Cytokine-modified VSV is attenuated for neural pathology, but is both highly immunogenic and oncolytic

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Abstract: Vesicular stomatitis virus (VSV), an enveloped, nonsegmented, negative-stranded RNA virus, is being tested by several laboratories as an antitumor agent. Unfortunately, viral infection of the central nervous system (CNS) has been observed by many groups following administration to tumor-bearing animals. In rodents, VSV encephalitis is characterized by weight-loss, paralysis, and high mortality. In order to provide protection from VSV infection of the CNS after therapeutic administration, we have attenuated VSV by the introduction of the gene encoding the proinflammatory cytokine interleukin (IL)-23, and designated the new virus VSV23. We hypothesize that while VSV23 is replicating within tumors, resulting in tumor destruction, the expression of IL-23 will enhance host antitumor and antiviral immune responses. In the event that the virus escapes from the tumor, the host’s immune system will be activated and the virus will be rapidly cleared from healthy tissue. Experimental VSV23 infection of the CNS is characterized by decreased viral replication, morbidity, and mortality. VSV23 is capable of stimulating the enhanced production of nitric oxide in the CNS, which is critical for elimination of VSV from infected neurons. Intraperitoneal administration of VSV23 stimulates both nonspecific natural killer cell, virus-specific cytolytic T lymphocyte and memory virus-specific proliferative T cell responses against wild-type VSV in splenocytes. Furthermore, VSV23 is able to replicate in, and induce apoptosis of tumor cells in vitro. These data indicate that VSV23 is immunogenic, attenuated and suitable for testing as an efficacious and safe oncolytic agent.

Keywords: IL-23, oncolytic, tumor, VSV

Introduction

Vesicular stomatitis virus (VSV), a prototypical member of the genus Vesiculovirus, is a naturally occurring virus which is transmitted by sand flies to livestock and causes the eponymous small oral rashes. VSV infections in humans tend to be asymptomatic and generally limited to researchers and livestock handlers. Therefore, most humans are seronegative.1-2 VSV is extremely sensitive to antiviral host cell interferon (IFN) responses.3-5 IFNs induced by VSV infection may help eliminate the virus and protect otherwise uninfected tissues, however blocking of host nuclear export by the VSV M protein is associated with viral evasion of IFN-induced cellular defenses.6-8 VSV rapidly replicates and induces apoptosis in cells that have compromised interferon pathways, a common characteristic of many tumor cell lines.9-15 These traits make VSV an attractive agent for utilization as an oncolytic agent.

Inoculation of immunocompetent mice with high doses of VSV by the intramuscular, subcutaneous, or intraperitoneal (IP) routes generally leads to limited viral replication and no apparent disease.16-18 Intravenous inoculation of immunocompetent
mice with high doses of VSV can lead to very limited viral replication in the periphery, but can cause central nervous system (CNS) pathology if the virus gains access to the brain despite the induction of antibody responses.\textsuperscript{17,19,20} Immunocompromising the IFN signaling cascade or T cell deficiency leads to death following host infection.\textsuperscript{18,21–23} Studies have shown that if VSV infection of tumors reaches the host’s CNS, viral encephalitis will result.\textsuperscript{9,24} Infection via intranasal (IN) administration results in severe pathology characterized by weight loss, hind-limb paralysis, and death.\textsuperscript{16,25,26} Immunocompetent mice develop high morbidity and mortality following IN infection at viral doses as low as 200 plaque-forming units (PFU), succumbing to infection between 6–11 days postinfection (PI). A recent study in nonhuman primates demonstrated significant neuropathology following intrathalamic inoculation.\textsuperscript{27} The neurotropism and neuropathology of VSV necessitates the development of an attenuated virus if VSV is to be used as a therapeutic.

During VSV infection of the CNS, neutrophils, natural killer (NK) cells, macrophages, and T cells are recruited to attack the virus at the site of infection.\textsuperscript{18,26,28–30} Plasmacytoid dendritic cells (pDC) in the lymph nodes and spleen respond to VSV infection through TLR 7.\textsuperscript{31,32} Within 36 hours of infection, neutrophils are recruited from circulation,\textsuperscript{26,29} NK cells are detected at day three and onward in the brains of VSV infected hosts.\textsuperscript{26,29,33} Neutrophils play an early role in control of the rate of infection, as they produce nitric oxide (NO), OONO•, lipid mediators, as well as pro-inflammatory chemokines and cytokines, critical components of immunity in controlling early infections of the CNS.\textsuperscript{28} VSV proteins, processed by antigen-presenting cells, result in the induction of the adaptive immune response: CD4+ and CD8+ T cells as well as anti-VSV antibodies.\textsuperscript{34–38} The essential role of MHC II-restricted viral glycoprotein CD4 effector cells in recovery from viral encephalitis was shown in the BALB/c\textsuperscript{H-2\textdagger} mouse strain.\textsuperscript{35} Neutralizing antibodies are directed against the glycoprotein with IgM produced first and IgG second.\textsuperscript{20,39} Interleukin (IL)-23 is a heterodimeric member of the IL-12 family of cytokines composed of a p40 subunit shared with IL-12 and a unique p19 subunit. A heterodimeric receptor IL-23R includes a unique chain and the IL-12R\textbeta\textsubscript{1} subunit.\textsuperscript{40} IL-23R is expressed on the surface of immune cells including T cells, NK cells, macrophages, and dendritic cells. Binding of IL-23 to IL-23R results in signaling through the Jak, Tyk, signal transducers and activators of transcription (STAT) pathways and the transcription factor RoRγT.\textsuperscript{41,42} IL-23 is produced by macrophages and dendritic cells.\textsuperscript{43} IL-23 also plays a role in the differentiation of proinflammatory T cells secreting IL-17.\textsuperscript{44} The IL-23/IL-17 cascade has been associated with several inflammatory illnesses including rheumatoid arthritis, psoriasis, and experimental allergic encephalomyelitis (EAE).\textsuperscript{45–48} Antimetastatic and antitumor properties have been associated with IL-23 and IL-12, however the therapeutic use of IL-12 in cancer treatment has been compromised by side-effects associated with the induction of IFN-γ.\textsuperscript{49–54} The antitumor effects associated with IL-23 are attributed to production of tumor necrosis factor-α (TNF-α) (not IFN-γ), and result in enhanced effectiveness of CD8+ T cells.\textsuperscript{55} Experiments indicated that depletion of NK cells had no affect on the antitumor activity of IL-23 as determined by tumor growth rate, however CD8+ T cell depletion significantly compromised the ability of naive mice to reject implanted tumors and limit tumor growth rates.\textsuperscript{56} Experiments conducted with IL-23-transduced CT26 cells and B16F1 tumor cells have shown increased antitumor memory T cell responses and decreased lung metastases in vivo.\textsuperscript{57} Experiments conducted with the murine mammary cancer cell line MA891, have shown that expression of IL-23 through retrovirus transduction resulted in increased antitumor CTL effectors.\textsuperscript{58} Based on IL-23’s proinflammatory activity in the CNS, we hypothesized that a recombinant VSV which expressed IL-23 would be attenuated in the CNS while still being fully immunogenic in the periphery. Additionally, this recombinant virus would maintain (and possibly enhance) its oncolytic capacity. To test this hypothesis we have generated a recombinant VSV that expresses a single chain IL-23 designated VSV23. Assays of innate and adaptive immune responses following parenteral challenge were performed to determine the immunogenicity of the virus. VSV23 has also been tested for attenuation in vitro and in vivo in the in model of VSV encephalitis. Finally the ability of VSV23 to infect and kill a mammary derived tumor cell line in vitro has been determined.

Materials and methods

Plasmid production

To produce a recombinant VSV that expresses IL-23, single-chain IL-23 (scIL23) comprised of the p40 and p19 subunits joined with a flexible linker [(Gly\_Ser)\_n] was amplified by PCR from plasmid pCEP4-scIL23lg, a generous gift from Dr Maria Laura Belladonna (University of Perugia, Italy).\textsuperscript{58} This reaction removed an Ig binding region from...
the 3’ end and introduced Xhol and SpeI restriction sites at the 5’ and 3’ ends respectively, as well as a stop codon at the 3’ end. The forward primer sequence was 5’-TAGTCCTCGAGATGTGTCCATCAAGCTAACCATCT-3’ and the reverse primer was 5’-TATGACTATGCTAAGCTTG-GCACTAAGGCCTGTT-3’. The amplified region was cloned into the VSV expression vector (pXN2) (the generous gift of Jack Rose, Yale University School of Medicine, New Haven, CT) and the resultant plasmid was designated pXN2-scIL23.  

To generate a control virus containing the IL-23 coding sequence, scIL23 was amplified from pCEP4-scIL23l, restriction digested with KpnI and XbaI, then ligated with the intermediate vector pSP73. Three stop codons were introduced into the p40 subunit using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) per manufacturer’s directions. The mutagenesis forward primer was 5’-ACTCCGGACGTTTCAGTATGATGACGT-GTGCAGAGAAGACATGG-3’ and the reverse primer was 5’-CCATGTTTCTTTCAGACCAGTCATCATCAGGT-GAACCCTCGAGGT-3’. XL1-Blue cells (Strategene) were transformed with the mutagenesis reaction polymerase chain reaction (PCR) product and plated on LB-Amp plates. Plasmids isolated from colonies and correct mutations were identified by sequencing at the New York University (NYU) Sequencing Core. Positive sequences were then subjected to PCR and subsequent cloning to the pXN2 plasmid as described above. The resultant plasmid was designated pXN2-scIL23ST.

Cell lines
BHK-21 baby hamster kidney cells, JC murine mammary gland adenocarcinoma-derived cells, L929 murine adipocytes and NB41A3 murine neuroblastoma cells were all purchased from the American Type Culture Collection (Manassas, VA). BHK-21 cells were grown in minimum essential media (Mediatech, Manassas, VA) with 1% nonessential amino acids, 1% penicillin-streptomycin (pen-strep) and 10% fetal bovine serum (FBS), JC cells grown in RPMI1640 (Mediatech) with 1% pen-strep and 10% FBS, L929 cells grown Dulbecco’s modified Eagles’ medium (Mediatech) with 1% pen-strep, 1% HEPES buffer, 1% L-glutamine and 10% fetal bovine serum (FBS), NB41A3 grown in F-12K media (Mediatech) with 2.5 FBS and 15% horse serum.

Recombinant VSV rescue
Recombinant VSVs (rVSVs) were rescued in BHK-21 cells using the previously described reverse genetics method.  

Briefly, cells were infected with vaccinia virus expressing the T7 RNA polymerase, then transfected with pXN2-scIL23, pXN2-scIL23ST, or pXN2 to produce VSV23, VSVST, and VSVXN2 respectively. In addition, plasmids encoding N, P, and L proteins were co-transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Vaccinia virus was removed by filtration through a 0.20 µm filter after 48 hours of incubation. Filtrate was added to fresh BHK-21 cells. Subsequently, individual clones were plaque purified and used for production of viral stocks. Titers of rVSV were determined by plaque assay on L929 cells.

One-step growth curve
L929 cells were grown to 90% confluence in 24-well plates and infected with VSV23, VSVST, VSVXN2, or VSVwt (Indiana serotype, San Juan strain, originally obtained from Alice S. Huang, then at The Childrens’ Hospital, Boston, MA) at a multiplicity of infection (MOI) = 1 for 30 minutes at room temperature (RT). Wells were washed with HBSS to remove unadsorbed virus and media was added to each well. Aliquots of media were removed at 1.5, 3, 6, 12, and 24 hours and stored at –80°C. Viral titers were determined by plaque assay on L929 cells. All samples were assayed in triplicate and the experiment repeated twice.

ELISA for virally produced IL-23
HK-21 cells were infected with VSV23, VSVST, or VSVXN2 at MOI = 0.1 and incubated overnight at 37°C and 5% CO₂. Uninfected BHK-21 cells were used as a negative control. Supernatants were harvested and subjected to ELISA analysis specific for the p40 subunit of IL-23 using the mouse IL-12/IL-23 Total p40 ELISA kit (eBioscience, San Diego, CA).

Animals
All procedures involving animals were approved by and performed according to the guidelines of The University Animal Welfare Committee of New York University. Six-week old male BALB/cAnNTac (BALB/c) mice were purchased from Taconic Farms, Inc. (Germantown, NY), housed under standard conditions and fed ad libitum. Mice were housed at NYU for one week prior to initiation of experiments.

Induction of IFN-γ
Spleens were aseptically harvested from eight-week old male BALB/c mice and teased into single cell suspensions. CD4⁺ cells were isolated using the DynaMAG Mouse CD4 Negative Isolation Kit (Oslo, Norway) and added to 6-well plates from Fisher Scientific (Pittsburgh, PA) with DMEM supplemented...
with 10% heat inactivated FBS and 1% pen/strep. CD4+ cells were treated with ultraviolet-inactivated supernatants from BHK-21 cells containing 500 pg of VSV23 induced IL-23 (vIL-23). UV-inactivated supernatants containing 500 pg of virally induced IL-12 (vIL-12) from BHK-21 cells infected with a rVSV expressing IL-12, [the generous gift of Dr Savio Woo (Mt Sinai School of Medicine, New York, NY)] were also tested.60 500 pg of recombinant IL-23 (rIL-23) or rIL-12 (R&D Systems Minneapolis, MN) were used to treat cells as positive cytokine controls and supernatant from untreated/uninfected BHK-21 cells was used as a negative control. Samples were incubated at 37 °C and 5% CO2 for six hours and RNA was isolated with Trizol® reagent (Invitrogen, San Diego, CA). RNA was subjected to reverse transcriptase-PCR for detection IFN-γ mRNA. The forward primer was 5'-GCTTTGCAGCTCTTCCTCAT-3' and the reverse primer was 5'-TGAGCTATTGAGCTTGGG-3'. Beta actin was used as a housekeeping control for the reaction. The forward primer was 5'-AAGAGCTATGAGCTGCTGTA-3' and the reverse primer was 5'-TACGGATGTCAACGTCACAC-3'.

**NK assay**

Cohorts of \(N=6\), six-week old male BALB/c mice were inoculated IP with \(1 \times 10^6\) pfu of VSV23, VSVST, VSVXN2, VSVwt, or mock infected as a control. Three days later, spleens from individual mice were harvested, teased into a single cell suspension, and resuspended in MEM supplemented with 10% FBS, 1% Pen-Strep, 1% Pen-Strep, 5 mM 2-mercaptoethanol, and 1% L-glutamine at 37 °C, 5% CO2. A20 cells (syngeneic H-2d MHC I and MHC II-expressing) were used as target cells. Target cells were either infected with VSVwt at multiplicity of infection (MOI) = 3 or mock-infected and plated in 96 well V-bottom plates at \(1 \times 10^5\) cells per well. Responder cells from individual mice of each treatment group were added to target cells in triplicate at effector to target ratios of 100:1, 50:1, 25:1, and 12:1. Plates were centrifuged for five minutes at \(200 \times g\) to improve cell contacts and incubated for four hours at 37 °C, 5% CO2. The CytoTox 96® nonradioactive cytotoxicity kit (Promega) was used per manufacturer’s instructions to determine T cell mediated cytolytic activity. Results were read on a Biorad 550 series microplate reader at 490 nm. Results are representative of two replicate experiments.

**CTL assay**

Cohorts of \(N=6\), six-week old male BALB/c mice were inoculated IP with \(1 \times 10^7\) pfu of VSV23, VSVST, VSVXN2, or VSVwt, to produce responder cells. Mock-injected mice were used as a negative control and as a source of naive controls for effector cells. Twenty days after immunization, spleens from individual mice were harvested, teased into a single cell suspension, and resuspended in MEM, 10% FBS, 1% Pen-Strep. Stimulator cells were prepared by infecting naive splenocytes with VSV tsG41 at MOI = 5 at the permissive temperature of 33 °C for one hour.7 Cells were washed in HBSS to remove unadsorbed virions. \(5 \times 10^6\) responder cells were cultured with \(1 \times 10^5\) VSV tsG41 stimulator cells or with uninfected stimulator cells for five days in DMEM, 10% FBS, 1% Pen-Strep, 5 mM 2-mercaptoethanol, and 1% L-glutamine at 37 °C, 5% CO2. A20 cells (syngeneic H-2d MHC I and MHC II-expressing) were used as target cells. Target cells were either infected with VSVwt at multiplicity of infection (MOI) = 3 or mock-infected and plated in 96 well V-bottom plates at \(1 \times 10^5\) cells per well. Responder cells from individual mice of each treatment group were added to target cells in triplicate at effector to target ratios of 100:1, 50:1, 25:1, and 12:1. Plates were centrifuged for five minutes at \(200 \times g\) to improve cell contacts and incubated for four hours at 37 °C, 5% CO2. The CytoTox 96® nonradioactive cytotoxicity kit (Promega) was used per manufacturer’s instructions to determine T cell mediated cytolytic activity. Results were read on a Biorad 550 series microplate reader at 490 nm. Results are representative of two replicate experiments.

**Proliferation assay**

Cohorts of \(N=6\), six-week-old male BALB/c mice were inoculated IP with \(1 \times 10^7\) pfu of VSV23, VSVST, VSVXN2, or VSVwt to produce responder cells. Mock-treated mice were used as a negative control. Twenty days later, spleens were harvested, teased into a single cell suspension, and resuspended in MEM supplemented with 10% FBS and 1% Pen-Strep. Stimulator cells were prepared by treating naive spleen cells with five pfu of VSVsG41 per cell at the permissive temperature of 33 °C for 1 hour. Cells were washed in HBSS to remove unabsorbed virions. \(1 \times 10^5\) responder cells from individual mice were seeded in a 96-well plate, in triplicate, with either \(1 \times 10^5\) of VSVsG41 infected or uninfected stimulator cells and allowed to incubate for 3 days in MEM supplemented with 10% FBS, 1% Pen-Strep, 2-mercaptoethanol, and L-glutamine at 37 °C, 5% CO2. The Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, Indianapolis, IN) was utilized per manufacturer’s instructions and the results read on a Biorad 550 series microplate reader at 490 nm. Results are representative of two replicate experiments.

**Neutralization assay**

Cohorts of \(N=10\), six-week old male BALB/c mice were infected with \(1 \times 10^7\) pfu of VSV23, VSVST, VSVXN2, or VSVwt. Uninfected animals were used as a control.
Blood samples were collected from the ocular plexus 20 days PI from surviving individual animals and allowed to clot overnight at 4 °C. Serum was diluted in PBS in serial five-fold steps. 1 × 10^6 pfu of VSVwt was added to each dilution and incubated at 37 °C 5% CO₂ for 1 hour. Plaque assays were performed in triplicate samples on L929 cells and neutralizing antibody titers were calculated. Results are representative of three replicate experiments.

**Viral titers and nitric oxide levels in CNS**

Cohorts of N = 6, six-week-old male BALB/c mice were infected IN with 1 × 10⁴ pfu or 1 × 10⁵ pfu of VSV23, VSVST, or VSVXN2. Individuals were sacrificed on days 1, 3, 6, and 9 PI and brains were divided sagittally. One half was reserved for immunohistochemical staining. The other brain half was individually homogenized, and an aliquot was serially diluted, and assayed in triplicate by plaque assay on L929 cells for the presence of VSV. Geometric mean titers were calculated for each cohort. Homogenate samples for NO assays were pre-cleared of solid material by centrifugation. The Total Nitric Oxide Assay Kit (Pierce, Rockford, IL) was used as per manufacturer’s instructions to convert nitrate to nitrite from individual samples. Equal volumes of experimental sample and Greiss reagent (1% sulfanilamide, 0.1% N-naphthylethylene-diamine, and 5% H₃PO₄; Sigma-Aldrich, St. Louis, MO) were incubated in the dark at room temperature for 10 minutes and results were read on a Biorad 550 series microplate reader at 540 nM.

**Morbidity and mortality assay**

Cohorts of N = 10, six-week old male BALB/c mice were infected IN with 1 × 10⁴ or 1 × 10⁵ pfu of VSV23, VSVST, VSVXN2, or VSVwt and monitored daily for 15 days. Mice were weighed daily. Hind-limb paralysis or weight loss that exceeded 30% of starting weight were considered end points for the experiment. Mice were individually scored blind on a subjective six point scale (0–5): “0” for no symptoms, “1” for lack of grooming behavior, “2” for hunched and severely lethargic mice, “3” for hind-limb paralysis, “4” for full paralysis, and “5” for deceased. The experiment was single-blinded.

**In vitro infection of target JC cell**

JC cells were seeded in 96-well plates at a concentration of 4.5 × 10⁴ and incubated overnight at 37 °C, 5% CO₂. Six replicate wells were used for each treatment condition and time point. Cells were then infected at MOI = 3.0 with VSV23, VSVST, VSVXN2, or VSVwt and incubated at 37 °C 5% CO₂ for 3, 6, 9, 12, 18, or 24 hours. Mock infected cells were used as a negative control. The TACS MTT Cell Proliferation Assay (R&D Systems Minneapolis, MN) was used per manufacturer’s instructions to conduct the assay. Samples were read at 540 nM on a Biorad 550 series microplate reader.

**Statistical analysis**

GraphPad Prism statistical software (GraphPad Software Inc., La Jolla CA) was used for ANOVA, Kruskal–Wallis, Student’s t-test, and Kaplan–Meier statistical analyses.

**Results**

**Recombinant VSV construction and characterization**

In order to produce a rVSV that expresses IL-23, the cDNA of a single chain IL-23 (scIL-23) comprised of the p40 and p19 subunits joined by a flexible linker was cloned into the pXN2 plasmid (Figure 1A) to produce the pXN2-scIL23 plasmid (Figure 1B). IL-23 was chosen for its proinflammatory and intrinsic oncolytic properties. The reverse genetics method described by the Rose lab was used to produce VSV23. To control for attenuation of VSV resulting from alterations in genome size and particle morphology, two control viruses were produced utilizing the same reverse genetics approach. The first, VSVST, was generated by insertion of a mutagenized cDNA for IL-23 that contained three stop codons in the p40 subunit into pXN2 (Figure 1C). The second, VSVXN2, was rescued with an unaltered pXN2 plasmid. If VSV23 were to have impaired replication kinetics, this would be adverse to the intended use for oncolysis. In order to determine if the kinetics of VSV23 infection were similar to VSVwt and controls, a one-step growth curve was conducted in L929 cells. At all time points, viral titers were comparable, indicating that genome alterations do not result in diminished infectivity or reduced replication capacity (Figure 2). Data are representative of three replicate experiments and expressed as means ± standard deviation.

To determine if IL-23 were secreted by VSV23 infected cells, but not by cells infected with control viruses, we tested supernatants with an ELISA specific for the p40 subunit if IL-23. BHK-21 cells were infected at MOI = 0.1 and incubated for 24 hours. Only supernatants from VSV23 infected cells contained detectable levels of IL-23 (Figure 3A). Stop codons inserted into the p40 subunit of IL-23 in the VSVST viral genome successfully prevented transcription, translation and secretion of IL-23, thus validating the use of this virus as a control.
Among concerns about the efficacy of VSV23, was the ability to produce an active cytokine. To determine the bioactivity of virally induced IL-23 (vIL-23), isolated CD4⁺ cells were treated with vIL-23 and probed for IFN-γ mRNA. CD4⁺ cells were isolated from spleens of male BALB/c mice and incubated with supernatants from BHK-21 cells that had been infected for 24 hours with VSV23. Supernatants from BHK-21 cells that had been infected with a rVSV that expresses IL-12 were used as a positive control, along with commercially available IL-23 and IL-12. Mock infected cells and BHK-21 cells which were infected with VSVST or with VSVXN2 did not induce IFN-γ mRNA (data not shown).

**Figure 1** Virus production plasmids. A) VSVXN2 is a plasmid map of the 14.6 kb pXN2 plasmid that serves as the cloning vector in the recombinant VSV system and used for the rescue of VSVXN2. B) VSV23. A single chain IL23 was created by linking the coding regions for the p40 and p19 subunit with a flexible linker and inserted in the pXN2 expression vector at the XhoI/NheI mcs. The resultant plasmid was designated pXN2scIL23 and used for the rescue of VSV23. C) VSVST. Point mutations were induced in the pXN2scIL23 plasmid to introduce 3 stop codons in the p40 subunit coding region. This plasmid was used in the rescue of VSVST. All three plasmids were subsequently co-transfected into BHK-21 cells with 3 helper plasmids individually coding N, P, and L. A previous infection of vaccinia virus provides the T7 polymerase for expression of the viral genes in the cytoplasm of the cells.

**Abbreviation:** MCS, multiple cloning sites.

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**Figure 2** One-step growth curve: VSV23 (diamond), VSVST (circle), VSVXN2 (square), and VSVw (triangle) were used to infect L929 cells at an MOI = 1 in triplicate. Media samples were removed at 1.5, 3, 6, 12, and 24 hours and stored at −80 °C. Virally infected supernatants were serially diluted and transferred to fresh L929 cells for plaques assay. Growth kinetics for all viruses are similar at all time points. Data presented are geometric means ± standard deviation and are representative of three replicate experiments.

**Abbreviations:** PI, post infection; MOI, multiplicity of infection; VSV, vesicular stomatitis virus.
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Figure 3 VSV23 Produces Active IL-23. A) vIL23 production by VSV23 infected BHK21 cells. BHK21 cells were infected with VSV23, VSVST, or VSVXN2 at MOI = 0.1 and incubated overnight at 37 °C. Virally infected supernatant was harvested and subjected to UV inactivation to inactivate virus. Supernatant from uninfected BHK21 cells was used as a negative control. Samples were subjected to the Quantikine Mouse IL-12/IL-23 p40 (non-allele-specific) Immunoassay ELISA kit (R&D Systems Minneapolis, MN). Supernatant from VSV23 infected cells contained 750 pg/ml of the p40 subunit. The experiment indicates that there are no detectable levels of p40 secreted by cells infected with VSVST or VSVXN2. BHK21 cells do not produce IL-23 and as expected control samples did not produce detectable levels of the cytokine component. B) IFN-γ is induced by IL-23: Lanes represent amplified mRNA specific for IFN-γ from CD4+ cells that were treated as follows – 1: rIL-12 for 6 hours, 2: rIL-23 for 6 hours, 3: rIL-12 for 12 hours, 4: rIL-23 for 12 hours, 5: rIL-12 for 18 hours, 6: rIL-23 for 18 hours, 7: vIL-12 for 6 hours, 8: vIL-23 for 6 hours, 9: vIL-12 for 12 hours, 10: vIL-23 for 12 hours, 11: vIL-12 for 18 hours, 12: vIL-23 for 18 hours, 13: untreated CD4+ T cells. Upper band represents IFN-γ signal at 400 bp, lower band is GAPDH loading control at 200 bp. Each reaction was run in triplicate, and the experiment was repeated twice. Image is typical of all results.

Abbreviations: IFN, interferon; mRNA, messenger ribonucleic acid.

IL-12 treated cells showed IFN-γ mRNA expression at 12 and 18 hours (lanes 3 and 5, respectively, Figure 3B), but IL-23 treated cells only showed a positive signal at 18 hours (lane 6). vIL-12 treatment induced a strong signal at 12 and 18 hours (lanes 9 and 11, respectively), while vIL-23 treated cells again induced IFN-γ message at 18 hours (lane 12). Data presented are representative of three replicate experiments. From this we conclude the VSV23 does encode a functional IL-23 cytokine.

**VSV23 induces robust immune responses in the periphery**

Use of VSV23 as an oncolytic agent requires that the virus be capable of inducing immune responses necessary to protect noncancerous tissues from viral infection. Immunization of groups of mice with the panel of viruses was performed to determine if VSV23 were immunogenic in vivo following parenteral exposure. Subsequently, we evaluated nonantigen restricted innate immune responses, as well as antigen-specific targeted CTL and memory responses, and antibody production induced by VSV23 inoculation and infection.

**NK assay**

Viral infection or immunization is one of the most effective ways to induce NK cells, an innate immune response to infection which is not antigen-specific nor histocompatibility-restricted.62 NK lytic activity can be quantified by measuring lysis of sensitive cells such as YAC-1.63 NK activation was
tested 3 days post-inoculation with the panel of viruses. Splenocytes from individual donors were tested separately. Both mock and uninfected splenocytes showed similar background levels of cell lysis indicating no NK mediated cell killing in either of these infection groups (Figure 4A). Data are representative of 3 replicate experiments; data are expressed as means ± standard deviation of 6 individuals per group. The NK cytolytic responses elicited by VSV23 inoculation were indistinguishable from those induced by VSVST, VSVXN2, and VSVwt.

**CTL assay**

The ability of viruses to elicit host CTL responses which control infection and promote recovery is a hallmark of host acquired immunity to infection. A secondary CTL experiment was conducted to determine VSV specific recognition of syngeneic target cells 20 days after immunization of mice with the panel of viruses. There was no lysis of uninfected A20 cells, and virus infection of stimulator cells was required to induce CTL activity (data not shown). All splenocytes cultured with VSV tsG41 (a temperature
sensitive VSV mutant) infected stimulators developed CTL activity against VSV-infected A20 cells, indicative of a memory response against VSV (Figure 4B). Data shown are representative of 3 replicate experiments and are expressed as means ± standard deviation of 6 individuals per condition. This experiment indicated that VSV23 was capable of inducing VSV-specific memory CTLs.

Proliferation assay
The ability of viruses to induce memory specific Th1 cell responses is often measured by the induction of antigen-specific proliferating cells with syngeneic antigen presenting cells (APCs). A proliferative response to VSV antigens by T cells was determined for the panel of viruses twenty days after injection. The ability of VSV23 and control viruses to induce memory responses was indistinguishable from VSVwt (Figure 4C). All responder CD4+ T cells incubated in the absence of VSVtsG41 showed no proliferation above background, similar to that seen in cells from naïve mice with VSV-infected APCs (data not shown). The data shown are representative of 3 replicate experiments. Data are expressed as means ± standard deviation of 6 individuals per condition. We interpret these data to indicate that VSV23 induced a robust CD4+ memory response.

Neutralizing antibody assay
The ability to induce the production of neutralizing antibody is critical for protection against secondary viral infections. Groups of mice were infected IN with the panel of viruses, and surviving individuals were bled twenty days after immunization to obtain serum. Serum of individual donors was diluted in PBS in serial five-fold steps. 1 pfu of VSVwt in PBS was added to each dilution and all samples were allowed to incubate for 1 hour before adsorption to L929 monolayers. Three replicate experiments were performed with similar results. Data are expressed as means ± standard deviation of six individuals per group. All 4 virus immunizations produced similar results (Figure 4D), which indicated that VSV23 was able to stimulate the production of neutralizing antibodies against VSVwt.

VSV23 is attenuated for encephalitis and induces no responses
A potential concern regarding the use of VSV as an oncolytic agent is the characteristic encephalitis that results from infection of the CNS in immunocompetent mice. Administration of VSV IN results in infection of olfactory sensory neurons in the nasal turbinates. The infection spreads along the olfactory nerve to the olfactory bulb and then caudally through synapses. Once VSV reaches the olfactory ventricle, virus spreads in cerebral spinal fluid to motor neurons in the lumbar-sacral spinal cord, giving rise to the symptoms of encephalitis and hind-limb paralysis in the infected animal.16,64,65

The ability of VSV23 to cause disease in mice when administered IN (the route which readily leads to viral encephalitis) was studied. VSV23 was compared with the rVSV controls and VSVwt for the ability to cause illness and death. Specific immune responses in the CNS in response to infection, as well as resultant viral titers, were determined.

Decreased morbidity and mortality at 1 × 10⁴ pfu
Cohorts of mice were infected IN with the panel of viruses and examined daily (by an observer who was blinded to the challenge viruses) for 15 days to monitor for weight loss and health. Hind-limb paralysis or weight loss that exceeded 30% of starting weight were considered end points for individuals in the experiment. Three replicate experiments indicated that VSV23 was completely attenuated in the CNS at 1 × 10⁴ pfu. Mice receiving the recombinant viruses at those doses exhibited 20% mortality while mice infected with VSVwt exhibited 70% mortality (Figure 5). Kaplan-Meier survival curve analysis indicated that VSV23 was different from the other rSVs by p < 0.05 and from VSVwt by p < 0.005.

Decreased morbidity and mortality at 1 × 10⁶ pfu
To determine the extent and mechanism of attenuation, the infection was performed at a much higher challenge, 1 × 10⁶ pfu. This dose was chosen based on pilot studies utilizing increasing log doses to determine the minimum pfu of VSV23 that could induce mortality.

Cohorts of mice were infected IN with the panel of viruses and examined daily (by an observer who was blinded to the challenge viruses) for 15 days to monitor for weight loss and health. Hind-limb paralysis or weight loss that exceeded 30% of starting weight were considered end points for individuals in the experiment. The nonparametric Kruskal-Wallis analysis of symptom data showed a significant difference in clinical scores among the groups (p < 0.05), indicative of the decreased morbidity characteristic of VSV23 infection (Figure 6A). Analysis of average percentage weight loss for all groups indicated no significant difference in weight among all infection groups (Figure 6B). VSV23 infection resulted in 25% mortality. Infection with VSVST and VSVXN2 resulted in 40% and 58% mortality, respectively (Figure 6C). Kaplan-Meier survival curve analysis utilizing the one-tail
VSV23 infection of the CNS induces increased levels of nitric oxide

Studies have shown that production of nitric oxide (NO) in the CNS promotes survival and recovery from VSV encephalitis.66–68 In addition, IL-12 promotes rapid containment of virus in the CNS and enhanced survival.69–71 In experiments in vitro, VSV23, but not the other viruses induced neuronal cells (but not fibroblasts) to produce NO which subsequently suppressed viral replication (data not shown). Therefore, it was important to determine if VSV23 were able to induce the production of NO in vivo during infection of the CNS. Mice were infected IN with the panel of viruses and on days 1, 3, 6, and 9 after infection, homogenates of brain tissue from individuals were examined for the presence of NO by a colorimetric test, the Greiss assay. Although there was no significant increase in NO levels day 1 PI, by day 3 VSV23 infection resulted in significantly increased NO levels, at both infection doses. Measurement of NO levels indicated a significant increase in NO in VSV23 infected animals compared to control viruses after infection with $1 \times 10^6$ pfu VSV23, $p < 0.05$ as determined by ANOVA analysis (Figure 7C). We interpret these data to be compatible with the hypothesis that IL-23 induces nitric oxide synthase (NOS) activation and NO production in vivo.

VSV23 replicates in tumor cells in vitro and induces apoptosis

VSV infection of susceptible cells rapidly leads to apoptosis due to both blockade of the nuclear pore complex and direct

**Figure 5** VSV23 infection is attenuated for lethal intranasal infection resulting in viral encephalitis at $1 \times 10^4$ pfu: Cohorts of $N = 10$, 6-week old BALB/c mice were infected intranasally with $1 \times 10^6$ pfu of VSV23, VSVST, VSVXN2, or VSVwt and monitored for 15 days. VSV23 infection resulted in no mortality, VSVST infection resulted in 20% mortality, VSVXN2 infection resulted in 20% mortality, and VSVwt resulted in 70% mortality. VSV23 is different from the other recombinant viruses by $p < 0.05$ and from VSVwt by $p < 0.005$ in Kaplan Meier analysis. Results were identical in three replicate experiments.
Cytokine-modified VSV: its utilization as an oncolytic agent

Figure 6

VSV23 infection is attenuated for lethal intranasal infection resulting in viral encephalitis at $1 \times 10^6$ pfu: Cohorts of $N = 10$, 6-week old BALB/c mice were infected intranasally with $1 \times 10^6$ pfu of VSV23, VSVST, or VSVXN2 and monitored for 15 days. A) Morbidity: Mice were weighed and scored daily to assess clinical symptoms: “1” for lack of grooming behavior; “2” for hunched and severely lethargic mice; “3” for hind-limb paralysis and “4” for full paralysis; “5” for death. Each data point represents the average score of the cohort. Kruskal-Wallis analysis of symptom data indicated that VSV23 exhibited a significant difference in clinical scores $p < 0.05$. B) Weight-loss: Weight-loss was comparable for all infection groups and is presented as mean ± standard deviation. C) Mortality: VSV23 infection resulted in 25% mortality, VSVST infection resulted in 40% mortality, VSVXN2 infection resulted in 58% mortality. VSV23 is different from the other viruses by $p < 0.05$ in Kaplan Meier analysis.

Abbreviation: PI, postinfection.
Figure 7 rVSV viral titers and NO production following intranasal infection: A) Viral titers at $1 \times 10^3$ pfu: Cohorts of $N = 6$, 6 week old male BALB/c mice were infected with $1 \times 10^3$ pfu of VSV23, VSVST, or VSVXN2. Brains were harvested on days 1, and 3 p.i., divided into hemispheres sagitally, and one half was homogenized. Samples were serially diluted and plated on L929 cells. Plaque assays were performed to determine viral titers. Data points represent titers in individual mice. Horizontal bars indicate the geometric mean titer of the cohorts. VSV23 titers as represented by the geometric mean were at least 1 log lower than all other viral treatment groups at both time points. B) Viral titers at $1 \times 10^6$ pfu: The experiment outlined above was repeated, but mice were infected with $1 \times 10^6$ pfu of VSV23, VSVST, or VSVXN2. Results were similar for all infections at both time points. Data shown are representative of 3 identical experiments. C) NO levels in the central nervous system: Brains were harvested on days 1, 3, 6, and 9 p.i. after infection with VSV23 (solid bars), VSVST (stippled bars), VSVXN2 (slashed bars), or mock infected (plaid bars) and homogenates were tested for the presence of NO using the Greiss assay. VSV23 induces greater amounts of NO compared to other rVSVs and does so at earlier time points. Data shown are means ± standard deviations. ANOVA analysis of days 3 and 6 data reject the null hypothesis with $p < 0.05$. This figure is representative of data from three identical experiments.

Abbreviation: NO, nitric oxide.
interactions with the mitochondria.\textsuperscript{6–8,72} In order to test the ability of VSV23 to infect and kill tumor cells, the panel of viruses was used in an in vitro growth study in a murine mammary derived JC tumor line. Cells were infected and an assay of apoptosis, the MTT assay, was conducted at 3, 6, 9, 12, 18, and 24 hours PI. Experimental results (Figure 8) indicated that VSV23 replicated and induced JC cell death that was indistinguishable from control rVSVs and VSVwt.

\textbf{Discussion}

VSV selectively destroys a wide variety of tumor cell lines including those with aberrant p53, Ras, or Myc function such as glioblastomas\textsuperscript{10,73,74} colorectal, pancreatic, and breast cancer cells.\textsuperscript{24,75–77} Other targets of VSV therapy include leukemia, hepatocellular carcinoma, nasopharyngeal carcinoma, and bladder cancer.\textsuperscript{75,78–85} Several characteristics of VSV make it particularly attractive as a candidate for development as an oncolytic virus. VSV has a rapid replication cycle and infection with VSV is generally both asymptomatic and very rare in humans.\textsuperscript{86} Potentially, VSV could replicate and exert its oncolytic effects prior to host development of a neutralizing serological response.\textsuperscript{75} Finally, the virus can easily be genetically engineered to express transgenes.\textsuperscript{59,87–90} However, administration of therapeutic doses of VSV in mice can lead to substantial “toxicities” including hind-limb paralysis and death.\textsuperscript{10,24} The need to create a safer oncolytic agent and our extensive prior studies on the pathogenesis of VSV encephalitis led to the hypothesis that a rVSV that expresses IL-23 would attenuate the virus in healthy tissue while maintaining and possibly enhancing oncolytic activity.\textsuperscript{16,26,65,91–93}

This hypothesis was based on several lines of research. In studies of VSV encephalitis, treatment of mice with IL-12 enhances viral clearance and survival.\textsuperscript{70,71,94} Unfortunately, administration of IL-12 in phase II clinical oncology trials has been associated with severe toxicity and death, partly due to the induction of high levels of IFN-\(\gamma\).\textsuperscript{53,54} Since IL-23 and IL-12 share the functional p40 subunit and a receptor chain component, IL-12R\(\beta 1\), IL-23 was anticipated to act in similar manner, however with reduced deleterious effects. This is due to the fact that IL-23 induces lower levels of IFN-\(\gamma\) than IL-12.\textsuperscript{43} Additionally, IL-23 is expressed in the CNS during inflammation.\textsuperscript{43,46,95} Finally, IL-23 has been reported to have antimetastatic and antitumor properties.\textsuperscript{49,52,55–57,96} Experiments in murine mammary cancer cells have shown that these IL-23 effects are mediated by increasing numbers of CD4\(^+\) T cells and induction of tumor specific CD8\(^+\) T cells.\textsuperscript{57}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{MIT_assay_MOI=3.png}
\caption{Infection with VSV23 and VSVXN2 induces mitochondrial dysfunction in JC cells: 4.5\times10^4 JC cells were plated in 96-well plates and incubated overnight. Cells were then infected at MOI = 3 and incubated for 3 (solid bars), 6 (stipled bars), 9 (slashed bars), 12 (horizontal striped bars), 18 (vertically striped bars), or 24 (plaid bars) hours with VSV23, VSVST, VSVXN2, VSVwt, or mock treated. MTT reagent was added per manufacturer’s instructions and samples were incubated at RT in the absence of light for 4 hours. Plates were read on an ELISA plate reader at 540 nm. All viruses are capable of disrupting mitochondrial function. Data are presented as mean \pm standard deviation and are representative of the replicate experiments.\textbf{Abbreviation:} RT, room temperature.}
\end{figure}
We presented data showing that a rVSV, designated VSV23, expresses functionally active IL-23 and maintains replicative capacity. A concern in using a scIL-23 cDNA is that translation of the subsequent mRNA might not result in an active protein. However, virally produced IL-23 readily induced IFN-γmRNA in CD4+ T cells (Figure 3B). Addition of 1.3 kb of RNA into the VSV genome could have resulted in significant deficiencies in viral replication; however one-step growth curve data showing similar viral titers between control viruses and VSV23 ameliorated this concern (Figure 2).

In order for VSV23 to be a safe oncolytic agent, the virus must be capable of inducing innate and adaptive host immune responses in order to prevent infection of the CNS and subsequently inducing encephalitis. Bystander effects including cellular immunity and antitumor responses that result in tumor destruction caused by VSV23 infection are also hypothesized to be beneficial in the treatment of cancer. To determine if VSV23 were sufficiently immunogenic, a series of experiments were conducted to examine innate and adaptive immune responses.

Innate NK cell activity does not require exposure to a specific antigen in order to elicit a cytolytic response. Activation only requires local immune responses that stimulate IFN-γ, IL-12, and other cytokines that result in positive regulation of NK cells. Inoculation of mice with VSV23 induced similar levels of NK mediated Y AC cell killing three days PI compared to control rSVs and VSVwt (Figure 4A). Studies have shown that IL-23 takes up to 2 weeks after the initiation of treatment to enhance the effect of NK cells.

Adaptive immune responses to viral infection can be detected in mice as early as 7 days PI. Memory responses in mice inoculated with the panel of viruses were assayed twenty days after inoculation and were indistinguishable (Figs 4B–D). Studies using unpimed lymphocytes indicated no memory response. This was due to insufficient precursors in naïve mice. There was a similar level of memory responses in VSV23 primed mice as indicated by comparable levels of CTL activity at all effector-to-target ratios. The proliferation of T cells is an important property of the adaptive immune system. The proliferative response to the VSV antigens by CD4+ T cells was determined. As expected, all virus-primed splenocytes cultured with VSV tsG41-infected stimulators showed similar levels of T cell proliferation (Figure 4B).

In addition to cell mediated responses to viral inoculation, we examined the production of neutralizing antibodies after IN infection. Twenty days PI, comparable levels of neutralizing antibodies were detected in blood serum of infected animals (Figure 4D).

The results of assessments of adaptive immunity to VSV indicated that there was no difference in the ability of any of the viruses to induce adaptive immune responses. There was a possibility that vIL-23 would induce altered T cell responses. The fact that no differences in CTL killing, T cell proliferation, nor neutralizing antibody titers were observed could be explained by rapid viral clearance. There was simply insufficient time to produce biologically relevant levels of vIL-23 following IP inoculation. In the case of antibody production, vIL23 did not enhance B cell clones specific for VSV. Alternatively, it is possible that the antibody responses to other viral antigens were altered but undetected as we only assessed neutralizing antibody.

In vivo studies showed that VSV23 is attenuated for induction of viral encephalitis compared to control rSVs and VSVwt. These results are consistent with previous findings that recombinant versions of VSV show greater attenuation in vivo compared to VSVwt. The reasons for attenuation are likely deletions and nucleotide variations characteristic of the cloned version of VSV. In order to control for the inherent differences in rSVs compared to VSVwt we utilized VSVXN2, a rVSV carrying no insert at the cloning site. VSVST was created to control for alterations in genome, and hence particle size associated with insertion of scIL-23. Stop codons were introduced into the p40 subunit coding region of IL-23, but not the p19 subunit. Infection of mice with VSV23 led to decreased morbidity and mortality compared to mice infected with control viruses at a very high challenge dose (Figure 6A–C), but virtually none at lower challenges (data not shown) indicating that the attenuation of VSV23 can be attributed to the effects of vIL-23. Despite increased survival (Figures 5 and 6C) and milder symptoms in VSV23 infected mice (Figure 6A, B), no difference was seen in weight loss indicating that infection with VSV23 results in some morbidity. This may be due to transient destruction of the olfactory neuroepithelium leading to a decrease in olfactory function and hence decreased feeding activity. Minor differences in the attenuation of VSVST compared to VSVXN2 are likely due to morphological and metabolic alterations associated with the additional genomic load. Alternatively, it is possible (but unlikely) that residual expression of p19 might have led to induction of IFN-γ resulting in some attenuation in vivo.

Measurement of viral titers in the CNS revealed a significant decrease in VSV23 compared to control viruses after infection with $1 \times 10^5$ pfu (Figure 7A). Animals infected with $1 \times 10^6$ pfu of VSV23 had less morbidity and mortality, however this did not correlate to a decrease in viral titers on...
days 1 and 3 PI (Figure 7B). It is possible that at this early stage in the infection innate immune responses are being overwhelmed, but are subsequently capable of controlling the infection at later time points. Another hypothesis is that the spread of the virus in the CNS is limited in VSV23-infected animals. This would allow for high viral titers without infection of caudal regions of the brain. Work is in progress to address this question.

We hypothesized that vIL-23 may share the ability of IL-12 to induce NOS in the CNS, resulting in enhanced NO production.\(^1\) This would provide a plausible mechanism of attenuation. After infection with \(1 \times 10^7\) and \(1 \times 10^6\) pfu there was no significant increase in NO levels day 1 PI as determined by the Greiss assay. However, by day 3 PI significantly increased levels were detected (Figure 7B). Preliminary immunohistological analysis of sections from the olfactory bulbs of infected animals show that NOSII expressing microglia were present in VSV23 infected mice as early as 1 day PI, but not in other infections until day 6 PI (data not shown). As such, increased levels of NOS II expressing cells between days 1 and 3 PI are likely to be responsible for the increase in NO levels in brain homogenates. Work continues at this time to determine whether increased NO levels can be attributed to any particular isoform of NOS in the CNS.

The purpose of creating VSV23 was to develop the virus as an oncolytic agent a vaccine carrier. To study the antitumor effects of the virus we have chosen the JC tumor cell, a murine mammary tumor cell line. In vitro, the ability of VSV23 to induce apoptosis in JC cells is indistinguishable from control rVSVs and VSVwt. Work is in progress to determine the whether VSV23 destroys tumors in vivo. Ideally vIL-23 produced within the tumor will induce a proinflammatory state that would increase oncolytic activity of the virus resulting in enhanced rates of tumor destruction. We will also determine if long-term memory responses are elicited specifically against JC tumor cells as a result of vIL-23. Examination of tumors to detect viral antigens and infiltrating immune cells will also help to determine the potential of VSV23 as an effective tumor treatment.

In conclusion VSV23 is an attenuated virus in vivo, but not in vitro. In the CNS, VSV23 infection is characterized by decreased morbidity and mortality, likely through the induction of early NO responses. In the periphery VSV23 promotes a strong antigenic response based on the results of the virus-specific proliferation, CTL, neutralizing antibody, and NK assays. In vitro VSV23 is capable of inducing apoptosis in tumor cells. This oncolytic capacity coupled with attenuation in the CNS makes VSV23 an attractive agent for further study as an oncolytic virus.

**Acknowledgments**

We are grateful for the generous gifts of essential reagents by Maria Louise Belladona (University of Perugia, pCEP4-sclL23Ig plasmid), Jack Rose (Yale, VSVXN2 and helper plasmids), and Savio Woo (Mt Sinai School of Medicine, VSV12). We have benefited by expert advice from Adolfo Garcia-Sastre (Mt Sinai School of Medicine), Savio Woo, Marcia Meseck (then at Mt Sinai School of Medicine), Oliver Ebert (then at Mt Sinai School of Medicine), Daniel Meruelo (NYU School of Medicine) and our colleague Paul M D’Agostino. This work could not have been accomplished without funding from the NIH to CSR, NS039746. The work described in this manuscript has been submitted to the US Patent and Trade Office US Provisional Patent Application Serial # 61/187,125.

**Disclosures**

The authors report no conflicts of interest in this work.

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