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Replication competent oncolytic herpes simplex viruses (HSV) exert a dual effect by their direct cytocidal action and by eliciting tumor specific immunity. These viruses can also deliver immunoregulatory molecules to tumors so as to enhance the cumulative antitumor response. This is particularly desirable for prostate cancers, which are usually poorly immunogenic. In order to generate a cytokine expressing oncolytic virus with enhanced cytotoxicity against prostate cancers and which can stimulate the immune system so as to overcome the immunological complacency observed in prostate cancer patients, we are genetically engineering PC-IL12 virus using BAC technology from the PC virus, which is an HSV 1 strain selected by screening for enhanced cytotoxicity against prostate cancer cells. The construction is progressing successfully. Additionally, we have described in this report that an IL-12 expressing oncolytic virus (NV1042) is superior to a GM-CSF expressing virus or a non-cytokine virus in its efficacy against both MHC class I positive (Pr14-2) and negative (TRAMP-C2) mouse prostate tumors. The NV1042 treated tumors exhibited increased immune cell infiltration and decreased levels of angiogenesis. Thus, an IL-12 expressing oncolytic herpes virus is an effective therapeutic vector against prostate cancers irrespective of their MHC class I status.

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Immunologic Approaches for Oncolytic Viral Therapy of Prostate Cancer

INTRODUCTION

The heterogeneity observed within most cancers, including prostate cancer, dictates a multimodal approach towards the treatment of these cancers. However, none of the current standard modalities of treatment for prostate cancer, including surgery, chemotherapy or androgen ablation therapy, has the potential to specifically activate the immune system. Thus, new multi-prong approaches which can eliminate overt tumors via oncolysis as well as induce tumor-targeted immune responses in the host to search and kill occult tumors is worthy of exploration. The strategy of coupling a direct cytotoxic effect by an oncolytic virus with an immune therapy approach for the treatment of cancers is enhanced via the use of viruses, which by virtue of infection of tumor cells may act as an adjuvant in sending a 'danger signal' to the host's immune system. However, many tumors, including prostate cancers, have developed molecular means to evade the immune system. Specific molecular adaptations exhibited by prostate cancers encompass escape from immune surveillance at all phases of recognition: at the level of innate immunity by NK cells and at the initiation and effector phases of adaptive immune response by DCs and T cells. Thus, strategies employed by prostate cancers include shedding of MHC class I chain-related molecules which results in deficient NK cell surveillance, inhibition of dendritic cell differentiation by PSA, down regulation of cell surface MHC class I molecules leading to escape from T cell recognition, deficiency in antigen processing, and secretion of immunosuppressive factors such as TGF-β. Most prostate cancers are thus either poorly or only moderately immunogenic. Nonetheless, we suggest that an intense oncolytic action mediated by a therapeutic viral vector could overcome this immunological complacency.

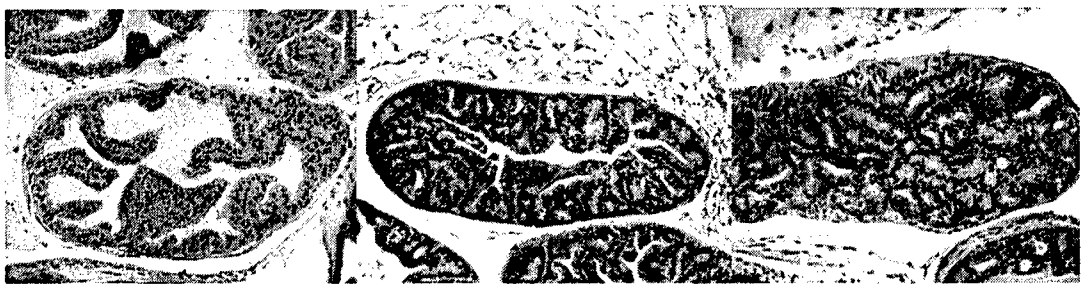
BODY

AIM 1. We hypothesize that efficacy of HSV therapy for prostate cancer can be enhanced by generating HSV vectors with improved replication and/or spread in prostate cancer cells.

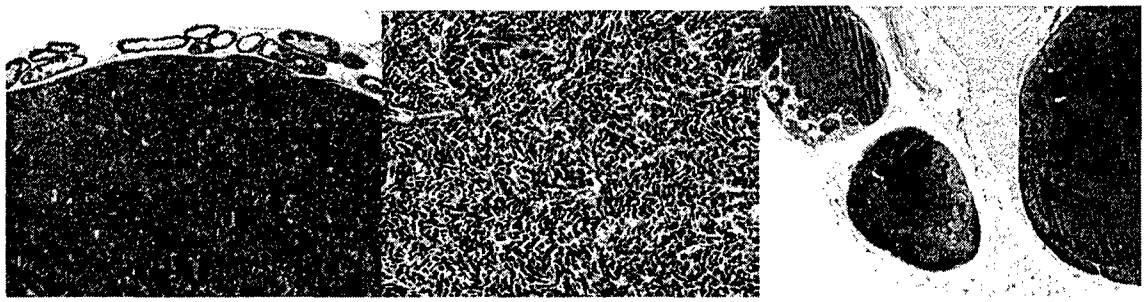
We initially tested multiple clinical isolates and laboratory strains for replication ability on prostate cancer cells and other cancer cell lines and determined that one lab strain (which we term PC) was consistently the best and was better than strain F, the wild type HSV-1 that our original G207 was derived. DNA from this strain was purified using standard techniques. In order to check the quality of purified viral DNA, the sample was subjected to restriction

analysis with Hind III. The sizes of restriction fragments obtained were identical to those calculated from the wild-type HSV genomic sequence in Genbank database. pSureBAC-ICP6 was used for the insertion of the BAC backbone sequence into the ICP6 locus of wild-type PC virus through homologous recombination. Recombinant ICP6$^-$ PC virus was generated by co-transfection of purified PC DNA with linearized recombination plasmid to Vero cells using LipofectaminePLUS (Invitrogen). The viral particles were released by three freeze/thaw cycles and sonication and titered on Vero cells. GFP-positive plaques were identified under the fluorescence microscope. Several GFP-positive recombinant virus isolates have been obtained and purified by limiting dilution. Several independent isolates of recombinant PC virus have now been isolated and were analyzed by viral replication assay, viral DNA restriction analysis and Southern blots. By these analyses, one recombinant clone, which grows best and contains confirmed BAC backbone insertion into the ICP6 locus, has been selected. The BAC clone was obtained by harvesting circular form of recombinant PC DNA from infected cells by Hirt extraction and by introducing the Hirt extract to E.coli by electroporation. This ICP6$^-$ PC BAC construct will then be available to develop for further constructs as described in the grant application.

As noted in the Grant proposal, we will test the recombinant PC viruses in the spontaneously developing prostate cancer mice model, TRAMP. During the submission of the Grant, we had indicated that the TRAMP mice we had established on C57Bl/6 strain background did not display the progression of prostate cancer as reported in the literature and therefore we were breeding TRAMP mice on an FVB/N background. We can now report that these mice mimic the progression of prostate cancer as seen in humans. By 8 weeks of age, they develop low grade PIN (prostatic intraepithelial neoplasia), which advances to high grade PIN by 10 weeks and to prostate carcinoma by 12 weeks of age (Fig 1). These mice also display metastasis to the periaortic lymph nodes and lungs by 20 weeks of age. Thus, we have a working model of spontaneously developing prostate cancer at this stage.



A. Normal B. Low Grade PIN C. High Grade PIN



D. Adenocarcinoma (low power) E. Adenocarcinoma (high power) F. Lymph Node Metastasis

Fig. 1: Photomicrographs of H&E stained prostates from TRAMP mice. Prostate tissues were analyzed by histology after formalin fixation and H&E staining from a non-transgenic mice showing normal prostate architecture (A), from an 8 week old TRAMP mice showing low grade prostatic intraepithelial neoplasia, PIN (B), from a 10 week old mice showing high grade PIN (adenoma) (C), a 12 week old mice showing adenocarcinoma (D,E), and a 24 week old mice showing metastasis in the peri-aortic lymph nodes (F).

AIM 2. In order to improve immunity against prostate cancer induced by HSV tumor therapy, we hypothesize that a recombinant virus expressing one or more cytokines will enhance the anti-tumor immune response.

As noted in the initial grant, we feel it is important to investigate whether the sub-optimal host immunity against poorly immunogenic prostate cancers could be stimulated and activated to trigger a more robust anti-tumor response by subjecting the tumors to a cytokine-expressing-oncolytic herpes virus. Our ultimate goal as described in the Grant is to test IL-12 expressing PC vectors on the spontaneous TRAMP model and as we have detailed in AIM 1, these vectors are currently under construction. Meanwhile, the selection of IL-12 as the cytokine of choice in an oncolytic vector in order to enhance the anti-tumoral response against prostate cancers is substantiated further through investigations conducted through another Grant mechanism. Since prostate cancers vary in the level of MHC class I expression and since IL-12 is a known inducer of IFN-γ, which can upregulate MHC class I molecules on tumor cells, we hypothesized that an IL-12 expressing virus would be more effective than a non-cytokine virus to treat prostate cancers independent of their MHC class I status.

To test this hypothesis, two implantable models of mouse prostate cancers that differed in both their genetic origin (i.e. different mouse strains) as well as their MHC class I expression levels were used. TRAMP-C2 cell line is derived from a spontaneously occurring prostate tumor in transgenic TRAMP mouse (C57Bl/6 strain background; H2-K^b, H2-D^b) [23] and expresses extremely low levels of MHC class I molecules with approximately 2% of cells displaying cell surface MHC class I staining. In contrast, Pr14-2 cell line, derived from the spontaneously

generated prostate cancer in the transgenic FVB/N C(3)1 Tag mouse (FVB/N background; H2-K^q, H2-D^q) [24] displays high level expression of MHC class I in over 91% of the cells.

In order to investigate whether the introduction of immune-stimulating cytokines concomitantly with viral destruction could modulate a more robust immune response against these mouse prostate tumors, two cytokine viruses, NV1034 (expressing GM-CSF) and NV1042 (expressing IL-12), were evaluated in comparison to their parental non-cytokine virus, NV1023. GM-CSF is a cytokine involved during the initiation phases of the immune response (differentiation, recruitment, and activation of macrophages and dendritic cells) where as IL-12 is a central regulator of the effector phase of the immune response (NK and T cell recruitment and activation). Thus, these cytokines are ideal candidates for evaluating a combined immuno-viral approach. Although both TRAMP-C2 and Pr14-2 tumors were not highly susceptible *in vitro* to the cytotoxic effect by these viruses in that significant killing occurred only at an MOI of 1.0 and not at lower MOI, the infected cells secreted substantial amounts of the appropriate cytokines, GM-CSF and IL-12. However, in spite of the secretion of cytokines by each of the cytokine containing viruses, only the IL-12 expressing NV1042 virus was significantly more efficacious than the other viruses, including the GM-CSF virus, NV1034, or the non-cytokine parental vector, NV1023, against both the low MHC class I expressing tumor, TRAMP-C2 as well as against the high MHC class I expressing tumor, Pr14-2 (Fig 2A, B). Thus, given that NV1023 and NV1042 exhibited similar levels of cytotoxicity against these tumors *in vitro*, it appears that the enhanced response of tumor inhibition observed with NV1042 is due to the activity of IL-12 expressed within the vicinity of tumor destruction.

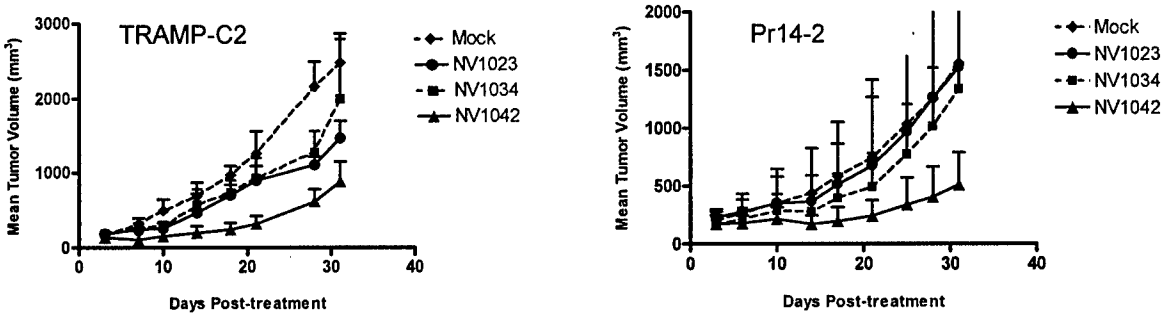


Fig 2: Efficacy of NV1042 (IL-12) virus irrespective of MHC class I status. Subcutaneous TRAMP-C2 and Pr14-2 tumors established in male C57Bl/6 mice and heterozygous FVB/N C3(1) T Ag mice were treated with $1x10^7$ pfu of each virus or Mock on days 0 and 3. Tumor growth was monitored by measuring tumor volume.

Interestingly, the GM-CSF expressing NV1034 vector was effective, albeit moderately, against

the low MHC class I expressing TRAMP-C2 tumors but not against the high MHC expressing Pr14-2 tumors. While the difference in the generation of the subcutaneous tumors (cell suspension for TRAMP-C2 versus tumor fragments for Pr14-2) might account for this moderate but non-significant response to GM-CSF in the Pr14-2 tumors, it also highlights the importance of using two or more models, when available, for evaluating a therapeutic agent. This is further substantiated by the response observed with the non-cytokine vector, NV1023, which was significantly effective for TRAMP-C2 tumors but not for Pr14-2 tumors.

The cause for the total lack of efficacy of NV1023 for Pr14-2 tumors as compared to TRAMP-C2 cells is uncertain but we speculate that it could be due to a limitation of the virus to spread intratumorally from cell to cell. This hypothesis is based on an important characteristic observed during the culture of these cells in vitro. Pr14-2 cells require higher concentrations (0.25%) of trypsin to dissociate as compared to TRAMP-C2 which dissociate more easily and with lower concentrations of trypsin (0.05%), suggesting that the Pr14-2 cells might contain higher amounts of extracellular matrix components. This feature is further amplified when using tumor transplants, where the Pr14-2 tumor extracellular architecture may inhibit the optimal spread of the virus.

The lack of an enhancement with the NV1034 virus as compared to the parental NV1023 virus as seen in this study with the TRAMP-C2 prostate tumor model is analogous to an earlier report with the murine SCC VII squamous cell carcinoma model, where both viruses exerted similar efficacy without additional enhancement with the NV1034 virus [1]. The superior efficacy of the IL-12 expressing NV1042 virus against both prostate tumor models in the present study is also consistent with prior studies in the SCC VII model. This earlier report and the current study are the only two reports where the cytokine viruses, NV1034 (GM-CSF) and NV1042 (IL-12) are compared against their non-cytokine parental vector, NV1023. NV1042 when compared against just NV1023 has been consistently demonstrated to be more efficacious in multiple tumor models, including colorectal cancer, and hepatic tumors [2,3]. It should also be noted that another replication competent IL-12 expressing oncolytic virus M002 has also been demonstrated to be more effective than a non-cytokine virus in treating gliomas in an immunocompetent model [4].

The contributory role of IL-12 to activate the host immune system in order to seek out distant tumors was also tested in both the low MHC expressing TRAMP-C2 and high expressing Pr14-2 tumors. Please note that the TRAMP-C2 data was included in the Preliminary data as Fig 3 when submitting the Grant. Intraneoplastic administrations of NV1042 but not NV1023 to one of the two Pr14-2 tumors established in parallel on either flanks of a mouse resulted in the inhibition of the growth of the second tumor on the contralateral side [Fig 3].

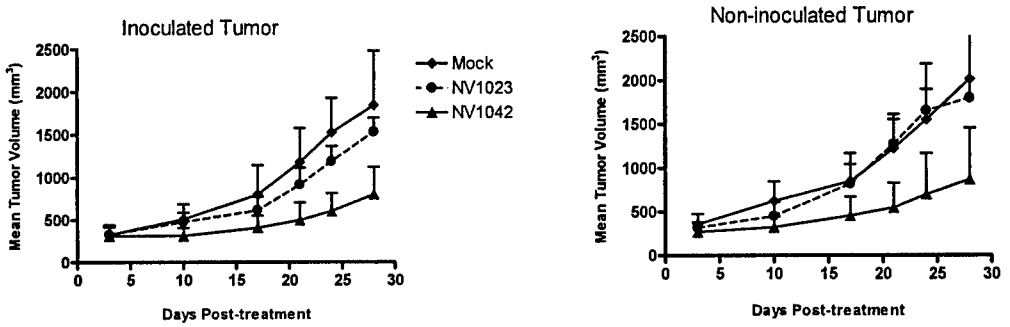


Fig 3: Efficacy of NV1042 (IL-12) virus on distant Pr14-2 tumors. One of the two subcutaneous Pr14-2 tumors established on either flanks of syngeneic mice were treated with $1x10^7$ pfu of each virus on days 0,3,7,and 10. Tumor growth of both tumors was monitored.

Since in the bilateral model, NV1042 virus was administered repeatedly four times intraneoplastically on days 0,3,7, and 10, one major concern was whether the IL-12 expressed by the virus is being retained locally within the tumor or whether it was seeping into the circulation. Systemic toxicity induced by recombinant IL-12 administered systemically or subcutaneously for the treatment of cancer has been a major concern in many prior studies, including phase I and II trials [5-7]. In the present study, however, no significant increase in the levels of IL-12 in the serum was observed when measured on days 4, 6, and 10 post-treatments in the TRAMP-C2 unilateral model suggesting that the expression of IL-12 from the NV1042 virus is a local effect and significant amounts of cytokine is not entering the systemic circulation. Furthermore, no toxicity was observed in the NV1042 treated mice. This is a beneficial feature of using a cytokine virus whereby the expression of the IL-12 is limited to the tumor microenvironment thus precluding any systemic cytokine-induced toxicity.

IL-12 is a central immune regulator acting as a cross-talk bridge between both the innate and the adaptive arm of the immune response. IL-12 secreted by antigen stimulated dendritic cells, macrophages, monocytes and B cells can effect the recruitment and activation of NK cells and the differentiation and functional response of T cells to the antigen. Pivotal to the efficiency of

these effector responses is the secretion of IFN-γ by IL-12 from antigen presenting cells or NK or T cells. One of the functions of IFN-γ is its ability to up regulate MHC class I expression in various cells, including tumor cells. This feature is especially relevant given that the low MHC class I expressing TRAMP-C2 tumor growth, tested in this study, was inhibited by the IL-12 virus (NV1042) to a significantly higher level as compared to the non-cytokine vector, NV1023. Given that the TRAMP-C2 prostate tumor model is representative of the poorly immunogenic prostate cancers commonly seen in humans, the enhanced efficacy observed with TRAMP-C2 model is an important one, thereby providing an additional benefit to the use of an IL-12 oncolytic virus for such cancers. Furthermore, IL-12 and IFN-γ also promotes a Th1 (cytotoxic T cell) response, which is critical for elimination of tumors and the development of long-term memory. In accordance with this expected function of IL-12, the NV1042 inoculated TRAMP-C2 tumors demonstrated higher infiltration of macrophages/ dendritic cells, CD4 and CD8 cells when examined on days 6 and 10 post-treatment as compared to the non-cytokine NV1023 virus or mock treated tumors [Table 1].

	Post-treatment Day	CD4	CD8	Mac-3
Mock		-	-	+
NV1023	Day 4	-	+	+
	Day 6	-	-	+
	Day 10	-	-	+
NV1042	Day 4	-	+	+
	Day 6	+	+	++
	Day 10	+	+	+

Table 1: CD4/ CD8/ Macrophage immunostaining of TRAMP-C2 tumors treated with viruses. Subcutaneous TRAMP-C2 tumors treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of viruses were sacrificed on day 10 and tumor sections were immunostained for CD4 or CD8 or Mac-3 molecules. Three random fields per section from 3 mice for each treatment group were scored and the average score recorded. [- : <5%, +: 5-25%, ++: 25-50% of field staining positive].

An increased influx of macrophages, CD4 and CD8 cells in tumors treated intraneoplastically with IL-12 expressing replication competent viruses (M002, NV1042) have also been previously reported with mouse models of glioma, and hepatic carcinoma [1,4].

In addition to its immunomodulatory effect, an ancillary mode of action of IL-12 is its anti-angiogenic effect, mediated by IFN-γ inducible protein-10 (IP10) and monokine-induced by IFN-γ (MIG). In the present study, TRAMP-C2 tumors treated with the IL-12 expressing NV1042

virus analyzed for the endothelial cell surface marker, CD31, exhibited significant reduction in staining for endothelial cells day 10 post-treatment, as compared to the NV1023 or mock treated tumors. This finding is in accordance with what has been previously reported after intraneoplastic treatment of squamous cell carcinoma with the NV1042 virus [8].

The significant inhibition of tumor growth observed in the inoculated tumors of both the TRAMP-C2 and Pr14-2 bilateral tumor models is thus consistent with the increased immune infiltration and anti-angiogenic effects mediated by the IL-12 expressed by the NV1042 virus in the microenvironment of the inoculated tumor. However, in light of the fact that there was no increase in systemic IL-12 levels and no inhibition of contralateral tumor growth observed until day 27 in the low MHC bearing TRAMP-C2 tumor bearing mice treated with the NV1042 virus, it could be hypothesized that this delayed tumor inhibition of the contralateral tumor might be a reflection of the possibility that only by day 27 post-treatment a threshold level of activated immune cells accumulated in this tumor to make a significant effect. Given that enough IFN-γ has to be secreted by the activated circulating immune cells within the local contralateral tumor environment in order to have sufficient MHC class I up-regulation and antigen presentation, this scenario is highly plausible. It has been reported previously that in order to reject an established tumor of about 5mm in diameter, an antigen-specific CTL response has to be maintained for approximately 3-4 weeks suggesting that the immune system has to be boosted for a protracted period of time [11]. In further support of the above mentioned hypothesis, a significant reduction of contralateral tumor volume is observed in the Pr14-2 tumor as early as day 10 post-treatment possibly suggesting that tumors with high levels of MHC class I expression is susceptible to even low levels of circulating activated immune cells.

In conclusion, the current study done during this past year demonstrates that irrespective of the level of MHC class I molecules on the surface of prostate tumors, the IL-12 expressing NV1042 virus was significantly more effective than either the GM-CSF expressing virus, NV1034, or the non-cytokine NV1023 parental virus. The enhanced response of these tumors to an IL-12 containing vector suggests that HSV vectors, such as NV1042, may demonstrate efficacy against prostate cancers through three mechanisms: direct viral oncolysis, anti-tumor immune activation by HSV infection which can be further amplified and sustained to render maximal benefit through expression of IL-12 by the virus, and anti-angiogenic effects of IL-12. For these reasons, we conclude that an IL-12 expressing oncolytic HSV vector is appropriate for further testing directed toward a clinical trial for the treatment of prostate cancer in man. These data

and model systems can also now be used to further develop studies using the constructs of Aim 1 to test for further improvements in efficacy and will serve as a comparison for the PC–derived vectors.

KEY RESEARCH ACCOMPLISHMENTS

- The ICP6$^-$ PC BAC has been genetically engineered successfully using PC virus, an HSV1 strain which replicates most efficiently in prostate cancer cells.
- An IL-12 expressing virus (NV1042) is effective against local and distant prostate cancers independent of their MHC class I status. IL-12 expression by this virus results in increased infiltration of macrophages and lymphocytes into the tumor and also acts as an anti-angiogenic agent within the tumor. This further substantiates the goal to use an IL-12 expressing PC virus construct, as described in Aim 2.

REPORTABLE OUTCOMES

Manuscript titled "Enhanced therapeutic efficacy of IL-12 expressing oncolytic herpes simplex virus for prostate cancers independent of MHC I status" has been submitted to *Molecular Therapy.*

CONCLUSIONS

Most prostate cancers are either poorly or moderately immunogenic. Therefore, oncolytic strategies that also include overcoming this immunological complacency is highly desirable. Oncolytic herpes viruses with improved cytotoxicity against prostate cancers (PC viruses), and which express IL-12, a central immunoregulatory cytokine, significantly improves the cumulative anti-tumoral efficacy against prostate cancers. Genetic engineering of these PC/ IL-12 viruses are currently under progress.

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APPENDICES

Manuscript titled "Enhanced therapeutic efficacy of IL-12 expressing oncolytic herpes simplex virus for prostate cancers independent of MHC I status" submitted to *Molecular Therapy.*

ENHANCED THERAPEUTIC EFFICACY OF AN IL-12 EXPRESSING ONCOLYTIC HERPES SIMPLEX VIRUS FOR PROSTATE CANCERS INDEPENDENT OF MHC I STATUS

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ABSTRACT

Replication competent oncolytic herpes simplex viruses (HSV) with broad-spectrum activity against various cancers, including prostate cancer, exert a dual effect by their direct cytocidal action and by eliciting tumor specific immunity. These viruses can deliver immunoregulatory molecules to tumors so as to enhance the cumulative antitumor response. This is particularly desirable for prostate cancers, which are usually poorly immunogenic. Initial studies described herein comparing the efficacy of three different oncolytic HSVs (G207, G47Δ, and NV1023) to inhibit the growth of the poorly immunogenic TRAMP-C2 mouse prostate tumors demonstrated that NV1023 was most effective in treating established tumors. In two murine prostate cancer models with highly variable MHC class I levels, Pr14-2 tumor with 91% and TRAMP-C2 with 2% of cells staining, the expression of IL-12 on an NV1023 background (NV1042) further enhanced the efficacy of NV1023 but not the expression of GM-CSF (NV1034). NV1042 also inhibited the growth of distant non-inoculated tumors in both prostate cancer models. The NV1042 treated tumors exhibited increased immune cell infiltration and decreased levels of angiogenesis. Thus, an IL-12 expressing oncolytic herpes virus (NV1042) is an effective therapeutic vector against prostate cancers irrespective of their MHC class I status.

Key Words: oncolytic virus, herpes simplex virus, cytokine virus, prostate cancer, immunotherapy, TRAMP-C2, Pr14-2, MHC-I, IL-12

INTRODUCTION

A major impediment to the effective therapy of most human cancers is the inherent heterogeneous nature of the tumor itself [1]. Tumors harbor populations of cells that vary in genes that are mutated or differentially regulated allowing subpopulations of cells to gain a survival advantage. Additionally, the tumor cells that survive have also developed sophisticated molecular mechanisms to evade the host immune system by down regulating molecules that mediate immune recognition [2]. Thus, the immune system can be blinded to seek and kill tumors as they arise because of the "self" façade displayed by the tumor cell. The ultimate effect of these strategies employed by the tumor cells result in aggressive tumors that are resistant to standard forms of therapy. A classic example of such a tumor is prostate cancer, where these slow growing, occult, poorly immunogenic tumors are able to evade immune surveillance early in their development, and with time accumulate additional genetic alterations that confer aggressiveness and resistance to most standard therapies [3,4].

The incidence of prostate cancer is rare until age 40, but increases to 30% of all males by age 50 and to 75% by age 80 [5]. With extension of the average life span, it has become the most common cancer among males of the Western world accounting for 35% of all cancers [5]. In the United States and Europe, it claims the life of 1 in 5 men diagnosed with the disease [6]. Current treatment modalities for localized prostate cancer include surgery and radiation therapy, both of which are associated with risks of nerve damage and sexual and/or bladder dysfunction, and other complications. Androgen ablation is initially beneficial but is significantly limited by the progressive outgrowth of therapy resistant disease [7]. Chemotherapy is used only as a palliative agent with no associated improvement in survival. Thus, there exists a paucity of novel treatment options for prostate cancer.

Replication competent oncolytic herpes simplex virus (HSV) vectors are innovative cancer therapeutic agents with a wide spectrum of activity against a variety of cancers [8,9]. Currently, three different HSV vectors (G207, 1716, NV1020) have been successfully tested for safety in humans [10-12] and have exhibited efficacy in pre-clinical animal models against various human cancers, including prostate cancer [12]. G207, a replication competent HSV-1 mutant with deletions of both copies of the *γ34.5* gene and a *LacZ* insertion inactivating the *ICP6* gene [13] has been demonstrated to be safe when inoculated intra-prostatically in both mice and *Aotus* monkeys [14]. G207 and 1716 has also been shown to be safe after inoculation at high-titers into human brain [10,11]. This neuron-sparing feature is an important characteristic in using oncolytic HSV vectors for the treatment of prostate cancer since the complications arising from surgery or radiotherapy of prostate cancer are often due to nerve damage. Furthermore, it has been demonstrated that G207 can act as an *in situ* tumor vaccine, whereby viral infection of tumor cells activates the host immune system to mobilize a tumor specific immune response against tumor antigens [15].

In an effort to maximize this immune effect conferred by HSV-1 vectors, replication competent viruses have been genetically engineered to contain cytokine transgenes. Two such viruses currently being investigated are NV1042, expressing mIL-12, and, NV1034, expressing mGM-CSF [16]. Both are derived from NV1023, which is a second-generation mutant of NV1020, an HSV1/2 intertypic recombinant virus [17]. NV1020 carries a deletion of one copy of *γ34.5* and the internal repeat, *UL24* and *UL56* genes, with addition of *gJ*, *gG*, *US2* and *US3* from HSV-2. NV1023 also has an insertion of *LacZ* and deletion of the *ICP47* and *US11* genes. The ICP47 deletion overcomes the down regulation of MHC class I expression normally observed in HSV-infected human cells [18]. This additional immune benefit resulting from the ICP47 deletion is

also observed with the non-cytokine expressing G47Δ virus, which is derived from G207 with an additional deletion of the *ICP47* gene and the *US11* promoter [19].

In this study we have compared various oncolytic HSV vectors (G207, G47Δ, NV1023, NV1034, and NV1042) in an effort to identify the most efficacious vector against prostate cancer and to test the hypothesis that an immunomodulating cytokine expressed concomitantly during viral destruction can enhance the cumulative anti-tumoral efficacy against poorly immunogenic prostate cancers. Our laboratory and others have previously reported that human prostate cancer xenografts established in athymic mice are sensitive to G207 and NV1020 [20,21]. A major caveat of these studies, however, is that the *in situ* vaccine effect or the enhancement mediated via cytokine expression by the oncolytic viruses is ineffective with the use of athymic mice. Therefore, as a means to evaluate the combined oncolytic and immune aspects in the efficacy of these various vectors we utilized two different immunocompetent mouse models of prostate cancer, TRAMP-C2 and Pr14-2. TRAMP-C2 and Pr14-2 cell lines are derived from spontaneously occurring prostate tumors in the transgenic TRAMP and C3(1)/T-Ag mice respectively, and can form subcutaneous tumors when implanted into male C57Bl/6 mice or heterozygous C3(1)/T-Ag mice respectively [22-25].

The advantage of using these two models is that each of these tumor cell lines represent two different variations of immunological status, in that the TRAMP-C2 cell line expresses virtually no MHC class I on its cell surface [26] where as the Pr14-2 cell line expresses high levels on its cell surface [25]. The expression of MHC class I molecules can be up regulated in these cells by treatment with IFN-γ, especially in the low expressing TRAMP-C2 cells [26]. Thus, these two tumor models reflect the variations of MHC class I levels seen *in vivo* with human prostate cancer

and serve as appropriate models to study the immunological response to oncolytic cytokine-expressing vectors.

Our studies demonstrate that the IL-12 expressing virus, NV1042 was the most effective virus for prostate cancer in both immunocompetent murine prostate cancer models irrespective of their MHC class I status. The enhanced efficacy observed with the NV1042 virus can be attributed to the immunoregulatory and anti-angiogenic effects of IL-12.

RESULTS

MHC Class I expression and up regulation by IFN-γ in mouse prostate cancer cell lines

The basal level of expression of MHC class I molecules on the surface of both mouse prostate cancer cell lines, TRAMP-C2 and Pr14-2, were analyzed by FACS staining using antibodies specific to H-2K^b and H-2K^q, respectively. As illustrated in Fig. 1, the TRAMP-C2 cell line expressed negligible levels of MHC class I molecules on its cell surface with approximately 2% cells staining where as the Pr14-2 cell line expressed high levels with approximately 91% of cells staining. After exposure to recombinant IFN-γ, a known inducer of MHC class I molecules on the surface of cells, the level of expression of these molecules in the TRAMP-C2 cell line increased dramatically with up to 94% of cells staining (Fig. 1A). Treatment of Pr14-2 cells with IFN-γ not only increased the number of cells staining for MHC class I from 91% to 98.5% but also increased the level of expression on the cells as indicated by the shift in the FACS intensity histogram (Fig. 1A,B).

In vitro and *in vivo* susceptibility of mouse prostate cancer cell lines to oncolytic HSVs

A major goal of these studies was to use mouse syngeneic prostate cancer models to evaluate the efficacy of various oncolytic HSVs, especially cytokine expressing viruses. Since mouse cells

are not the natural host of HSV and are generally more resistant to HSV replication than human cells, we evaluated the *in vitro* susceptibility of the mouse prostate cancer cell lines. Initial cytotoxicity studies were conducted using the low MHC expressing TRAMP-C2 cell line to evaluate three non-cytokine expressing viruses, G207 (ICP34.5$^{-/-}$ ICP6^{-}), G47Δ (ICP34.5$^{-/-}$ ICP6^{-} ICP47^{-}) and NV1023 ICP34.5$^{+/-}$ ICP47^{-}). The results demonstrate that in general these cells were relatively resistant to killing at "low" MOIs (0.01 or 0.1) with G47Δ being the most cytotoxic, and were uniformly susceptible at the "high" MOI of 1.0 (Fig 2A). Pr14-2 cells showed similar susceptibility, with G47Δ also being the most cytotoxic (data not shown, [27]).

An *in vivo* comparison of these viruses was also conducted using TRAMP-C2 and Pr14-2 tumors established in the appropriate syngeneic hosts, C57Bl/6 and C3(1)/T-Ag mice. Subcutaneous tumors were treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of G207, G47Δ or NV1023 or virus buffer (Mock) and tumor growth monitored. As illustrated in Fig 2B, NV1023 was significantly more effective than G207, G47Δ or mock ($p<0.05$, student's t test; days 19,23) at inhibiting the growth of the TRAMP-C2 tumors. The NV1023 treated mice also exhibited a significant extension in median survival of 49 days as compared to 27 days for the other viruses ($p<0.05$, Logrank (Mantel-Cox) test); Fig 2B). In contrast, none of the three viruses were effective in inhibiting the growth of Pr14-2 tumors as compared to the mock treatment (data not shown).

***In vitro* susceptibility and cytokine expression in mouse prostate cancer cell lines**

The *in vitro* susceptibility of TRAMP-C2 and Pr14-2 cells to killing by NV1042 was similar to that seen with NV1023 (data not shown). As expected, the insertion of the IL-12 gene in NV1023 (NV1042) did not affect viral cytotoxicity. The synthesis of the appropriate cytokines from TRAMP-C2 and Pr14-2 cells infected with NV1023 (no cytokine), NV1034 (expressing GM-

CSF) and NV1042 (expressing IL-12) *in vitro* was assayed 24 hours post-infection. Both cell lines expressed comparable levels of the cytokines with infected TRAMP-C2 and Pr14-2 cells secreting 1.75 and 6.6 ng/ml of GM-CSF (NV1034) and 52 and 41 ng/ml of IL-12 (NV1042) respectively.

Efficacy of oncolytic cytokine HSVs in syngeneic prostate cancer models

While our initial *in vivo* studies demonstrated that NV1023 virus was effective in inhibiting the growth of TRAMP-C2 tumors, it was of interest to determine whether immunomodulatory cytokines, such as GM-CSF or IL-12, expressed *in vivo* concomitantly with viral destruction of tumors would elicit a more robust anti-tumor response. For this, we compared the efficacy of cytokine viruses NV1034 (GM-CSF) and NV1042 (IL-12) against their non-cytokine parental vector NV1023, using both the low MHC class I expressing TRAMP-C2 and high MHC class I expressing Pr14-2 models. Subcutaneous TRAMP-C2 and Pr14-2 tumors established in the appropriate syngeneic hosts, C57Bl/6 and C3(1)/T-Ag mice, were treated on days 0 and 3 intraneoplastically with $1x10^7$ pfu of NV1023, NV1034 or NV1042 and tumor growth monitored. In both models, the IL-12 expressing virus, NV1042 was the most effective virus in inhibiting the progression of tumor growth ($p<0.05$, student's t test; NV1042 vs. NV1034, NV1023, Mock; Fig. 3A,B) and also in extending the median survival of these mice ($p<0.05$, Logrank (Mantel-Cox) test; NV1042 (38 days) vs. Mock (31 days) for TRAMP-C2 tumors, and NV1042 (75 days) vs. all groups (48-54 days) for Pr14-2; Fig. 3A,B). In both models, NV1034 (GM-CSF) was no more effective than its non-cytokine parent, NV1023, with significant inhibition as compared to mock treatment seen in TRAMP-C2 tumors ($p<0.05$, days 18-28) but not in Pr14-2 tumors.

Inhibition of distant non-inoculated tumors with NV1042 virus

In order to examine the immune contribution of IL-12 in the efficacy of NV1042 virus in inhibiting the growth of the two mouse prostate tumor models with differing MHC class I status,

investigations were conducted using a bilateral tumor model. TRAMP-C2 and Pr14-2 subcutaneous tumors were established on the right and left flanks of C57Bl/6 or C3(1)/T-Ag mice respectively and only the left tumors were treated intraneoplastically on days 0, 3, 7, and 10 with $1x10^7$ pfu of either NV1023, NV1042 or virus buffer (mock). The growth of tumors on both flanks was monitored. As illustrated in Fig 4A-B, NV1042 was effective in both prostate tumor models at inhibiting the growth of not only the inoculated (left) tumors but also the non-inoculated (right) tumors. It is relevant to note that although the implanted TRAMP-C2 tumor cells express virtually no MHC class I, the inoculated ipsilateral tumor responded dramatically to NV1042 administration, with significant inhibition observed as early as day 7 post-treatment ($p<0.05$ vs. Mock, NV1023, student's t test), where as significant inhibition of contralateral tumor growth was delayed until day 27 (Fig 4A). In this model, one inoculated tumor out of 11 from each of the NV1023 and NV1042 treated groups showed a complete response and there were significant but non-durable responses (no measurable tumors for up to 2 weeks) in 2 inoculated tumors from the NV1023 group (18%) and 4 from the NV1042 group (36%). In contrast, Pr14-2 tumors with high levels of MHC class I on their surface were inhibited by NV1042 to a similar degree in both inoculated and non-inoculated tumors with statistical significance ($p<0.05$, student's t test; vs. Mock, NV1023) observed from day 10 and 21, respectively (Fig 4B). Interestingly, doubling the number (and dose) of virus injections had a large effect on TRAMP-C2 tumor growth (compare Fig 3A with Fig 4A), whereas it had no effect on Pr14-2 tumors.

Mechanism of NV1042 efficacy in the TRAMP-C2 model

To investigate the mechanism of tumor growth inhibition of the prostate tumors by NV1042, unilateral TRAMP-C2 tumors established subcutaneously in C57Bl/6 mice were inoculated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of NV1023, NV1042 or virus buffer. Three mice from each group were sacrificed on days 4, 6, and 10 post-treatment and the sera and tumor

lysate collected for IL-12 measurement. Tumors from another set of 3 mice were also evaluated for *LacZ* expression from virus, immune cell infiltration and anti-angiogenesis.

a). IL-12 levels in the serum and tumor

In order to determine whether IL-12 induced locally within the tumor, either by virus infection/inflammation or expression from NV1042, is entering systemic circulation, sera was analyzed for IL-12 by ELISA. Two mice from the NV1023 treated group and 1 from the NV1042 group on day 4 post-treatment and 2 mice from the NV1023 treated group on day 10 post-treatment displayed levels greater than twice the Mock values (Fig 5A). However, serum IL-12 levels in the virus treated groups were not statistically significant on any day post-treatment, suggesting that the amounts of IL-12 expressed locally from NV1042 were not sufficient to alter serum levels. In contrast, measurable levels of tumor IL-12 were observed only in the NV1042 treated tumors on days 4 and 6 post-treatment and not in any of the other treatment groups (Fig 5B).

b). X-gal staining of the tumors: Both NV1023 and NV1042 have an insertion of the *E.coli LacZ* gene driven by the immediate-early ICP47 gene promoter and therefore virus-infected cells can be detected by X-gal staining. TRAMP-C2 tumors treated with NV1023 and NV1042 exhibited X-gal staining post-treatment. Representative tumor sections after NV1042 infection are shown in Fig 6, with staining observed on all days examined, but the highest levels were observed on day 6 followed by decreased staining on day 10 post-treatment. On days 6 and 10, *LacZ* staining was detected mostly around dying cells. None of the mock-inoculated tumors displayed *LacZ* staining (data not shown).

c). CD4/CD8/Mac-1 staining of tumors: Since IL-12 can activate and recruit T lymphocytes, tumors inoculated with NV1023, NV1042 or virus buffer on days 0 and 3 were immunostained on

day 4, 6 and 10 for $CD4^+$ and $CD8^+$ T cells and macrophages. The mock treated tumors did not exhibit any infiltration of $CD4^+$ or $CD8^+$ cells but both NV1023 and NV1042 treated tumors exhibited increased infiltration of $CD8^+$ cells on day 4 (Table 1). However, by days 6 and 10 post-treatment only the NV1042 inoculated tumors contained $CD8^+$ and $CD4^+$ cells. On day 6, a marked increase of macrophages is observed in the NV1042 treated tumors with up to 50% of the field staining, which interestingly corresponds to the day when maximal *LacZ* expression of the virus is also observed. Thus, NV1042 expressing IL-12 is able to attract and retain immune cell populations including macrophages, and $CD4^+$ and $CD8^+$ cells at the site of tumor destruction.

d). CD31 staining: New vasculature established during tumor growth can be identified by staining for CD31 molecules on the surface of endothelial cells within the tumors. In order to determine if IL-12 expressed by the virus within the tumor can inhibit this neo-angiogenesis, TRAMP-C2 tumors inoculated with NV1023, NV1042 or virus buffer on days 0 and 3 were immunostained for CD31 on day 10. The results show that in keeping with the highly vascular nature of these tumors, the mock tumors display extensive CD31 staining and although the NV1023 tumors exhibited a decrease in CD31 staining, the NV1042 treated tumors displayed a dramatic reduction in CD31 staining as compared to either NV1023 or mock treated tumors (Fig 7). In all these tumors there were some interspersed unidentifiable cells (most likely histiocytes), which also stained for CD31. Thus, the NV1042 treated tumors display significantly lower CD31 staining, which can be attributed to the anti-angiogenic effects of IL-12 expression.

DISCUSSION

The heterogeneity observed within most cancers, including prostate cancer, dictates a multimodal approach towards the treatment of these cancers. However, none of the current standard modalities of treatment for prostate cancer, including surgery, chemotherapy or androgen ablation therapy, has the potential to specifically activate the immune system. Thus, new multi-pronged

approaches which can eliminate overt tumors via oncolysis as well as induce tumor-targeted immune responses in the host to search and kill occult tumors is worthy of exploration.

Oncolytic HSV vectors are one such novel treatment strategy [8,9]. In addition to their proven efficacy against a variety of tumors through a direct cytotoxic effect, these viruses can also activate the immune system [15]. This was first demonstrated with the prototypic oncolytic HSV vector G207, whereby infection and destruction of colon cancer tumors in immunocompetent mice resulted in the activation of tumor specific T cells, which not only destroyed distant tumors but also conferred long term protection against re-challenge [15]. Furthermore, the strategy of coupling a direct cytotoxic effect with an immune therapy approach for the treatment of cancers is enhanced via the use of oncolytic viruses, which by virtue of infection of tumor cells may act as an adjuvant in sending a 'danger signal' to the host's immune system.

Many tumors, including prostate cancers, however, have developed molecular means to evade the immune system. Specific molecular adaptations exhibited by prostate cancers encompass escape from immune surveillance at all phases of recognition: at the level of innate immunity by NK cells and at the initiation and effector phases of adaptive immune response by DCs and T cells. Thus, strategies employed by prostate cancers include, shedding of MHC class I chain-related molecules which results in deficient NK cell surveillance [28], inhibition of dendritic cell differentiation by PSA [29], down regulation of cell surface MHC class I molecules leading to escape from T cell recognition [30-32], deficiency in antigen processing [33], and secretion of immunosuppressive factors such as TGF-β [34]. Most prostate cancers are thus either poorly or only moderately immunogenic. Nonetheless, we suggest that an intense oncolytic action mediated by a therapeutic viral vector could overcome this immunological complacency.

The overarching goal of this study, therefore, was to investigate whether this otherwise sub-optimal host immunity against poorly immunogenic prostate cancers could be stimulated and activated to trigger a more robust anti-tumor response by subjecting the tumors to a cytokine-expressing-oncolytic HSV. As a direct comparison for this hypothesis, two implantable models of mouse prostate cancers that differed in both their genetic origin (i.e. different mouse strains) as well as their MHC class I expression levels were used. The TRAMP-C2 cell line is derived from a spontaneously occurring prostate adenocarcinoma in a transgenic TRAMP mouse (C57Bl/6 strain background; H2-K^b, H2-D^b) [23] and expresses extremely low levels of MHC class I molecules with approximately 2% of cells displaying cell surface MHC class I staining. In contrast, the Pr14-2 cell line, derived from a spontaneous prostate cancer arising in the transgenic C3(1)/T-Ag mouse (FVB/N background; H2-K^q, H2-D^q) [24,25] displays high level expression of MHC class I in over 91% of the cells.

Initial studies comparing three genetically different oncolytic viruses, G207 (ICP34.5$^{-/-}$ ICP6$^-$), G47Δ (ICP34.5$^{-/-}$ ICP6$^-$ ICP47$^-$) and NV1023 (ICP34.5$^{+/-}$ ICP47$^-$) using the poorly immunogenic TRAMP-C2 implantable model demonstrated that NV1023 was superior to the other viruses in inhibiting the growth of this tumor. In contrast, the Pr14-2 tumors were not susceptible to any of these viruses. It is noteworthy that although both TRAMP-C2 and Pr14-2 cells exhibited similar cytotoxicity profiles *in vitro* to NV1023, TRAMP-C2 tumors but not Pr14-2 tumors established *in vivo* were susceptible to this virus. Interestingly, TRAMP-C2 cells originate from C57Bl/6 mice, a strain highly resistant to HSV with an LD50 of greater than $1x10^6$ pfu, as compared to BALB/c with an LD50 of $1x10^2$ pfu [35]. It is possible that the different viral mutations in NV1023 versus G207 and G47Δ contribute to the increased efficacy of NV1023 in this model. G207 and G47Δ lack both copies of ICP34.5 as compared to NV1023 in which one copy is retained [13,16,19]. Further, only NV1023 has a deletion of UL56, and it has been reported recently that deletion of UL56 in a spontaneously generated virus, HF10, might contribute towards its increased potency

against a poorly immunogenic metastatic peritoneal tumor as compared to hrR3, a UL56^{+} virus [36]. The cause for the total lack of efficacy of NV1023 for Pr14-2 tumors as compared to TRAMP-C2 tumors is uncertain but we speculate that it could be due to a limitation of the virus to spread intratumorally from cell to cell. This hypothesis is based on an important characteristic observed during the culture of these cells *in vitro*. Pr14-2 cells require higher concentrations (0.25%) of trypsin to dissociate as compared to TRAMP-C2 which dissociate more easily and with lower concentrations of trypsin (0.05%), suggesting that the Pr14-2 cells might contain higher amounts of extracellular matrix components. This feature might be further amplified when using tumor transplants, thus Pr14-2 tumors may be less susceptible to optimal spread of the virus.

In order to investigate whether the expression of immune-stimulating cytokines concomitantly with viral destruction could modulate the immune response against these mouse prostate tumors, two cytokine viruses, NV1034 (expressing GM-CSF) and NV1042 (expressing IL-12), were evaluated in comparison to their parental non-cytokine virus, NV1023. GM-CSF is a cytokine involved during the initiation phases of the immune response (differentiation, recruitment, and activation of macrophages and dendritic cells), whereas IL-12 is a central regulator of the effector phase of the immune response (NK and T cell recruitment and activation) [37]. Thus, these cytokines are ideal candidates for evaluating a combined immuno-viral approach. Although both TRAMP-C2 and Pr14-2 cell lines were not very susceptible to the cytotoxic effect of NV1034 or NV1042 *in vitro*, with significant killing occurring only at an MOI of 1.0 and not at lower MOIs, the infected cells secreted substantial amounts of the appropriate cytokines, GM-CSF and IL-12. However, only NV1042 was significantly more effective against both the low MHC class I expressing tumor, TRAMP-C2, as well as the high MHC class I expressing tumor, Pr14-2, as compared to either NV1034 or the non-cytokine parental vector, NV1023. Given that NV1023 and NV1042 exhibited similar levels of cytotoxicity against these tumor cells *in vitro*, it appears

that the enhanced tumor growth inhibition observed with NV1042 is due to the activity of IL-12 expressed within the vicinity of tumor destruction. Interestingly, NV1034 when compared to NV1023 was effective, albeit moderately but not significantly, against the low MHC class I expressing TRAMP-C2 tumors but not against the high MHC expressing Pr14-2 tumors.

The lack of an effect of GM-CSF (NV1034 as compared to NV1023) in the prostate tumor models described here is analogous to an earlier report with the murine SCC VII squamous cell carcinoma model, where both viruses exerted similar efficacy without additional enhancement with the NV1034 virus [16]. The superior efficacy of the IL-12 expressing NV1042 virus against both prostate tumor models in the present study is also consistent with prior studies in the SCC VII model [16]. This earlier report and the current study are the only two reports where the cytokine viruses, NV1034 (GM-CSF) and NV1042 (IL-12) are compared. NV1042 when compared to NV1023 has been consistently more efficacious in multiple tumor models, including colorectal cancer, hepatic tumors and breast tumors from C3(1)/T-Ag mice [38,39,27]. In addition, another replication competent IL-12 expressing oncolytic HSV vector, M002 was more effective than its parent virus in treating gliomas in an immunocompetent model [40].

The contributory role of IL-12 to activate the host immune system in order to seek out distant tumors was also tested in both the low MHC expressing TRAMP-C2 and high expressing Pr14-2 tumors in this study. Intraneoplastic administrations of NV1042 but not NV1023 to one of two tumors established in parallel on either flank of a mouse resulted in inhibition of growth of the second tumor on the contralateral side. Systemic toxicity induced by recombinant IL-12 administered intravenously or subcutaneously for the treatment of cancer has been a major concern in many prior studies, including phase I and II trials [41-43]. In the present study, however, no significant increase in the levels of IL-12 in serum was observed in the TRAMP-C2 unilateral model when measured on days 4, 6, and 10 post-treatments, even though significant

levels were detected in the tumor on days 4 and 6 post-treatments, suggesting that expression of IL-12 from NV1042 is local and not entering the systemic circulation. Furthermore, no toxicity was observed in the NV1042 treated mice. This is a beneficial feature of using a cytokine virus whereby the expression of IL-12 is limited to the tumor microenvironment thus precluding any systemic cytokine-induced toxicity.

IL-12 is a central immune regulator acting as a cross-talk bridge between both the innate and the adaptive arm of the immune response [44]. IL-12 secreted by antigen stimulated dendritic cells, macrophages, monocytes, and B cells can effect the recruitment and activation of NK cells, and the differentiation and functional response of T cells to antigen. Pivotal to the efficiency of these effector responses is the secretion of IFN-γ from antigen presenting cells, NK, or T cells [44,45]. One of the functions of IFN-γ is its ability to up regulate MHC class I expression in various cells, including tumor cells [46]. This feature is especially relevant given that the low MHC class I expressing TRAMP-C2 tumor growth, tested in this study, was inhibited by NV1042 (IL-12) virus to a significantly higher level as compared to the non-cytokine vector, NV1023. In light of the fact that the TRAMP-C2 prostate tumor model is representative of poorly immunogenic prostate cancers commonly seen in humans, the enhanced efficacy observed with TRAMP-C2 is an important one, thereby providing an additional benefit to the use of an IL-12 oncolytic virus over a non-cytokine virus for such cancers. IL-12 and IFN-γ also promotes a Th1 (cytotoxic T cell) response, which is critical for elimination of tumors and the development of long-term memory. In accordance with this expected function of IL-12, the NV1042 inoculated TRAMP-C2 tumors demonstrated higher infiltration and retention of macrophages, $CD4^+$ and $CD8^+$ cells when examined on days 6 and 10 post-treatment as compared to the non-cytokine NV1023 virus or mock treated tumors. Similar elevated immune cell infiltration has been reported after M002 and NV1042 treatment of glioma and hepatic carcinoma, respectively [39,40].

In addition to its immunomodulatory effect, an ancillary mode of action of IL-12 is its anti-angiogenic effect, mediated by IFN-γ inducible protein-10 (IP-10) and monokine-induced by IFN-γ (MIG) [47]. In the present study, TRAMP-C2 tumors treated with NV1042 exhibited significant reduction of CD31 immunostaining on day 10 post-treatment, as compared to NV1023 or mock treated tumors. This finding is similar to a previous report of NV1042 treatment of squamous cell carcinoma [48]. Interestingly, the non-IL-12 expressing NV1023 treated tumors also exhibited a reduction in angiogenesis, and while there was no measurable levels of IL-12 detected in these tumors, it is possible that viral infection *per se* activates and attracts immune cells into the tumor. This is supported by the finding that an increased infiltration of CD8 cells (which can secrete IFN-γ) is observed in the NV1023 tumors on day 4 post-treatment.

The significant inhibition of tumor growth observed in both the TRAMP-C2 and Pr14-2 bilateral tumor models is thus consistent with increased immune infiltration and anti-angiogenic effects mediated by IL-12 expressed from NV1042 in the microenvironment of the inoculated tumor. However, in light of the fact that there was no increase in systemic IL-12 levels and no inhibition of contralateral tumor growth observed until day 21 in Pr14-2 and day 27 in TRAMP-C2 tumor bearing mice treated with NV1042, it could be hypothesized that the delayed contralateral tumor inhibition might be a reflection of the time needed for sufficient numbers of activated immune cells to accumulate in this tumor. Given that enough IFN-γ has to be secreted by the circulating activated immune cells within the local contralateral tumor environment in order to have sufficient MHC class I up-regulation, especially in the low MHC class I expressing tumors such as TRAMP-C2 model, this scenario is plausible. It has been reported that in order to reject an established tumor of about 5mm in diameter, an antigen-specific CTL response has to be

maintained for approximately 3-4 weeks suggesting that the immune system has to be boosted for a protracted period of time [49].

In conclusion, the current study demonstrates that irrespective of the level of MHC class I expression on the surface of prostate tumors, IL-12 expressing NV1042 was significantly more effective than either GM-CSF expressing NV1034, or the non-cytokine NV1023 parental virus. The enhanced response of these tumors to an IL-12 containing vector suggests that HSV vectors, such as NV1042, may demonstrate efficacy against prostate cancers through three mechanisms: direct viral oncolysis, anti-tumor immune activation by HSV infection which can be further amplified and sustained to render maximal benefit through expression of IL-12, and anti-angiogenic effects of IL-12. For these reasons, we conclude that an IL-12 expressing oncolytic HSV vector is appropriate for further testing directed towards a clinical trial for the treatment of prostate cancer in man.

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 Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/l) (Mediatech Inc., Herndon, VA) supplemented with 5% inactivated fetal calf serum (IFCS) (Hyclone Laboratories, Logan, UT), 5% Nu-Serum IV (Becton-Dickinson, Bedford, MA), 10^{-8}M dihydrotestosterone (Sigma-Aldrich, St. Louis, MO), 5μg/ml insulin (Sigma-Aldrich, St. Louis, MO), and 25u/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA). Pr14-2 cells were a kind gift from Dr. Jeffrey Green (NCI, Bethesda, MD) and were cultured in DMEM supplemented with 10% calf serum (Hyclone Laboratories) and 25u/ml penicillin-streptomycin (Invitrogen). Vero (African green monkey kidney) cells (ATCC) were cultured in DMEM with glucose (4.5 g/l) supplemented with 10% calf serum.

Viruses

Purified virus stocks of G207, G47Δ, NV1023, NV1034, and NV1042 were obtained from MediGene Inc (San Diego, CA). Construction of these viruses have been described previously: G207 [13], G47Δ [19], NV1020 [17], NV1034 and NV1042 [16]. The viruses were individually titered on Vero (African green monkey kidney) cells by plaque assay.

MHC Class I Analysis and IFN-γ Treatment of Cell Lines

Single cell suspensions of TRAMP-C2 and Pr14-2 cell lines were incubated with their respective MHC class I specific antibodies, H-2K^b (clone AF6-88.5, BD Biosciences, San Jose, CA) or H-2K^q (clone KH114, BD Biosciences) or the isotype control antibodies for 30 min on ice. The cells were then washed with PBS three times, fixed in PBS with 1% paraformaldehyde and analyzed by FACS (BD FACScan). Each cell line was also treated with recombinant murine IFN-γ (Peprotech Inc., Rocky Hill, NJ) at 100u/ml for 72 hours to up-regulate MHC class I expression, which was analyzed by FACS staining as above.

***In vitro* Cytotoxicity Assays**

Monolayers of TRAMP-C2 and Pr14-2 cells at approximately 20% confluency were washed with PBS containing 1% IFCS and infected with viruses at multiplicity of infection (MOI) of 0.01, 0.1, and 1.0 for 2 hours at 37^{0}C. The virus inoculum was removed and replaced with DMEM supplemented with 1% IFCS. Cells were counted using a Coulter Counter on days 1, 2, and 3 post-infection.

***In vitro* Cytokine Expression**

Logarithmically growing monolayers of TRAMP-C2 and Pr14-2 cells were infected with either NV1034 (GM-CSF) or NV1042 (IL-12) viruses at MOI of 1.0 for 2 hours at 37^0C. The virus inoculum was removed and replaced with DMEM supplemented with 1% IFCS. Twenty-four hours post-infection, the supernatants were collected and assayed for murine GM-CSF or murine IL-12 by ELISA using Quantikine M kits (R&D Systems, Minneapolis, MN).

In vivo Tumor Models and Treatment

Animal procedures were conducted with approval from the Massachusetts General Hospital Subcommittee on Research Animal Care. All animal studies were blinded.

TRAMP-C2: $5x10^6$ TRAMP-C2 cells were implanted subcutaneously into the left flank of male C57Bl/6 mice and when the tumors grew to approximately 80-100 mm^3 in volume, they were treated intraneoplastically with $1x10^7$ plaque forming units (pfu) of either G207, G47Δ, NV1023, NV1034, NV1042 or virus buffer (Mock) on days 0 and 3. For the bilateral tumor model, $5x10^6$ cells were implanted in both the left and right flanks and the left tumors were treated as above, except on days 0, 3, 7 and 10. Tumor growth was monitored by measuring the length (l), width (w) and height (h) of the tumors and the tumor volume calculated as l x w x h. Mice were sacrificed when the tumor volume reached or exceeded 2400 mm^3.

Pr14-2: Tumor fragments of approximately $2mm^3$ were implanted subcutaneously into the left flank of heterozygous male C3(1)/T-Ag mice (National Cancer Institute, Frederick MD) or both the left and right flanks for the bilateral tumor model, and treated as above in the TRAMP-C2 model.

Efficacy Mechanism studies

Mice with established TRAMP-C2 unilateral subcutaneous tumors were treated with $1x10^7$ pfu of either NV1023, NV1042 or virus buffer (Mock) on days 0 and 3. Six mice from each group were sacrificed on days 4, 6, and 10. Blood and tumors were collected from 3 mice to determine the

levels of IL-12 in serum and the tumor. Another 3 mice were perfused with Zamboni's fixative (1.8% paraformaldehyde, 7.5% picric acid, 0.19% EGTA, and 2mM Magnesium chloride, pH 7.3) and tumors excised and immediately frozen in dry ice with isopentane. 10μm thick cryostat sections were prepared for X-gal and immunohistochemistry.

a. **Serum and tumor IL-12 ELISA:** Blood was allowed to clot for 2 hours at room temperature and then centrifuged at 16,000g for 15 min to collect serum. Tumors resuspended in 2 volume of cold PBS were homogenized thoroughly and centrifuged to obtain cellular lysate. Sera and cellular lysate were assayed for IL-12 by ELISA using Quantikine M kits (R&D Systems, Minneapolis, MN).

b. **X-gal staining:** Sections were fixed with 2% paraformaldehyde in PBS for 10 min and the fixative washed away thoroughly with PBS. Following incubation with PBS containing 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 (NP-40) at 4°C for 10 min, the sections were stained with substrate solution (PBS, pH 7.2, containing 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, 1 mg/ml), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% NP-40) at 34^0C for 4 hr. Sections were washed with PBS/ 0.2mM EDTA and counterstained with eosin before mounting.

c. **CD4/CD8/Macrophage Staining:** Cryostat sections were fixed in cold acetone for 15 minutes and then treated with 0.3% hydrogen peroxide in methanol for 5 min. Following pre-treatment with PBS containing 20% IFCS and 0.1% Triton X-100 for 10 min, the sections were incubated with biotin conjugated antibodies to either CD4 (clone RM4-5, BD Biosciences), or CD8 (clone 53-6.7, BD Biosciences) or Mac-3 (clone M3/84, BD Biosciences) for 2 hours at room temperature. The antibodies were washed off with PBS and incubated for 30 min with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA). The sections were washed again with PBS and developed using diaminobenzidine tetrahydrochloride (DAB) as substrate for 3-5 minutes. Excess substrate was washed off and

the sections counterstained with hematoxylin for 20 seconds. Evaluation of various immune populations was performed by a pathologist in a blinded manner. Three random fields per section from three mice from each treatment groups were analyzed and the average quantitative score for each group recorded.

d. **CD31 Staining:** Cryostat sections were fixed in 4% paraformaldehyde for 10 minutes, washed with PBS and incubated with 3% goat normal serum for 30 min at room temperature. The sections were incubated with antibody to CD31 (rat anti-mouse CD31, clone MEC13.3, BD Biosciences) for overnight in a humidified chamber at 4^0C. Following thorough washing with PBS, the sections were incubated with biotinylated secondary anti-rat IgG (Vector Laboratories) for 30 min at room temperature. The sections were processed further as above with Vectastain Elite ABC reagent kit as described above.

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LEGENDS

Figure 1: MHC Class I expression and induction by IFN-γ in mouse prostate cancer cell lines. TRAMP-C2 and Pr14-2 cell lines were grown in the presence or absence of IFN-γ (100u/ml of growth medium) for 72 hours and analyzed for MHC class I molecules, H-2K^b and H-2K^q, respectively by FACS analysis. **(A)**: The basal levels of expression of MHC class I molecules on TRAMP-C2 cells is very low (~2%) whereas Pr14-2 cells express high levels (~91%). IFN-γ induces the expression of Class I molecules to greater than 94% on both cell lines. **(B):** FACS histogram of Pr14-2 cells in the absence (dotted line) and presence (solid line) of IFN-γ treatment showing an increase in the level of expression of MHC class I molecules post-treatment, as indicated by the shift in the curves. The shaded area represents the isotype antibody control.

Fig 2: *In vitro* and *in vivo* susceptibility of TRAMP-C2 cells to oncolytic HSVs. (A): TRAMP-C2 cells at 20% confluency were infected with G207, G47Δ or NV1023 or virus buffer (control) at various MOIs and cell viability assessed on days 1, 2, and 3 post-infection. Data shown are percent of uninfected control. These cells were generally resistant to all the viruses except at an MOI of 1.0. **(B):** Subcutaneous TRAMP-C2 tumors established in male C57Bl/56 mice were treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of G207, G47Δ or NV1023 or virus buffer (Mock); n = 7/group. NV1023 was most effective in inhibiting the growth of the tumors as compared to Mock, G207 and G47Δ ($p<0.05$, student's t test; days 19, 23) and extending survival of the treated mice ($p<0.05$ vs. Mock, G207 and G47Δ; Logrank (Mantel-Cox) test).

Fig. 3: Efficacy of oncolytic cytokine HSVs in syngeneic mouse prostate cancer models. TRAMP-C2 **(A)** and Pr14-2 **(B)** tumors were established subcutaneously in male C57Bl/6 and heterozygous C3(1)/T-Ag mice respectively, and treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of NV1023, NV1034 (GM-CSF), NV1042 (IL-12) or virus buffer (Mock); n = 7/ group. In both tumor models, NV1042 virus was significantly more effective in tumor growth inhibition ($p<0.05$, student's t test; from day 7 (TRAMP-C2) and day 17 (Pr14-2) vs. Mock, NV1023 and NV1034) as well as prolonging median survival. (TRAMP-C2: 38 days vs. 31 days with other treatments; Pr14-2: 75 days vs. 54 days with other treatments) ($p<0.05$, Logrank (Mantel-Cox) test).

Fig 4: Efficacy of NV1042 in bilateral mouse prostate cancer models. Subcutaneous TRAMP-C2 **(A)** and Pr14-2 **(B)** tumors were established on both flanks of male C57Bl/6 and heterozygous C3(1)/T-Ag mice respectively and treated intraneoplastically on days 0, 3, 7, and 10 with $1x10^7$ pfu of NV1023, NV1042 (IL-12) or virus buffer (Mock); n = 11 (TRAMP-C2) or 8 (Pr14-2)/ group. In both tumor models, NV1042 significantly inhibited the growth of the inoculated (from day 7 in TRAMP-C2 and day 10 in Pr14-2) and non-inoculated tumor (from day 27 in TRAMP-C2 and day 21 in Pr14-2) ($p<0.05$ vs. Mock, NV1023; student's t test).

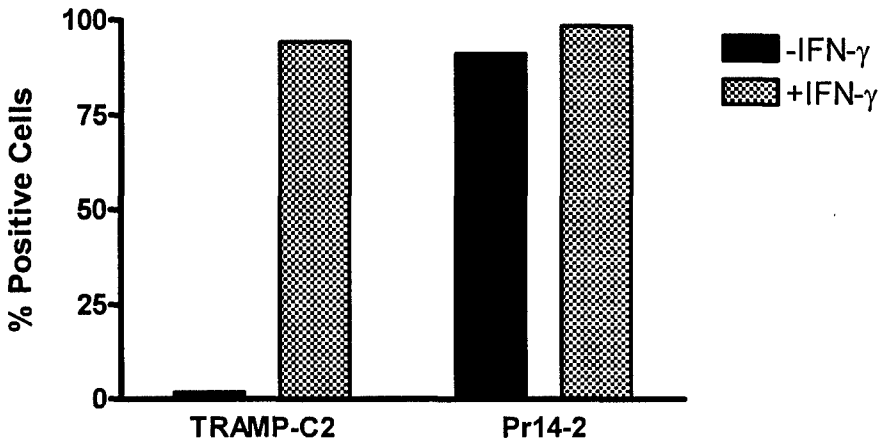
Fig 5: Serum and tumor IL-12 levels in virus treated TRAMP-C2 tumors. Subcutaneous TRAMP-C2 tumors were treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of NV1023 or NV1042 (IL-12), or virus buffer (Mock); n = 18/ virus treatment group; n = 6/ Mock. Groups of 3 mice were sacrificed on day 4, 6 and 10 (Mock only on day 4) and IL-12 levels measured by ELISA from serum **(A)** and tumor **(B)**. Data shown is the mean of 3 mice from each group and the error bars represent S.D. values. Serum IL-12 levels in the virus treated groups on any day post-treatment were not significantly different (NV1042 or NV1023 vs. Mock; student's t test). Measurable levels of IL-12 were detected only in NV1042 treated tumors.

Fig 6: X-gal staining of TRAMP-C2 tumors inoculated with NV1042. Subcutaneous TRAMP-C2 tumors were treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of NV1042 and sacrificed on day 4 **(A)**, day 6 **(B)** and day 10 **(C)**. Tumors sections (10μm) were stained for Lac Z expression by X-gal histochemistry. Maximal staining was observed on day 6 post-treatment, adjoining areas of significant cell death. Magnification: Day 4- 200X; Day 6- 125X; Day 10- 125X.

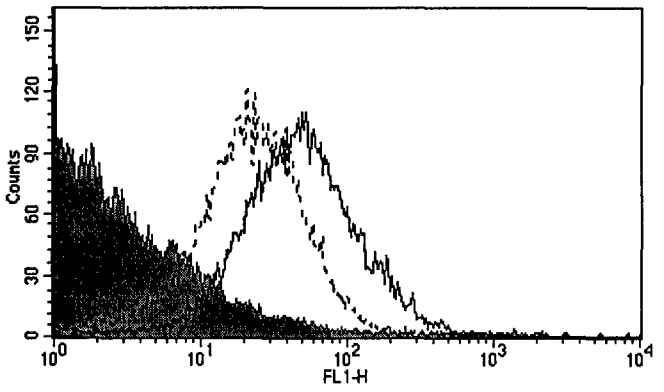
Fig 7: CD31 immunostaining of virus treated TRAMP-C2 tumors. Subcutaneous TRAMP-C2 tumors were treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of NV1023 or NV1042, or virus buffer (Mock). Tumor sections (10μm) obtained from mice sacrificed on day 10 were immunostained for CD31 molecules. Photomicrographs (400X) illustrate that while NV1023 treatment **(B)** led to a decrease in CD31 staining as compared to the Mock treated group **(A)**, a dramatic reduction in CD31-positive cells was observed in tumors treated with NV1042 **(C)**.

Table 1: CD4/ CD8/ Macrophage immunostaining of TRAMP-C2 tumors. Subcutaneous TRAMP-C2 tumors were treated intraneoplastically on days 0 and 3 with $1x10^7$ pfu of NV1023 or NV1042, or virus buffer (Mock). Tumor sections (10μm) obtained from mice sacrificed on day 10 were immunostained for CD4 or CD8 or Mac-3 molecules. Three random fields per section from 3 mice for each treatment group were scored and the average score recorded. [- : <5%, +: 5-25%, ++: 25-50% of field staining positive].

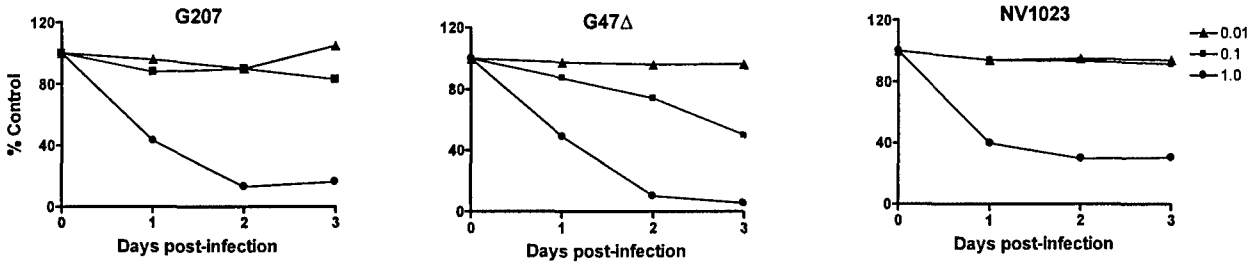
Varghese et al
Fig 1A



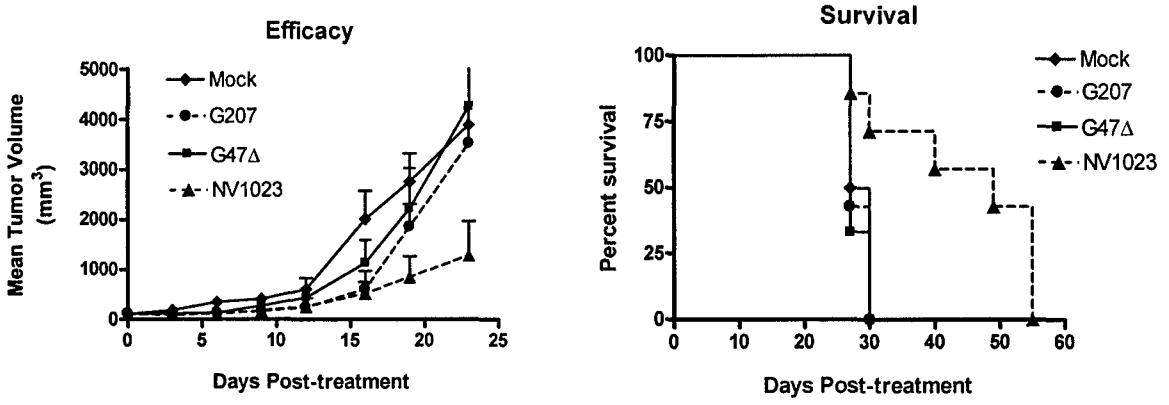
Varghese et al
Fig 1B



Varghese et al
Fig 2A

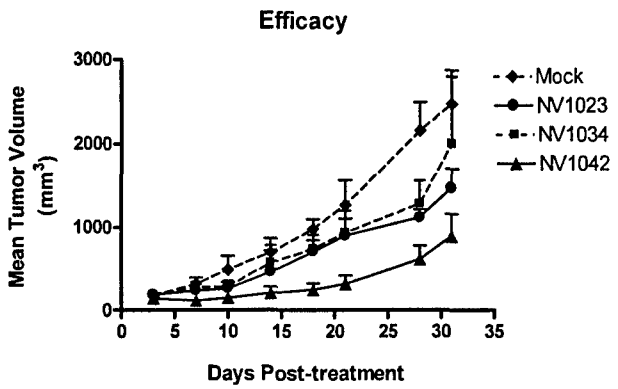


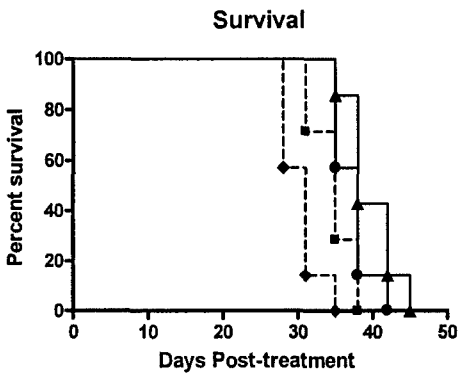
Varghese et al
Fig 2B



Varghese et al
Fig 3A

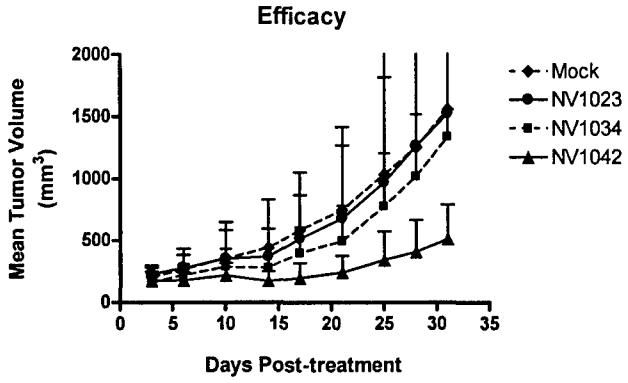
A: TRAMP-C2

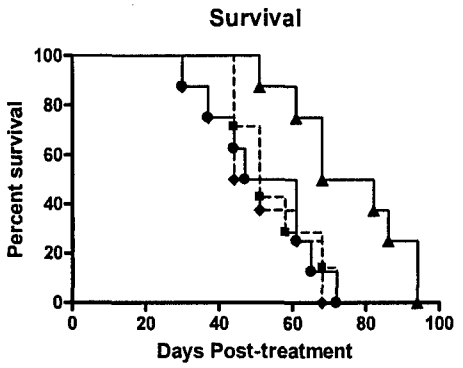




Varghese et al
Fig 3B

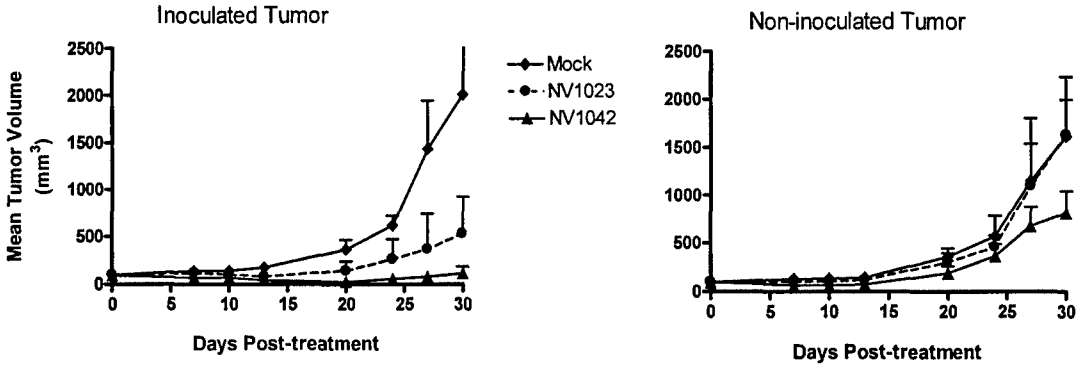
B: Pr14-2





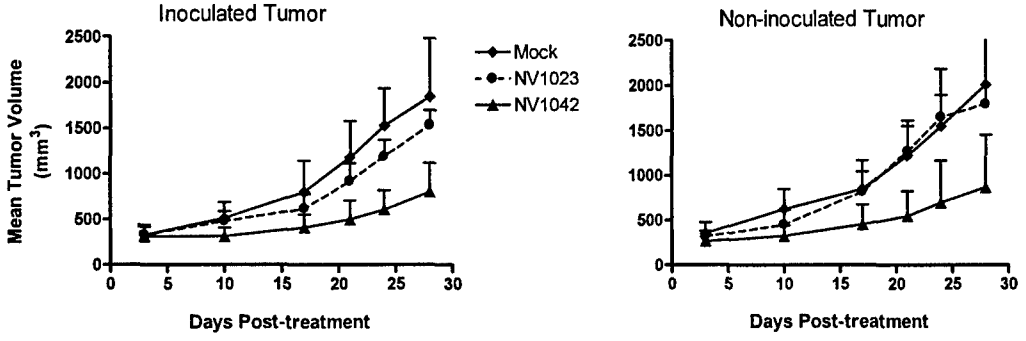
Varghese et al
Fig 4A

A: TRAMP-C2



Varghese et al
Fig 4B

B: Pr14-2



Varghese et al
Fig 5A

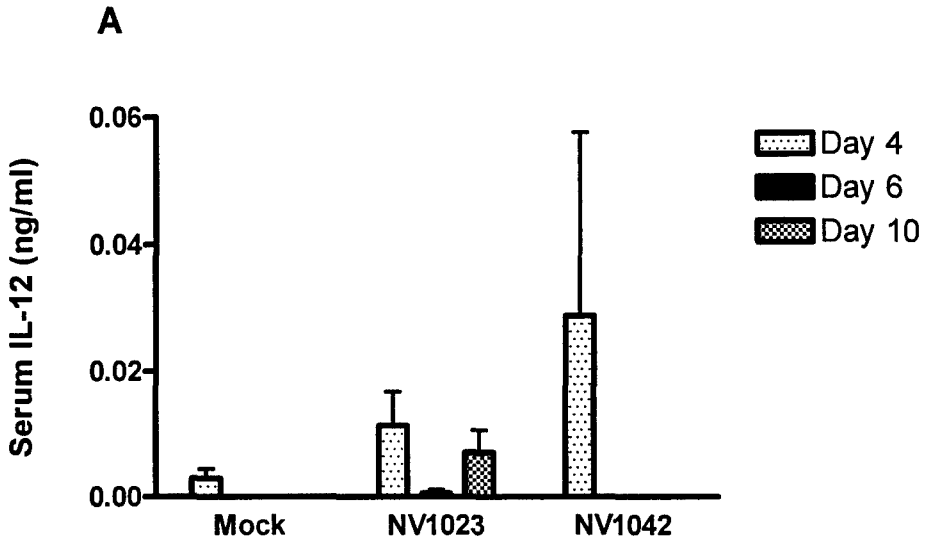


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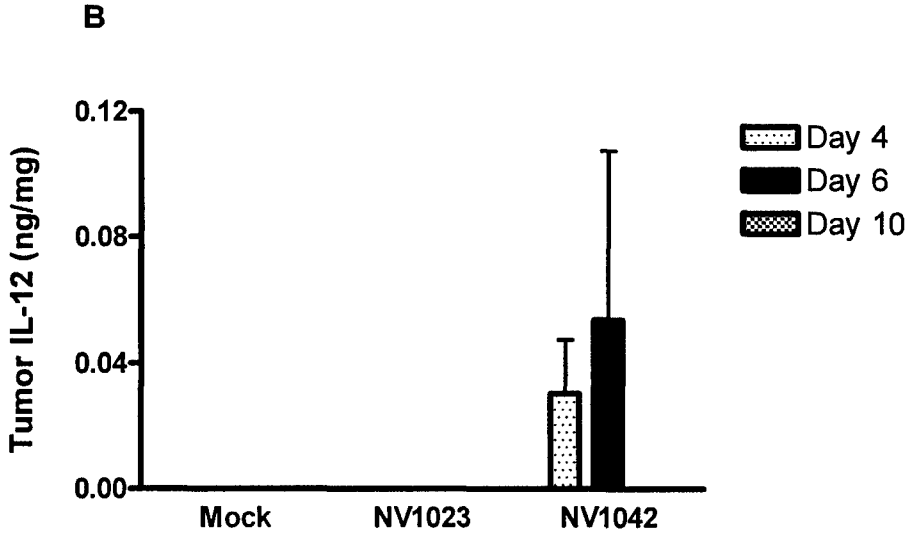
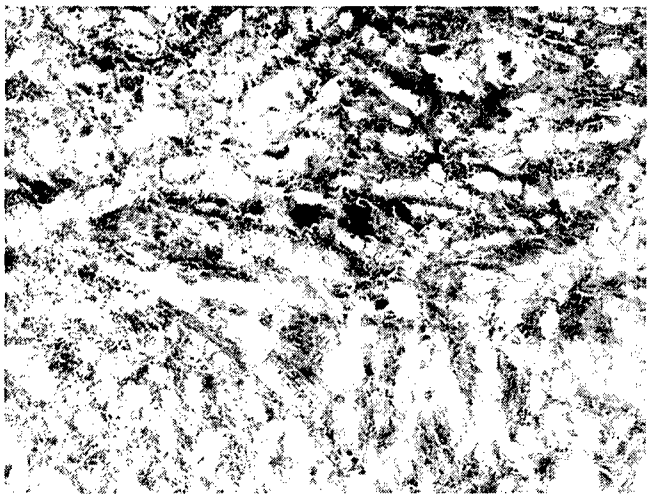
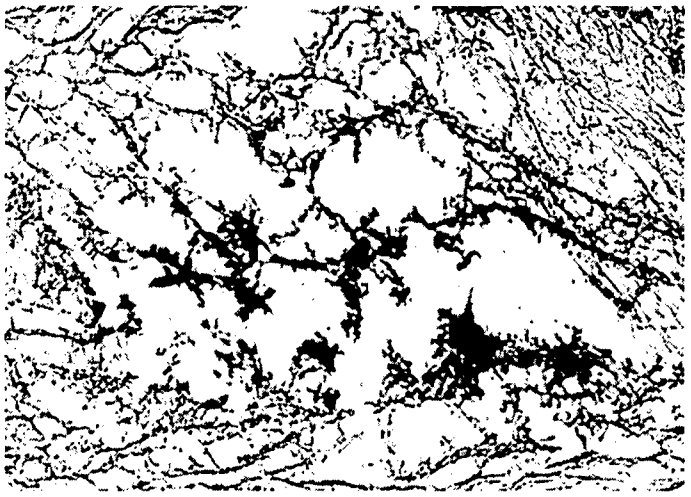
Fig 5B



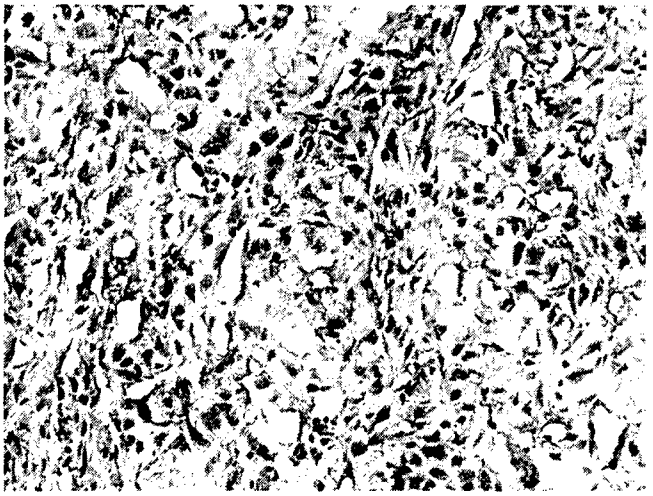


Day 4

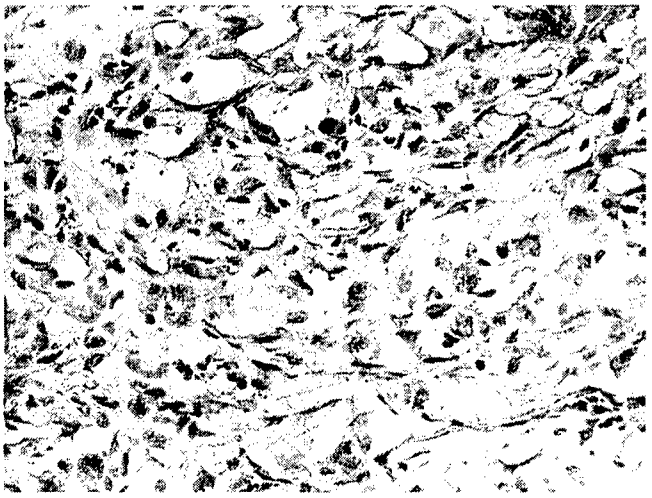
Day 6



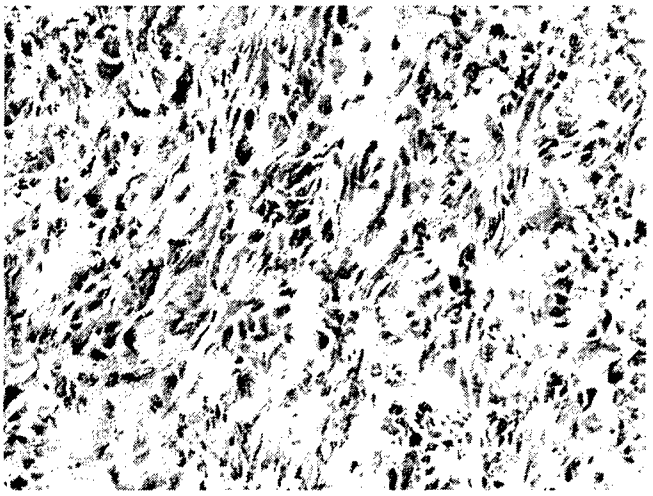
Day 10



Mock



NV1023



NV1042

Table 1

	Post-treatment	CD4	CD8	Mac-3
Mock		-	-	+
NV1023	Day 4	-	+	+
	Day 6	-	-	+
	Day 10	-	-	+
NV1042	Day 4	-	+	+
	Day 6	+	+	+ +
	Day 10	+	+	+