collect serum. Lungs were excised and resuspended in 2 volumes of cold PBS and then homogenized thoroughly to obtain cellular lysate. IL-12 was assayed from sera and lung lysates by ELISA, using Quantikine M kits (R&D Systems). Therefore, in this study, we evaluated whether NV1042 given systemically would be effective against advanced prostate cancer. As a direct comparison for this cytokine-expressing virus, we used its parental noncytokine NV1023 virus as a control. Because human prostate cancer is known to metastasize to the lungs, we established a metastatic lung tumor model with the poorly immunogenic TRAMP-C2 cells as shown in Fig. 1A. TRAMP-C2 cells (5 $\times$ 10⁴) were inoculated by tail vein into male C57Bl/6 mice. Preliminary analysis showed multiple microscopic and surface tumor nodules in the lung by 21 days (Fig. 1B), at which time the mice were treated with $2 \times 10^7$ pfu of NV1042, NV1023, or virus buffer (mock) by i.v. administration (tail vein) on days 21, 24, 28, and 31. Mice were sacrificed on day 42, and lungs were excised, weighed, and stained with India ink to count surface tumor nodules. The diameter of the surface tumor nodules was measured using a vernier caliper. A photomicrograph of the lungs from each of the treated groups is illustrated in Fig. 2A, with surface tumor nodules seen as white masses. There were two clear visual differences between the virus- and mock-treated lungs: (a) the mock-treated mice had extrapleural tumors that were attached to the rib cage, as shown in Fig. 2A; and (b) the mock-treated mice had large tumor filled hemorrhagic lobes (seen as red in Fig. 2A) with some mice having lobes that were completely hemorrhagic. The volume of these hemorrhagic nodules varied between 144 and 540 mm³ for a total volume of 2,607 mm³ in the mock group.

A comparison of the distribution of lung weight from these mice (Fig. 2B) show that the NV1042-treated mice had an average lung weight of 0.43 ± 0.01 g, resembling that of a normal nontumorigenic lung (~0.44 g), and was significantly different from either the mock-treated mice with 0.92 ± 0.07 g (P < 0.0001, Student’s t test) or NV1023-treated mice with 0.73 ± 0.08 g (P = 0.0026, Student’s t test). NV1042-treated mice had a significant reduction in lung weight compared to mock-treated mice, suggesting a possible therapeutic effect.

**Results**

Efficacy of systemic oncolytic virus therapy on preestablished metastatic lung tumors. Through prior studies, we had identified NV1042 (IL-12) to be the most effective virus against mouse prostate tumors when given intraneoplastically (23). Therefore, in this study, we evaluated whether NV1042 given systemically would be effective against advanced prostate cancer. As a direct comparison for this cytokine-expressing virus, we used its parental noncytokine NV1023 virus as a control. Because human prostate cancer is known to metastasize to the lungs, we established a metastatic lung tumor model with the poorly immunogenic TRAMP-C2 cells as shown in Fig. 1A. TRAMP-C2 cells (5 $\times$ 10⁴) were inoculated by tail vein into male C57Bl/6 mice. Preliminary analysis showed multiple microscopic and surface tumor nodules in the lung by 21 days (Fig. 1B), at which time the mice were treated with $2 \times 10^7$ pfu of NV1042, NV1023, or virus buffer (mock) by i.v. administration (tail vein) on days 21, 24, 28, and 31. Mice were sacrificed on day 42. A comparison of the distribution of lung weight from these mice (Fig. 2B) show that the NV1042-treated mice had an average lung weight of 0.43 ± 0.01 g, resembling that of a normal nontumorigenic lung (~0.44 g), and was significantly different from either the mock-treated mice with 0.92 ± 0.07 g (P < 0.0001, Student’s t test) or NV1023-treated mice with 0.73 ± 0.08 g (P = 0.0026, Student’s t test). NV1042-treated mice had a significant reduction in lung weight compared to mock-treated mice, suggesting a possible therapeutic effect.

**Fig. 1.** i.v. treatment of established metastatic lung tumors with oncolytic HSVs. A, scheme of TRAMP-C2 metastatic lung tumor treatment: 5 $\times$ 10⁷ TRAMP-C2 cells were implanted via tail vein into male C57Bl/6 or nude mice and treated with 2 $\times$ 10⁷ pfu of NV1023, NV1042, or virus buffer (mock) on days 21, 24, 28, and 31. Mice were sacrificed on day 42. B, H&E staining of lung harboring a TRAMP-C2 tumor nodule at the initiation of virus treatment on day 21.
mice also had a significantly lower number of tumor nodules on the surface, with 11 ± 2.26 compared with either 21 ± 3.98 for NV1023-treated mice (P = 0.036, Student’s t test) or 32 ± 2.24 for mock-treated mice (P < 0.0001, Student’s t test) as shown in Fig. 2B (top line). It should be noted that the tumor nodule number in the mock-treated mice is highly conservative, as each of the hemorrhagic lobe tumors were counted as a single nodule because the hemorrhagic tumors had coalesced into one another, and it was difficult to count them separately. Because the tumor nodules also varied in size, the diameter of each surface nodule was measured and used to estimate the mean surface tumor burden in these mice (data not shown).

NV1042 virus–treated mice had a significantly lower mean surface tumor burden per mouse of 20.9 ± 4.3 mm compared with mock-treated mice with 69.5 ± 6.1 mm (P < 0.0001, Student’s t test) or NV1023-treated mice with 46.25 ± 10.7 mm (P = 0.0365, Student’s t test). A significant reduction in tumor nodule number was also observed in NV1023-treated mice when compared with mock-treated mice (P = 0.03, Student’s t test) but not when mean surface tumor burden or lung weights were compared.

The efficacy of tumor growth inhibition with NV1042 virus translated also into significant prolongation of survival of these mice. Both NV1023 and NV1042 treatment extended the median survival of mock mice from 35 to 42 days (P = 0.02, log-rank test) and 60 days (P < 0.0001, log-rank test), respectively. NV1042 also significantly prolonged survival of treated mice compared with NV1023 (P = 0.0007, log-rank test).
mice, as shown in Fig. 2C. The median survival of NV1042-treated mice was 60 days compared with 35 days for mock (P < 0.0001, log-rank test) and 42 days for NV1023 (P = 0.0007, log-rank test). Interestingly, in agreement with the significant reduction in tumor nodule number observed in NV1023-treated mice, a significant extension in their survival was also observed when compared with mock-treated mice (P = 0.02, log-rank test).

### Biodistribution and virus activity in the metastatic lung tumor model

TRAMP-C2 lung tumor nodules established in C57Bl/6 mice were treated on days 21, 24, 28, and 31 with 2 × 10^7 pfu of NV1042 by i.v. administration (tail vein), and three mice from each group were sacrificed on days 32, 34, and 38. Various tissues (lung, liver, spleen, and brain) were collected to assess biodistribution of the virus following i.v. delivery. From a second set of three mice treated identically with NV1042, NV1023, or virus buffer, blood, lungs, and splenocytes were harvested to evaluate serum and lung IL-12 levels and immune activity.

#### Biodistribution of virus

Because the virus was delivered i.v. via tail vein, it was important to determine the distribution of the virus in these mice. NV1042 has an insertion of the *Escherichia coli* LacZ gene, the expression of which can be detected by 5-bromo-4-chloro-3-indolyl-D-galactopyranoside staining. Therefore, various tissues that may potentially harbor the virus were analyzed for LacZ staining. Table 1 lists the results of LacZ staining and show that on day 32 (1 day after all four treatments), the lungs, liver, and spleen had positive staining, with the most staining observed in the liver section (2+). By day 34 (2 days after the treatments), expression in the lung increased from 1+ to 2+, where as expression in liver and spleen decreased from 2+ to 1+ and 1+ to negative, respectively. By day 38, none of the sections from any of the organs examined had any positive staining. It is important to note here that following i.v. delivery of NV1042, LacZ staining was not detected in the brain on any of the days tested. Although it is known that liver and spleen might harbor HSV, it is noteworthy that LacZ expression in these organs quickly subsides, whereas in the tumor-bearing lungs, it increases over several days. All of the organs examined maintained their normal structure, as noted by H&E staining (data not shown).

#### Serum and lung IL-12

To assess the levels of IL-12 in the blood and lungs of mice treated by i.v. inoculation with NV1023, NV1042, or virus buffer, ELISAs were conducted. Results as shown in Fig. 3A demonstrate that IL-12 levels increased in the serum of NV1042-treated mice on day 32 but not on days 34 or 38. Mock- and NV1023-treated mice did not show any increase in serum IL-12 levels on any of the days tested. On the other hand, measurement of IL-12 levels in the lungs of mice showed a substantial increase in the NV1042-treated mice from day 32 until day 34, which then tapered off by day 38 to basal levels, similar to that observed in mock-treated or NV1023-treated mice (Fig. 3B). Thus, whereas there was a transient increase of IL-12 in the serum of NV1042-treated mice, the levels in the tumor-bearing lungs continued to persist at least until day 34.

#### Immune cell activation

IL-12 functions in the effector phase of the immune response and activates various immune populations to secrete IFN-γ, which therefore can be used as an indicator of immune cell activation in the treated mice. Pooled splenocytes isolated from NV1042, NV1023, or mock mice on days 32, 34, and 38 were stimulated *in vitro* with mitomycin-treated TRAMP-C2 cells and the supernatant assayed for IFN-γ. Figure 4A shows that only splenocytes from

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**Table 1. NV1042 virus biodistribution as determined by LacZ expression**

<table>
<thead>
<tr>
<th></th>
<th>Day 32</th>
<th>Day 34</th>
<th>Day 38</th>
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<tbody>
<tr>
<td>Lung</td>
<td>+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
<td>++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Brain</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
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**NOTE:** –, no staining in the entire section; +, one to five spots in a section under <10 magnification; ++, 5 to 10 spots in a section under <10 magnification.
highest levels of lysis was observed immediately following viral treatments on day 32, and it gradually dropped by day 38 to that observed for mock- or NV1042-treated mice. Thus, these data suggest that at least one of the populations activated by IL-12 expressed from NV1042 virus includes NK cells.

**Efficacy of systemic oncolytic HSVs for metastatic lung tumors in nude mice.** To ascertain the role of T cells in the inhibition of metastatic tumor growth observed in NV1042-treated mice, we established TRAMP-C2 lung tumors in nude mice and treated groups of mice each with NV1042, NV1023, or mock. Mice were sacrificed on day 42, and the weight of lungs and tumor nodule number were determined. Results show that the mean lung weight of both NV1023- and NV1042-treated mice were similar with each weighing $0.37 \pm 0.01$ g, respectively (Table 2), and were significantly different from mock with a mean weight of $0.58 \pm 0.01$ g ($P < 0.0001$ versus NV1023 or NV1042, Student’s t test). When the mean lung tumor nodule numbers were compared between the groups, the mock mice harbored $16.5 \pm 1.02$, whereas NV1023- and NV1042-treated mice harbored $5.5 \pm 0.6$ and $4.7 \pm 0.53$, respectively (Table 2). Both NV1023 and NV1042 were equally effective in reducing the tumor nodules ($P < 0.0001$ versus mock, Student’s t test). Thus, in the nude mice, NV1042 was significantly effective in inhibiting the growth of TRAMP-C2 lung tumors compared with mock but not when compared with NV1023.

**Discussion**

Prostate cancer is expected to claim the life of ~30,000 men in the United States in 2005 (1). The majority of these deaths will be due to the metastatic nature of prostate cancer, for which the only available treatment of androgen ablation is a temporary option. Hormone-refractory prostate cancer is an inevitable outcome following androgen ablation, which is due to the heterogeneous nature of metastatic prostate cancer. A gene expression analysis study showed that metastatic hormone-refractory prostate cancers are heterogeneous in their morphology, immunophenotype, and genotype, even within an individual patient (27). These tumor cells, therefore, tend to be more aggressive in nature and more resistant to standard therapy than the original localized tumor, highlighting the importance of developing alternate methods of treatment that are less subject to tumor heterogeneity.

Oncolytic HSV vectors act through their ability to kill the cells they infect and replicate in and have been designed to differentially target tumor cells while sparing normal cells (5, 6). Because of the widespread occurrence of HSV receptors

![Graph A](image1.png)

**Fig. 4.** Immune cell activation in NV1042 (IL-12) – treated metastatic lung tumors. A, IFN-γ secretion: splenocytes obtained from NV1023-, NV1042-, or mock-treated mice on the days indicated were stimulated in vitro with TRAMP-C2 cells and IFN-γ secreted into the supernatant were assayed by ELISA. Columns, mean of triplicates; bars, SD. Splenocytes from only NV1042-treated mice secreted increased amounts of IFN-γ on days 32 and 34 ($P < 0.005$, Student’s t test versus NV1023 or mock). B, NK cell activity: YAC-1 cell lysis by splenocytes obtained from NV1023-, NV1042-, or mock-treated mice on the indicated days. Columns, mean of triplicates; bars, SD. Increased lysis was observed with splenocytes from NV1042-treated mice on days 32 and 34 ($P < 0.005$, Student’s t test) compared with NV1023- or mock-treated mice.

![Graph B](image2.png)

Table 2. Efficacy of systemic oncolytic HSV treatment of metastatic TRAMP-C2 lung tumors in nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor nodules no.</th>
<th>Lung weight (g)</th>
</tr>
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<tbody>
<tr>
<td>Mock ($n = 8$)</td>
<td>$16.5 \pm 1.02$</td>
<td>$0.58 \pm 0.01$</td>
</tr>
<tr>
<td>NV1023 ($n = 9$)</td>
<td>$5.3 \pm 0.6^*$</td>
<td>$0.39 \pm 0.01^*$</td>
</tr>
<tr>
<td>NV1042 ($n = 9$)</td>
<td>$4.7 \pm 0.53^*$</td>
<td>$0.37 \pm 0.01^*$</td>
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$^*P < 0.0001$ vs mock, Student’s t test.

the NV1042-treated mice exhibited increased levels of IFN-γ release in response to TRAMP-C2 cell stimulation and not from NV1023-treated or mock mice. In the NV1042-treated mice, IFN-γ secretion peaked at day 32, which then tapered off to levels similar to mock- or NV1023-treated mice by day 38. Splenocytes from NV1042-treated mice obtained on day 32 were also stimulated in vitro with mitomycin-treated B16-F10 cells, and the levels of IFN-γ secreted were similar to that observed with mock mice (data not shown).

**NK cell activation.** To determine which immune cells are being activated by IL-12 in the NV1042-treated mice, splenocytes obtained on days 32, 34, and 38 from various treatment groups were also tested in a YAC-1 assay. Lysis of YAC-1 cells is a reflection of NK cell activity. The results show increased lysis of YAC-1 cells by splenocytes from NV1042-treated mice compared with mock- or NV1023-treated mice (Fig. 4B). Lysis by splenocytes from NV1042-treated mice was time dependent in that the
on many types of cells, oncolytic HSVs are not limited by the
heterogeneity observed in various tumors or within an
individual tumor. Thus, these oncolytic viruses represent a
viable option for the treatment of advanced prostate cancer.
Furthermore, oncolytic HSVs have been shown to activate the
immune system so as to elicit a tumor specific immune
response (7–10). This is a highly beneficial feature to treat
advanced prostate cancer because of its prolivity to metastasize
to various sites, including bone, lymph nodes, and lung.
However, there have been no studies conducted to date to
evaluate the efficacy of oncolytic HSVs for metastatic prostate
cancer.

To assess the efficacy of oncolytic HSVs for prostate cancer in
immunocompetent animals, we are using TRAMP-C2 cells
established from the transgenic TRAMP mouse, which develops
prostate cancer spontaneously. Because mouse cells are not very
susceptible to HSV infection, our initial studies focused on
identifying the most effective oncolytic HSV in immunocom-
petent mouse models. We showed that IL-12-expressing NV1042
was the most efficacious virus against s.c. mouse prostate
tumors, independent of the levels of MHC class I expression in
tumors cells, and that both inoculated and distal s.c. tumor growth was inhibited (23). The advantages of using
NV1042 for prostate cancer treatment are many: (a) IL-12 is an
effector cytokine acting as a cross-bridge between both innate
and adaptive immune responses (28); therefore, the concom-
itant expression of IL-12 at the site of tumor destruction leads
to an enhancement of antitumoral immune effects. (b) IL-12
activates various immune cells to secrete IFN-γ, which is
capable of up regulating MHC class I molecules on the surface
of cells, including prostate and other tumor cells (29), many of
which down-regulate these molecules to evade the host
immune system. Thus, prostate cancers that are generally
poorly immunogenic can be potentially transformed to a more
immunogenic phenotype, allowing them to be detected by
the immune system through the indirect action of IL-12. (c) IL-12
also has antiangiogenic activity (30), thereby directly cutting off
the blood supply to tumors.

Although bone is the most common site of metastasis in
human prostate cancer, evaluation of new therapies for bone
metastasis is hampered by the lack of appropriate models. It has
been reported that in experimental models of prostate cancer
using smaller mammals, including mice, metastasis to the bone
is rare (31). Therefore, in an effort to evaluate the efficacy of
NV1042 in a more realistic setting, and because prostate cancer
is known to metastasize to the lungs in humans, we used a lung
metastatic model with TRAMP-C2 prostate cancer cells estab-
lished in syngeneic C57Bl/6 mice (26). Mice were treated i.v. at
21 days after tumor cell inoculation, when the lungs harbored
well-established TRAMP-C2 tumors. At the time of sacrifice
(day 42), mock mice exhibited significantly enlarged lungs and
had several extrapleural tumors and hemorrhagic lobes. These
mice become severely morbid within the following week. Thus,
this highly aggressive metastatic tumor represents a realistic
model to evaluate oncolytic viral therapy. Both NV1042 and its
noncytotoxic parental vector NV1023 were effective in comp-
pletely eliminating the extrapleural and hemorrhagic tumors
observed in mock mice. However, NV1042 was more effic-
cacious than NV1023 in reducing the number and size of tumor
nodules, which was also reflected in lung weight. The augmented efficacy observed with NV1042 when compared
with NV1023 was correspondingly reflected in a significant
prolongation of survival in the NV1042-treated mice. Interest-
ingly, whereas NV1023 treatment reduced the total number of
surface tumor nodules and extended survival, it was not
significantly different from mock when mean surface tumor
burden or lung weights were compared.

I.v. given NV1042 for the treatment of metastatic lung
tumors of squamous cell carcinoma has been previously
reported (32); however, in that study, treatment was initiated
1 day after tumor cell inoculation, at which time it is unlikely
that tumor nodules were established. Here, we have studied
animals with established tumor nodules, which better reflects
the clinical situation, where there is an initial increase in
prostate-specific antigen but still small or radiologically non-
detectable metastases.

In this study with established lung tumors, viruses were given
four times over a 10-day period without any observable toxicity
in the animals, although serum IL-12 levels on day 32 (1 day
posttreatment) were significantly higher in NV1042-treated
mice than in the NV1023-treated mice. This transient increase
of IL-12 in the serum could be due to the presence of virus in
the liver and spleen in addition to tumorous lung on that day.
Although the liver continued to harbor virus on day 34 (2 days
posttreatment) as shown by LacZ staining, serum (systemic)
IL-12 levels decreased to basal levels similar to that observed
with NV1023-treated mice. The lack of viral gene expression in
the liver after day 34 is consistent with the normal histologic
structure maintained by the liver on all days tested. In contrast
to levels in the serum, IL-12 levels in the tumor harboring lungs
persisted further 2 days, which correlated with the increased
levels of LacZ staining on day 34. Thus, lack of any toxicity in
the mice and the detection of virus and IL-12 in tumor-bearing
lungs suggest that the enhanced efficacy of NV1042 virus over
NV1023 is attributable to the local expression of IL-12 within
the lung tumors.

Local expression of the cytokine in the vicinity of tumors is
important due to the toxicity often observed with systemic
treatment using recombinant IL-12 in various clinical trials
(33–35). Furthermore, because of the inherently poor immu-
nogenicity of metastatic cancers, including prostate
cancers, administration of these cytokines in the absence of
sufficient tumor antigens may not bring about the desired
results. In this regard, cytokine expressing oncolytic viruses
provide an additional yet vital benefit with their combination
of cytolytic activity and cytokine expression within the vicinity
of tumors.

IL-12 is a cytokine that is involved in functional activation of
various immune populations, including NK and T cells. A
consequence of this activation is the induction and secretion of
IFN-γ from these cells. Activation of the various immune cells
occurs within spleens and lymph nodes; therefore, evaluation of
splenocytes for immune activity would be indicative of the
immune effect. The results showed that only splenocytes from
NV1042-treated but not NV1023-treated mice secreted IFN-γ in
response to specific stimulation with TRAMP-C2 cells in vitro.
The activated immune cells within the splenocyte population
progressively declined with time, to basal levels by day 38,
which correlated with the level of IL-12 expression observed in
the lungs. Among the cell types present in the splenocyte
population were NK cells, as reflected by the increased lysis of
YAC cells. Spleen cells from NV1023-treated mice had basal
levels of lytic activity, similar to mock-treated mice. This might suggest that the tumor growth inhibition observed after NV1023 treatment is a result of direct cytolitic action and not any immune effect.

In an effort to test whether splenocytes from NV1042-treated mice also contain CTLs, we attempted numerous in vitro CTL assays under various conditions, including the addition of IL-2 to amplify the CTL population and MHC class I up regulation by in vitro treatment with IFN-γ, without success. Reasons for the lack of in vitro CTL activity are unclear but could include: (a) no TRAMP-C2–specific CTLs were generated in this model system. A recent study reported that in addition of IL-2 to amplify the CTL population and MHC class I up regulation by anti–class I MHC antibody, the CTL assay was again unsuccessful. (b) At the time at which CTL activity was evaluated (day 17), sufficient CTLs may not have been generated in vivo to be detected by this assay. Although in most systems, CTLs are activated within 14 to 21 days after antigenic stimulation, it is possible that in this model the strength of the antigenic stimulus was weak and/or required longer periods of stimulation.

Despite the lack of a direct confirmation for the presence of CTLs in the TRAMP-C2 syngeneic model, the role of T cells in NV1042-mediated inhibition of lung tumors were indirectly ascertained in nude mice. Both NV1023 and NV1042 were equally and significantly effective against TRAMP-C2 lung tumors in the nude mice when compared with mock, without additional enhancement by NV1042, as was observed in the syngeneic model. Thus, the tumor growth inhibition observed with both viruses in nude mice was likely attributable to their oncolytic activity and potential NK, macrophage, or B cell activity, with IL-12 expression from NV1042 virus providing no enhanced benefit.

A concern with systemic/i.v. delivery of oncolytic HSV is the presence of anti-HSV antibodies in seropositive individuals, which could neutralize the virus. Although this study did not examine efficacy in immunized mice, it has been previously reported with NV1020 that giving multiple doses of the virus and in close proximity to the tumor (such as hepatic artery for hepatic metastatic cancer) can overcome the effects of preexisting immunity (37). In this study, administration of four doses of 2 × 10^7 pfu of NV1042 or NV1023 via tail vein had substantial effects in treating metastatic prostate cancer in lungs.

In summary, we have shown that an IL-12 expressing oncolytic HSV (NV1042) given i.v. is more effective than a noncytokine expressing vector (NV1023) in a mouse model of lung metastasis established from poorly immunogenic prostate cancer cells. It is worth noting that although in this study, NV1042 virus was tested in a C57Bl6 strain – derived tumor, we have previously shown that this virus was also more effective than NV1023 in FVB/N-derived prostate cancers (23), while another report has shown enhanced efficacy of an IL-12-expressing virus in an A/J-derived neuroblastoma (38). Thus, IL-12-expressing oncolytic herpes viruses have consistently exhibited superior antitumor effects in a wide range of tumor models and inbred mice strains, thereby making them highly valuable agents for cancer treatment.

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References


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