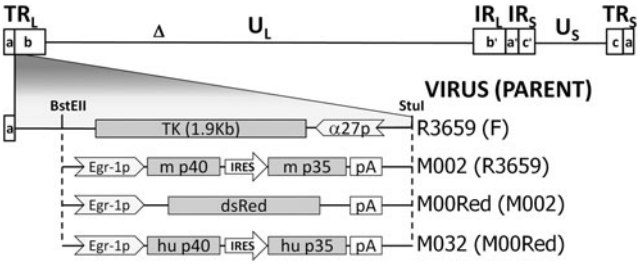
Supplementary Information

Materials and Methods

Investigational agent (M032 construction and cGMP preparation)

Construction of M032, which expresses hIL-12 under the transcriptional control of the murine early-growth response-1 promoter (Egr-1), is described below. This strategy is identical to that used to construct the murine IL-12-expressing virus M002 (Parker *et al*., 2000). Complementary DNA was obtained by oligo-dT RT-PCR of total mRNA isolated from NC37 cells stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin. The hIL12 p40 and p35 subunits were PCR-amplified using sequence-specific primers (p40: sense, 5′-gctctagaccATGggtcaccagcagttggt; antisense, 5′-gcggatcc taactgcagggcacagatg; p35: sense, 5′-gtccctgcag tgccggctcacc atgggtccagcg; antisense, 5′-ggagctcttttaggaagcattcagatagc). The 5′ *Xba*I and 3′ *Bam*HI sites incorporated into the 1000 bp p40 PCR product were digested and cloned into the corresponding sites in pBS-SK$^+$. A 5′ *Pst*I and a 3′ *Sst*I site were incorporated into the 691 bp p35 PCR product. The EMCV-IRES sequence was obtained from pBS-mIL-12 (previously described by Parker *et al*., 2000), which was liberated by digestion with *Bam*HI and *Nco*I. The *Xba*I-hP40-*Bam*HI, *Bam*HI-IRES-*Nco*I, and *Nco*I-hp35-*Sst*I fragments were mixed for a 4-piece ligation into pBS-SK$^+$ digested with *Xba*I and *Sst*I. The resulting bicistronic cassette was liberated from pBS-SK + with *Spe*I and *Sst*I and ligated into the HSV shuttle plasmid pRB4878 (kindly provided by Bernard Roizman), modified to contain an *Spe*I-*Sst*I-*Xho*I polylinker, resulting in the hIL-12 shuttle vector (pH002.2). In order to generate the recombinant virus, a dsRED-expressing intermediate HSV (M00Red) was derived from M002 by recombination with pCK1136 (Parker *et al*., 2000). Cotransfection of M00Red and pH002.2 into rabbit skin cells generated recombinant HSV expressing hIL-12. Recombinant plaques were identified and sequentially purified based on loss of dsRED expression.



SUPPLEMENTARY FIG. S1. Schematic representation of the double-copy hIL12-expressing HSV (M032). Top, schematic representation of the HSV-1 (F) Δ305 genome, including the long (L) and short (S) regions defined as terminal repeats (TR_L and TR_S), inverted repeats (IR_L and IR_S), and Unique (U_L and U_S). Sequences a, b, and c are repetitive regions in the forward or inverse (′) orientation, with the 501 bp deletion (Δ) in the *tk* gene. R3659 is a recombinant derived from HSV-1 F with the *Stu*I-*Bst*EII fragment, encompassing the ORF P and γ_1*34.5* domains, replaced with a chimeric *α27-tk* gene in both the ab and a′b′ domains. M002 is derived from R3659 and contains an Egr-1-driven bicistronic transcript encoding the murine p40 and p35 subunits of IL12. M00Red was created by replacing the mIL12 sequences with dsRED, and was used as an intermediate virus for generation and selection of M032. M032 was generated by replacing the dsRED sequence with those encoding the human p40 and p35 IL12 sequence.

The resulting recombinant HSV (M032) was validated by Southern analysis and sequencing. M032 DNA was provided to SAIC-Frederick, Inc., and NCI-Frederick for sequence verification and large-scale production of clinical-grade M032. Briefly, M032 vDNA was transfected into a certified working cell bank (WCB) of African green NHP kidney (Vero) cells. Plaques were purified and amplified in the WCB Vero cells to generate M032 seed stocks before large-scale amplification. For large-scale production WCB Vero cells were seeded into five 10-tier cell factories and 5 T-225 cm^2 flasks at a density of 3×10^4 cells/cm^2. At 95% confluence the cells were infected for 4 hr with M032 at 0.01 pfu/cell. At 47 hr postinfection the cells were lysed with 0.4 *M* NaCl in PBS, pH 7.4. The cell lysate was treated with benzonase and purified over Q-Sepharose XL chromatography columns. The virus eluate was then pooled and loaded onto A Q-Sepharose XL column for concentration. Buffer exchange of the virus peak from the Q-Sepharose XL column into DPBS, 0.4 *M* NaCl, and 10% glycerol was performed using 4-Sepharose fast flow chromatography. The final product was brought to 150 ml DPBS, 0.4 *M* NaCl, and 10% glycerol; sterile filtered through a Millipak 0.45 μm filter; and aliquoted into vials at a volume of 0.4 ml/vial and a concentration of 3.46×10^9 pfu/ml (1.38×10^9 pfu/vial). Before administration to animals, the virus stock was diluted with sterile 0.9% saline to 1×10^7 or 1×10^9 pfu/ml.

Assays

Animals. A total of 15 male and 15 female *Aotus nancymae* were used in this study. Among these animals, 14 male and 15 female NHPs were received from the University of Texas MD Anderson Cancer Center (Bastrop, TX) and 1 male NHP was received from Alpha Genesis, Inc. (Yemassee, SC). Animals were assigned to respective dose groups using a computer-generated randomization procedure. After randomization, 15 NHPs/sex were assigned to one of two M032-injected groups consisting of 6 NHPs/sex/group or to one vehicle control group consisting of 3 NHPs/sex/group. Male weights ranged from 766 to 1216 g, and female weights ranged from 796 to 1112 g. The animals were housed individually in stainless steel, slat-bottom cages in an environmentally monitored, well-ventilated room that was maintained at a temperature of 64.1–85.1°F and a relative humidity of 21.8–79.9% during the quarantine and study periods. NHPs were sedated for procedures involving

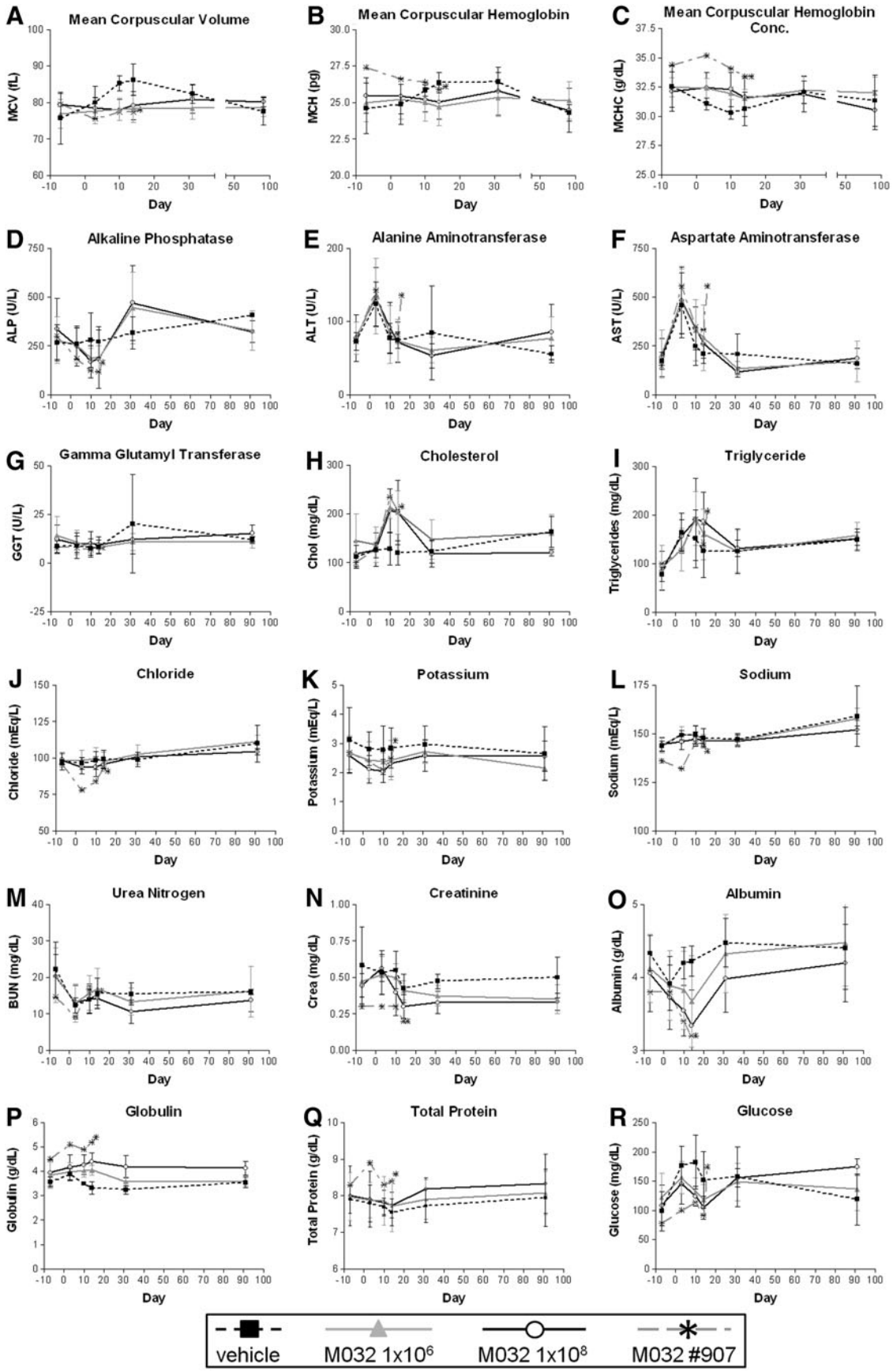
removal from the cages and included, but were not limited to, detailed clinical observations, body weight measurements, blood collections for clinical pathology and/or qPCR determinations, and preparation for euthanasia. NHPs were sedated using ketamine administered intramuscularly at a dose of 10–60 mg/kg per animal. Doses were calculated based on the most recent available body weights. Each NHP was euthanized by intravenous administration of a barbiturate. All animal studies were carried out at Southern Research Institute (Birmingham, AL). Cage size and animal care conformed to the guidelines of the Guide for the Care and Use of Laboratory Animals, to the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99–198), and to the applicable Standard Operating Procedures of Southern Research Institute. Procedures performed and data collected at Southern Research Institute were inspected and audited by a quality assurance unit (Supplementary Fig. S4).

Intracerebral administration. Each NHP was anesthetized before dosing by the administration of ketamine (10–30 mg/kg) given intramuscularly in combination with diazepam (1–2 mg/kg). Intracerebral dosing was performed with the aid of a stereotactic device (Stoelting, Kiel, WI). After appropriate surgical preparation of the adjacent area, the injection site was determined by locating the sagittal suture at the junction of the parietal bones and the coronal suture of the skull, and an incision was made at the site. A hole was bored in the skull 3 mm anterior to the coronal suture and 7 mm lateral and to the right of the sagittal suture. The depth of the drilled bur hole was such that it just penetrated the calvarium. Vehicle and M032 formulations were drawn into a 0.5 ml tuberculin syringe equipped with a 27-gauge, 0.5-inch needle (Becton Dickinson, Franklin Lakes, NJ). The syringe/needle was positioned in the stereotactic device, and the needle was inserted into the hole in the skull up to the hub. The dose (0.1 ml/NHP) was delivered over $\sim$2–3 min, at a target rate of $\sim$15 sec per 10 μl. After delivery of the entire dose volume, the dosing needle was maintained in place for $\sim$2–3 min and then slowly withdrawn. After the completion of dosing, the needle entry hole was sealed with sterile bone wax, and the skin over the injection site was closed with sutures and/or tissue glue. Postsurgical monitoring was under the supervision of the attending Veterinarian. Animals were monitored until sufficiently recovered from anesthesia to allow for postural changes within the home cage. For analgesia, each animal received buprenorphine at 0.03 mg/kg by intramuscular injection. Animals were provided buprenorphine at a dose of 0.01–0.05 mg/kg in some form of consumable item twice daily from day 1 to day 3.

Clinical observations. All NHPs were observed twice daily throughout the quarantine and study periods for signs of morbidity, mortality, or adverse clinical signs. Detailed clinical observations of each NHP were performed on days 1 (before dosing), 3, 8, 10, 14, 22, 31, 36, 43, 50, 57, 64, 71, 78, 85, 91, or before moribund euthanasia (1 animal). The detailed clinical examination included, but was not limited to, observations of the general condition, skin, fur, eyes, nose, oral cavity, thorax, abdomen, external genitalia, limbs, feet, and neurological status, as well as evaluation of respiration and palpation for tissue masses. Each animal was weighed on day $-6/-7$ (randomization) and on days 1 (before dosing), day 3 (only animals scheduled for euthanasia), 8, 14, 22, 31, 36, 43, 50, 57, 64, 71, 78, 85, and 91. Clinical observations were carried out by veterinarians or veterinary assistants trained in *Aotus* husbandry.

Hematology and clinical chemistry. Blood samples for hematology and clinical chemistry determinations were collected from each NHP during week -1 and on days 3, 10, 14, 31, and 91. In addition, blood samples for coagulation determinations were collected from each NHP during week -1 and from those animals scheduled for euthanasia on day 3, 31, or 91. Blood samples (hematology, clinical chemistry, and coagulation samples) also were obtained from one male NHP before moribund euthanasia on day 16. Animals were fasted overnight before baseline clinical pathology blood collections and for variable (as short as 20 min) time periods after intracerebral injection. The blood samples were collected into tubes containing EDTA, no anticoagulant ($\sim$1.0 ml, clinical chemistry samples), or sodium citrate ($\sim$1.8 ml, coagulation). Coagulation parameters included prothrombin time, activated partial thromboplastin time, and fibrinogen concentration. The Provantis application (Version 7; Instem Life Sciences Systems, Ltd., Staffordshire, United Kingdom) was used for the direct online capture of most in-life data. For clinical chemistry measurements, Provantis was interfaced with the Hitachi 911 Clinical Chemistry Analyzer (Version 4.32; Roche Diagnostic; Indianapolis, IN). Hematology and coagulation measurements were carried out using the ADVIA 120 Hematology Analyzer (Version 3.1.8; Bayer Diagnostics, Tarrytown, NY), and the ACL Elite Pro Coagulation Analyzer (Rev 3.0.5; Beckman Coulter, Inc., Miami Lakes, FL), respectively, both of which were also interfaced with Provantis. Chemistry measurements included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), urea nitrogen (BUN), creatinine (Crea), glucose (Gluc), cholesterol (Chol), triglycerides (Trig), total protein (TP), albumin (Alb), globulin, albumin/globulin ratio (A/G ratio), sodium (Na), potassium (K), and chloride (Cl). For coagulation, Prothrombin and Activated partial thromboplastin times, and Fibrinogen levels were measured. All samples were processed

SUPPLEMENTARY FIG. S2. Hematology. Blood samples for hematology were collected from each NHP during the week before intracerebral injection of saline or M032. After injection on day 1, samples were collected on days 3, 10, 14, 31, and 91. Among the measurements carried out **(A)** white blood cells, **(B)** red blood cells, **(C)** hematocrit, **(D)** hemoglobin, **(E)** platelets, **(F)** reticulocytes, **(G)** neutrophils, **(H)** lymphocytes, **(I)** monocytes, **(J)** eosinophils, **(K)** basophils, and **(L)** large unstained (myeloperoxidase negative) cell counts are presented. Blood samples obtained from one male NHP before moribund euthanasia on day 16 are depicted as ''X'' in each plot. Error bars represent mean $\pm$ SD. Preadministration to day 3; vehicle ($n=6$), M032 ($n=12$/dose). Days 4–31; vehicle ($n=4$), 1E6 M032 ($n=8$), 1E8 M032 ($n=7$ or 8; 1 animal euthanized moribund on day 16). Days 32–91; vehicle ($n=2$), 1E6 M032 ($n=4$), 1E8 M032 ($n=3$).


A
Mean Corpuscular Volume
MCV (fL)
Day
B
Mean Corpuscular Hemoglobin
MCH (pg)
Day
C
Mean Corpuscular Hemoglobin Conc.
MCHC (g/dL)
Day
D
Alkaline Phosphatase
ALP (U/L)
Day
E
Alanine Aminotransferase
ALT (U/L)
Day
F
Aspartate Aminotransferase
AST (U/L)
Day
G
Gamma Glutamyl Transferase
GGT (U/L)
Day
H
Cholesterol
Chol (mg/dL)
Day
I
Triglyceride
Triglycerides (mg/dL)
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BUN (mg/dL)
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Crea (mg/dL)
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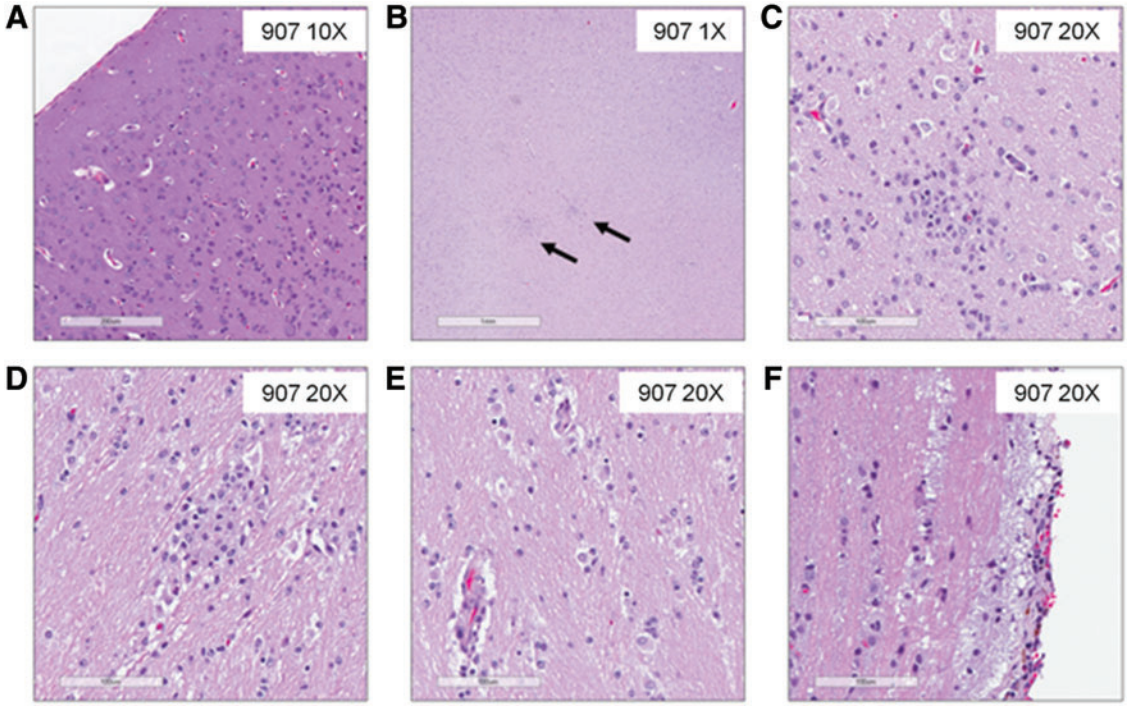
vehicle
M032 1x10^6
M032 1x10^8
M032 #907



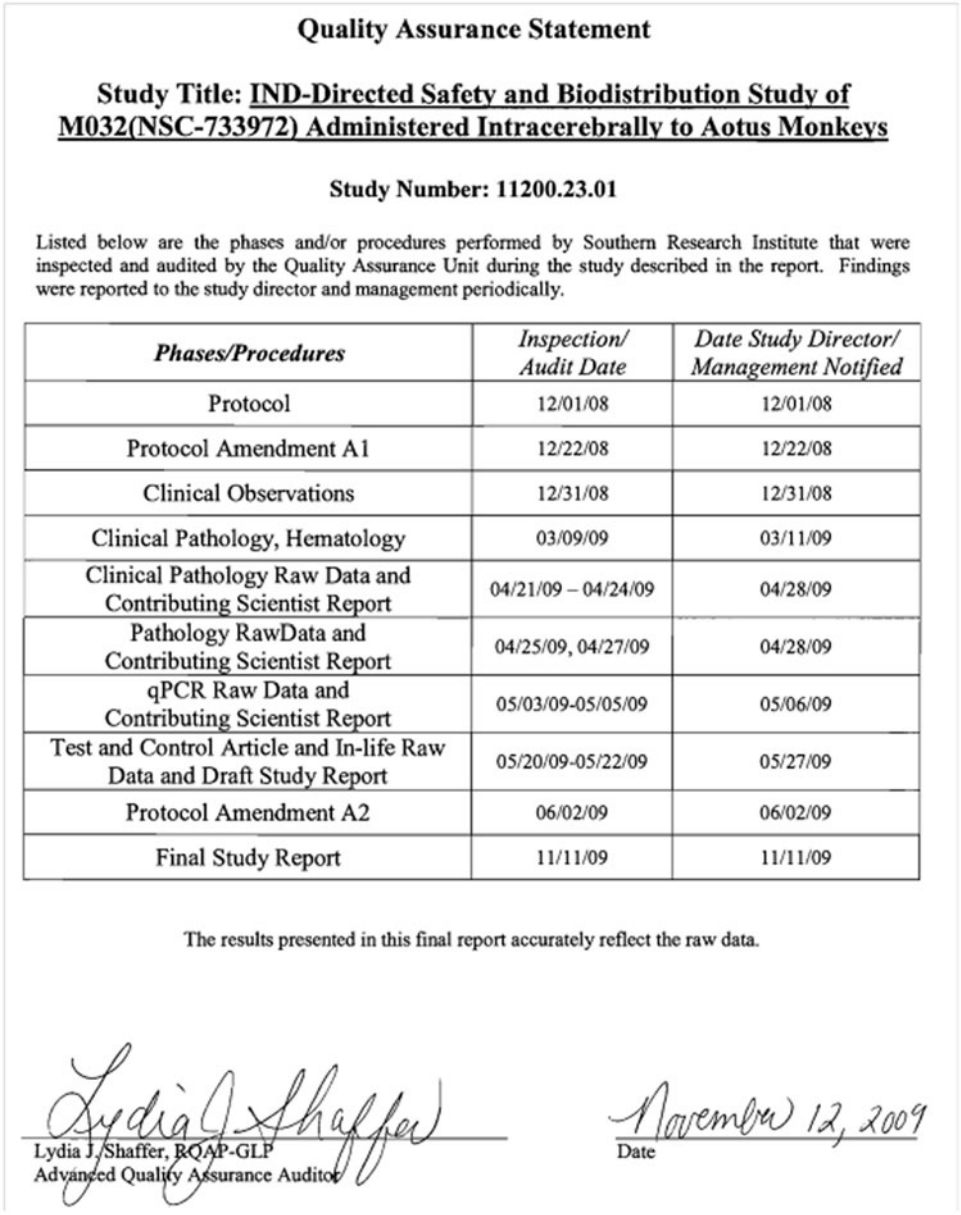
SUPPLEMENTARY FIG. S3. Inflammatory sites observed in the NHP (#907) euthanized early on day 16. **(A)** Representative parasagittal section similar to those presented in Fig. 5. **(B)** Rather than widespread, inflammation was multifocal **(B–D)**, primarily consisting of macrophage infiltrates, gliosis, and mild leptomeningitis **(F)**.

and analyzed at Southern Research. Results were sent to Dr. Ronna Fulton at Fulton Veterinary Clinical Pathology Consulting, L.L.C. (Birmingham, AL) for evaluation.

Macroscopic and microscopic evaluations. On days 3, 31, and 91, one NHP/sex in the vehicle control group and two NHPs/sex in the 1×10^6 and 1×10^8 pfu/NHP dose groups were euthanized, with the exception of day 91, on which only one male NHP in the 1×10^8 pfu/NHP dose group was euthanized because of the one male NHP euthanized moribund on day 16. Before euthanasia, terminal body weights and clinical observations were recorded, and blood samples for clinical pathology evaluation were collected. In addition, blood and CSF for qPCR analysis were collected from NHPs in the vehicle control and 1×10^8 pfu/NHP dose groups. Each NHP was euthanized by intravenous administration of a barbiturate. After euthanasia, each NHP received a complete postmortem examination. The postmortem examination included, but was not limited to, examination of the external surfaces of the body, all orifices of the body, and the cranial, thoracic, abdominal, and pelvic cavities and their contents. For euthanized animals tissues/organs were collected in partial ($\leq$200 mg in weight) for qPCR analysis. Subsequently, samples of tissues/organs were saved in 10% neutral buffered formalin, except the eyes and testes/epididymides. Eyes were fixed in Davidson's solution, and the testes/epididymides were fixed in modified Davidson's solution. The animal identification was retained with tissues collected for histopathology.

Histology. Sections of the fixed tissues from all animals in each dose group were processed for histopathological examination. Samples were obtained from CNS tissues, including the spinal cord (cervical), the injection site, and the pons/medulla and motor cortex regions of the brain (injection side). Lymphatic tissues collected included the bronchial, mandibular, and mesenteric lymph nodes, as well as the spleen, and right tonsil. Peripheral tissues were also obtained from the, colon, heart, ileum, and right kidney. The fixed tissues were trimmed, processed, and sectioned by microtomy ($\sim$5-μm-thick sections). The tissue sections were mounted on glass slides, stained with hematoxylin and eosin. Special stains (silver, acid fast, Congo red, trichrome, and Prussian blue stains) were applied at the discretion of the pathologist when necessary to establish a diagnosis.

Microscopic observations. All slides were submitted to a veterinary pathologist for evaluation and diagnosis. Records of gross findings for a specimen from postmortem observations were available to the pathologist when examining that specimen histopathologically. All findings were categorized either as drug related or nondrug related, and listed and coded by the most specific topographic and morphologic diagnoses, severity, and distribution using the Toxicology Data Management System nomenclature. Inflammation in the CNS was graded based upon the following criteria designated by the study pathologist: minimal, $<$100 inflammatory WBCs in the tissue examined; mild, $\geq$100 and $<$500 inflammatory WBCs in the tissue examined; moderate, inflammation in $>$25% and $\leq$50% of tissue examined; marked, inflammation in $>$50% of the tissue examined. Gliosis was graded based upon similar criteria for glial cells. Grading criteria for the meningeal fibrosis were the following: minimal, focal to multifocal areas of fibrosis involving



Quality Assurance Statement

Study Title: IND-Directed Safety and Biodistribution Study of M032(NSC-733972) Administered Intracerebrally to Aotus Monkeys

Study Number: 11200.23.01

Listed below are the phases and/or procedures performed by Southern Research Institute that were inspected and audited by the Quality Assurance Unit during the study described in the report. Findings were reported to the study director and management periodically.

Phases/Procedures	*Inspection/ Audit Date*	*Date Study Director/ Management Notified*
Protocol	12/01/08	12/01/08
Protocol Amendment A1	12/22/08	12/22/08
Clinical Observations	12/31/08	12/31/08
Clinical Pathology, Hematology	03/09/09	03/11/09
Clinical Pathology Raw Data and Contributing Scientist Report	04/21/09 – 04/24/09	04/28/09
Pathology RawData and Contributing Scientist Report	04/25/09, 04/27/09	04/28/09
qPCR Raw Data and Contributing Scientist Report	05/03/09-05/05/09	05/06/09
Test and Control Article and In-life Raw Data and Draft Study Report	05/20/09-05/22/09	05/27/09
Protocol Amendment A2	06/02/09	06/02/09
Final Study Report	11/11/09	11/11/09

The results presented in this final report accurately reflect the raw data.

Lydia J. Shaffer, RQAP-GLP
Advanced Quality Assurance Auditor

November 12, 2009
Date

SUPPLEMENTARY FIG. S4. Quality assurance statement by Southern Research Institute.

≤10% of the meninges; mild, focal to multifocal areas of fibrosis involving >10% and ≤25% of the meninges; moderate, focal to multifocal areas of fibrosis involving >25 and ≤50% of the meninges; marked, >50% fibrosis of the meninges. Slides of parasagittal cerebrum sections were scanned by Charles River Pathology Associates using an Aperio image capture device. The scanned slides were further assessed in ImageScope by a neuropathologist at University of Alabama at Birmingham (UAB; Dr. Steven Carroll), and to generate publication quality images.

qPCR analyses. Peripheral blood, CSF, and tissue samples specified under ''histology'' (above) were subjected to qPCR analysis. Before blood and CSF collection, the animals were anesthetized with ketamine (≤60 mg/kg) given intramuscularly; in addition, isoflurane was administered (inhaled), as needed. Each blood and CSF sample was collected into a tube containing EDTA, gently inverted, immediately snap-frozen, and then stored at or below −70°C before analysis. Genomic DNA (gDNA) was isolated from 18 *Aotus* NHPs dosed with 0 pfu/NHP or 1×10^8 pfu/NHP using a Qiagen DNeasy Blood and Tissue Kit or QIAamp Viral RNA Mini Kit (CSF samples). After the gDNA was extracted from the tissues or fluid, DNA concentration was determined using a PicoGreen Kit (Invitrogen Corp., Carlsbad, CA) and a Verasflur (Bio-Rad, Hercules, CA). This concentration was then used to determine the volume of gDNA needed to reach the 1 μg/reaction used in the qPCR assay.

Primer and probe sets were designed using Beacon Designer software (PREMIER Biosoft International; Palo Alto, CA). The HSV-1 Primer probe set detected the UL27 (gB)

gene (accession number E03114.1) on the HSV-1 genome. The internal amplification control (IAC) primers and probe detect a section of the PMP1 metalloproteinase gene (accession number AJ005052.1) on the *Podocoryne carnea* (jellyfish species) genome. All primers, probes, and the internal amplification control (IAC) oligo were manufactured by Operon Inc. (Huntsville, AL)—HSV-1 primers: sense, 5′-gcttcgccgacatcgacacg; antisense, 5′-cccacgatgcccatcaccac; probe, 5′-(6-FAM)-tcatccacgccgacgccaacgcc-(BHQ-1); IAC primers: sense, 5′-aaaattgcttatcaaattgaacgg; antisense, 5′-tgtttatggtattgtgcgatgg; probe, 5′-(HEX)-agctgttcttccgc-cacttccaat-(BHQ-1).

qPCR was carried out using Roche LightCycler TaqMan Master Mix (Roche Applied Science, Indianapolis, IN) and the custom primers, probes, and oligo listed above. Sample analysis was done in a Roche LightCycler 2.0 (software version 4.05) using the following PCR run parameters: initial heating cycle at 95°C for 10 min, 45 cycles of two steps consisting of denaturing at 95°C for 60 sec, and annealing and elongation at 62°C for 60 sec. A final cooling cycle was then performed for 30 sec at 40°C. Fluorescence was monitored in the end of each cycle at the end of the annealing and elongation step. The copy number for each sample was calculated from the crossing threshold (Ct) values against the validated standard curve, created by serially diluting M032 viral DNA.

A total of 3 μg of gDNA per sample (except CSF) was run in triplicate (1 μg per replicate, unless noted otherwise). The third replicate was spiked with 50 copies of M032 vDNA to determine if the amplification of the HSV-1 primer set was inhibited. Each of the 38 runs of qPCR contained a positive control, run in duplicate (also used as the LightCycler Calibrator), and a no template control, run in duplicate to detect any cross contamination. An IAC was used to determine the presence of inhibitors within the samples that could cause false negatives. The data produced using the IAC were then used to interpret the levels of inhibitors present in the gDNA sample. If 2/3 IAC Ct values for the samples were within 3 Ct values of the mean no template controls IAC Ct value, and then the sample's HSV-1 data were used to determine the levels of viral DNA present in the gDNA. If samples were determined to have inhibitors present, they were retested.

Statistical analysis. Mean values were calculated for qPCR data. Because of the small number of animals per dose group per day of necropsy, no other statistical analyses of the data were performed.