Published in final edited form as:

Vaccine. 2011 November 21; 29(50): 9345–9351. doi:10.1016/j.vaccine.2011.09.130.

Rapid, non-invasive imaging of alphaviral brain infection: Reducing animal numbers and morbidity to identify efficacy of potential vaccines and antivirals

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Abstract

Rapid and accurate identification of disease progression are key factors in testing novel vaccines and antivirals against encephalitic alphaviruses. Typical efficacy studies utilize a large number of animals and severe morbidity or mortality as an endpoint. New technologies provide a means to reduce and refine the animal use as proposed in Hume's 3Rs (replacement, reduction, refinement) described by Russel and Burch. *In vivo* imaging systems (IVIS) and bioluminescent enzyme technologies accomplish the reduction of animal requirements while shortening the experimental time and improving the accuracy in localizing active virus replication. In the case of murine models of viral encephalitis in which central nervous system (CNS) viral invasion occurs rapidly but the disease development is relatively slow, we visualized the initial brain infection and enhance the data collection process required for efficacy studies on antivirals or vaccines that are aimed at preventing brain infection. Accordingly, we infected mice through intranasal inoculation with the genetically modified pathogen, Venezuelan equine encephalitis, which expresses a luciferase gene. In this study, we were able to identify the invasion of the CNS at least 3 days before any clinical signs of disease, allowing for reduction of animal morbidity providing a humane means of disease and vaccine research while obtaining scientific data accurately and more rapidly. Based on our data from the imaging model, we confirmed the usefulness of this technology in preclinical research by demonstrating the efficacy of Ampligen, a TLR-3 agonist, in preventing CNS invasion.

Keywords

IVIS; Luciferase; in vivo; VEEV; TC83; neuroinvasion; encephalitis

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Introduction

Quick and accurate identification of disease progression are key factors in testing novel vaccines and antivirals. Preclinical efficacy and viral pathogenesis studies utilize a large number of animals [1–3]. These studies involve distinct endpoints, primarily mortality or largely decreased body weight to identify severe illness that is often lethal [3]. The large numbers are needed to statistically confirm viral replication sites, time points of organ infection, and overall timeline for disease progression. During many studies for viral pathogens the animals develop illnesses in which anorexia and hyperthermia or hypothermia followed by paralysis are a few of the morbidity indicators [1]. These viral studies also pose increased risk to researchers due to increasing disease development in the animals. High virus titers in infected animals, irritable animals, and organ collection all pose potentially higher risks for researchers when working with animals. New methodologies and technologies can be utilized to reduce animal numbers, animal morbidity, experimental time, and researcher exposure risk while improving our capability to localize to specific organs or within organs regions of viral replication all in a single study with the same animals.

In vivo imaging systems (IVIS) generate these desirable experimental qualities utilizing either fluorescent proteins [4] or bioluminescent enzymes [5] to visualize the signal. Utilizing bioluminescent enzymes, such as firefly luciferase, provides many benefits compared to fluorescent proteins, including lower background in animals while providing sufficient spread within an animal for visual identification [6]. Technological developments have greatly increased the ability to detect minute levels of emitted light assisted with real-time detection of the reactive vector in living animals [7]. Previously published studies have presented the effectiveness of IVIS in studying multiple viral pathogens and their progression in animal models [8–11].

We propose the utilization of IVIS to rapidly visualize the penetration of the CNS by Venezuelan equine encephalitis virus (VEEV) in the murine model. This is an alphavirus known to cause periodic, large outbreaks in both humans and equines. Humans can develop severe acute, encephalitic disease followed by muscle weakness with an increased mortality rate in pediatric cases [12]. The attenuated IND vaccine strain of VEEV, TC83, was passaged 83 times and has several mutations [13, 14], however, TC83 replicates to very high titers in the brains of several murine species while causing high mortality in some [2, 15] especially following intra-nasal inoculation. Previously, the use of TC83 as a model for VEEV encephalitis has been proposed as a means to study possible antivirals against VEEV [16, 17].

Our goal was to further develop this murine model for the usage in the preclinical setting for 3 equally important reasons: 1) to minimize the regulatory burden that relates to biosafety level 3 work and select agent regulations required for the experiments with wild type VEEV; 2) to shorten the experimental time needed to accurately demonstrate the brain infection as an experimental endpoint and 3) to reduce the animal numbers and suffering that relate to studies of encephalitic diseases.

In this study, we were able to identify the invasion of the CNS by VEEV at least 3 days before any clinical signs of disease, which allows for reduction of animal morbidity providing a humane means of disease and vaccine and/or antiviral research while obtaining scientific data accurately and more rapidly. In addition to identification of viral replication we were able to accurately identify the movement of viral replication from the nasal region to the fore region of the CNS through 3-Dimensional IVIS. To demonstrate the usefulness of this technology in preclinical research, we tested the efficacy of Ampligen, a TLR-3 agonist [1], in preventing CNS invasion.

Materials and Methods

Cells and Viruses

Baby hamster kidney (BHK-21) and Vero cells (American Tissue Culture Collection, Manassas, VA) were maintained in minimal essential medium (MEM) supplemented with 10% FBS, L-glutamine and vitamins.

VEEV TC83 viral stock was produced in Vero E6 cells and stored at -80°C in 1-mL aliquots until use. All work with infectious virus was performed at UTMB Bio-Safety Level 2 (BSL-2) in accordance with institutional health and safety guidelines.

Construction of VEE recombinant virus

DNA work was accomplished using standardized cloning techniques [18] with commercially available enzymes. The competent *Escherichia coli* strain of cells DH5 α (Invitrogen) was used for all cloning and maintenance of the recombinant constructs. Platinum *Pfx* and *Taq* (Invitrogen) polymerases were utilized for all polymerase chain reactions (PCR). Sequencing of all cDNA fragments and plasmids was completed at the UTMB sequencing core facility.

The TC83 VEEV vector, two helper plasmids, and the firefly luciferase vector (provided by Dr. Frolov, University of Alabama) used to package TC83 into infectious virions, are all described elsewhere [19, 20].

Animals

Six to eight week female ICR mice were purchased from Charles River (Wilmington, MA) and housed in the Galveston National Laboratory ABSL-2 facility. All animals were given a minimum of 2 days to become acclimated to the environment before any study manipulations. All animal studies were approved by the UTMB Institutional Animal Care and Use committee (IACUC) and carried out according to NIH guidelines.

Telemetry

All animals were implanted subcutaneously with a BMDS IPTT-300 transponder (chip) purchased from Bio Medic Data Systems, Inc., with a trocar needle assembly. The animals were monitored for signs of infection or migration of the transponder for at least 24 hours prior to further progression into the study. All chips were scanned using a DAS-6007 (Bio Medic Data Systems, Inc.) and temperature data downloading was performed in accordance with the manufacturer's protocol.

In vivo imaging

ICR mice were used for all imaging experiments. Mice were shaved prior to inoculation to allow better detection of the bioluminescent signal. All mice were inoculated through intranasal exposure of 4×10^6 to 1×10^7 pfu in 40 μL volumes. Prior to imaging mice were given luciferin through either intraperitoneal injection (10 μL /gram body weight of a solution containing 15mg/mL Luciferin) or intranasal inoculation (10–20 μL /nare of a solution of 3mg/mL of Luciferin). *In vivo* images were acquired with the IVIS charge-coupled-device camera system and analyzed with the LivingImage 3.0 and 4.0 software package. Exposure times used were 1–5 seconds per image. Three dimensional imaging was accomplished utilizing the LivingImage 4.0 software package. Default wavelength and auto exposure detection software defaults were selected through the Wizard bioluminescent selection tool in which 5 still images were completed at increasing wavelength filters (560,580,600,620,640 nm). Surface topography was generated automatically through the

software with minimal user changes and final DLIT reconstruction and organ fitting was accomplished utilizing a non-linear transformation.

Infectious virus titration in organs

To quantify VEEV replication within the CNS, specimens were dissected at necropsy and homogenized in MEM containing 1% penicillin-streptomycin solution (50,000units of penicillin and 50,000µg of Streptomycin in 500mL media volumes). Suspensions were clarified by centrifugation and the supernatants were harvested and frozen at -80C until analysis was performed. The titer of infectious virus was determined using a plaque assay in Vero cells.

Results

Rescue of TC83-Luciferase

To generate the engineered TC83-Luciferase virus, we utilized a previously designed VEEV rescue system [19] based upon the recombinant TC83 vaccine strain [21]. A firefly luciferase gene was cloned into the TC83 cDNA plasmid directly downstream of the sub-genomic promoter (Figure 1). The recombinant virus was rescued in Vero cells at a comparable titer to wild type TC83. Plaque size analysis showed some differences from wild type TC83 virus [19]. Luminometer detection confirmed luciferase activity *in vitro* following virus infection and replication in Vero cells. Visualization of bioluminescent signal from cells infected with TC83-Luciferase confirmed our successful rescue of an infectious recombinant TC83 virus which we can further utilize for *in vivo* studies.

Visualization of alphavirus in vivo CNS invasion

To confirm that recombinant VEEV could be visualized with the IVIS system, ICR mice were infected through intranasal inoculation with TC83-Luciferase. Two different luciferin administration methods, intraperitoneal (IP) and intranasal (IN), were also analyzed to determine the most efficient means of producing a strong bioluminescent image early in the infection. Images taken 1 day post infection (dpi) showed the strongest bioluminescent signal in those mice receiving IN luciferin (Figure 2B). By 3dpi signal strength was strongest in mice receiving IP luciferin and by 4dpi no signal was visible in mice receiving IN luciferin (Figure 2A). IVIS analysis showed maximum bioluminescent signal strength at 3 and 4dpi with maximum brain virus titration at 4 and 5dpi (Figure 2D/E). Histopathological analysis identified inflammation and cellular invasion correlated to encephalitic diseases (Figure 2C) and antigen detection specific to VEEV identified high levels of virus antigen throughout the mice brains (Figure 2C). Clinical analysis of temperature and weight change in the mice during the study showed no significant changes from negative control non-infected mice for the first 5dpi (Figure 1B/C) nor from wild type infection with TC83 up to 6 dpi (data not shown). Imaging confirmed that we were able to identify CNS invasion and progression of TC83Luciferase before any clinical symptoms develop. We were able to visualize this progression in living animals over multiple days without the need to sacrifice the animals.

Ampligen antiviral treatment and vaccine analysis

The IVIS system is an ideal system to identify and complete initial tests of potential vaccines and antiviral candidates against VEEV. Due to CNS invasion being a key aspect for VEEV encephalitic disease, further development of the TC83-Luciferase infection model is a perfect test system to develop a non-invasive means to test therapeutics. Accordingly, ICR mice were given Ampligen, a TLR-3 agonist that protects against lethal TC83 challenge [22–24] through either an IP or IN route of administration. Mice in the group receiving IP

Ampligen at -4 and +24 hours showed no bioluminescent signal throughout the period of the study (Figure 3A) and viral titer analysis from the brain showed 2 out of 3 mice with no detectable virus load by 6 DPI. Histochemical analysis confirmed no signs of encephalitis or meningitis at 6 DPI. Mice receiving IP Ampligen at +24hpi showed a significantly reduced ($p<.05$) signal compared to our control TC83-Luciferase infected mice as well as an increased time of clearance from the nasal cavity (Figure 3B). Mice that were previously immunized with TC83 (subcutaneous injection 21 days prior to TC83-Luciferase challenge at a dose of 5×10^5 pfu) had no signal throughout the entirety of the study. (Figure 4D). Analysis of brain slides identified minimal pathologic changes pertaining to wild type disease and TC83 specific antibody staining showed reduced viral antigen present in the brain at 6 DPI (Figure 3A/B).

Mice receiving IN Ampligen at -4 and +24 HPI failed to show a significant reduction of bioluminescent signal compared to untreated mice or to mice receiving IP Ampligen at +24 HPI (Figure 4D). Pathological analysis did identify reduced levels of encephalitic disease reduced levels of viral antigen than those compared to wild type infection but still greater than levels seen with IP Ampligen treatment (Figure 4D). In addition, two mice were given a vaccine dose of TC83 21 days prior to TC83-Luciferase challenge. They were imaged following IP luciferin injection and presented with no signal throughout the study (Figure 4A). Pathological analysis of the brain identified no signs of disease or viral antigen present in the slides at 6 DPI (Figure 4B). Following therapeutic and prophylactic treatments with an immune enhancer, our model allowed rapid efficacy assessment. The IVIS system was able to quickly and accurately identify signal reduction or complete inhibition provided by Ampligen and TC83 immunization before any clinical disease development was detected in unprotected mice.

Three-Dimensional localization and analysis

To confirm localization of bioluminescent signal we utilized the LivingImage 4.0 software system to generate 3-dimensional images. Using these images we were able to demonstrate the signal within the brain, most likely from the olfactory bulb and progressing more caudal with time, which is consistent with the previously described entry of VEEV into the CNS [2, 25, 26, and 27]. Further imaging (Figure 5) confirmed this progression from the nasal region to a strong CNS signal as the study progressed.

Discussion

We present an improved experimental model in which research studying brain infection by VEEV may be accomplished rapidly and more effectively than before. This is different from other efficacy studies commonly utilized to research VEEV infection which normally include large animal numbers requiring daily sacrifice and potentially high morbidity development. This leads to increased risk for researchers, increasing research costs, and extended study timelines which may all be reduced with the utilization of new IVIS models.

To further murine VEEV models we have shown the capability to visualize CNS invasion following intranasal inoculation. We were able to detect bioluminescent signal from virus within the brain at titers as low as 500pfu/gram of brain tissue through IVIS imaging. This allows us to track the progression of viral replication from its earliest time point in the nasal cavity to the virus invasion of the brain, supporting data previously described [25]. As technology improves we expect our detection capabilities to also improve, including 3-dimensional identification of locations within the brain showing viral replication, inflammation response, and chemokine production at the site of replication. Future camera capabilities to detect low virus loads in the brain and other organs will further prove the

benefit of IVIS models in testing vaccines and therapeutics in a quicker and more accurate method.

Our study has shown IVIS modeling to be a simpler and faster research methodology than current *in vivo* studies. IVIS allows us to collect multiple data points from an individual animal, reducing the required number of animal sacrifices normally [1, 2]. We have shown the ease of tracking the virus into the CNS and the ability to identify key infection endpoints without waiting for development of disease and morbidity in the animals. In addition, the capability and speed of 3-dimensional imaging allows for the localization of replication and infection to specific organs and tissues as a means to confirm *in vivo* these sites as never before. Also, we were able to accomplish this with increased safety to the researcher as it required minimal interaction with ill animals at the biosafety level 2, reduced viral loads in the blood of the animals, and decreased total number of animal necropsies.

While a bioluminescent signal was detectable through the white-pigmented hair of the ICR mice, shaving assisted with detection and will probably be required for all darker haired animal models. Due to our utilization of firefly luciferase instead of a fluorescent protein, minimal background was observed in our images providing clear and precise points of viral replication. This decreased background promotes the usage of luciferase genes as a means to visualize viral replication in future studies compared to other proteins. The insertion of a luciferase gene into the viral genome did result in some attenuation visible in both titer load and plaque size. However, the attenuation is not important due to the short period of time it takes to detect viral invasion of the CNS in which there is no discernable difference between TC83-Luciferase and wild type TC83.

As modeling technologies, IVIS and other *in vivo* systems are becoming more prominent as more researchers are fully utilizing these developing technologies. Infectious disease research is not fully utilizing these technologies compared to other fields but within the next decade we expect more and more researchers will come to utilize these systems which increase scientific research efficiency while decreasing animal use. Current political and social pressure will continue to be applied to research to reduce or eliminate animal use and sacrifice. IVIS is one step in reducing this pressure while increasing the quality of the research being accomplished. Our study provides a scientific basis to standardize and create key endpoints for rapid screening of anti-VEEV drugs or vaccines and potentially might be easily translated to other CNS invading viruses.

Acknowledgments

We would like to acknowledge Dr. Ilya Frolov for providing the initial TC83 plasmid and plasmid containing firefly luciferase, and to Dr. Nadezda Yun for assistance in writing this manuscript. Michael Patterson was supported in part by the Institute for Translational Sciences UTMB-NIH grant 1UL1RR029876-01 and SP was supported in part for this research by the IHII grant.

References

1. Julander JG, Skirpstunas R, Siddharthan V, Shafer K, Hoopes JD, Smee DF, et al. C3H/HeN mouse model for the evaluation of antiviral agents for the treatment of Venezuelan equine encephalitis virus infection. *Antiviral Research*. 2008; 78(3):230–41. [PubMed: 18313150]
2. Steele KE, Davis KJ, Stephan K, Kell W, Vogel P, Hart MK. Comparative Neurovirulence and Tissue Tropism of Wild-type and Attenuated Strains of Venezuelan Equine Encephalitis Virus Administered by Aerosol in C3H/HeN and BALB/c Mice. *Veterinary Pathology Online*. September; 1998 35(5):386–97.
3. Ludwig GV, Turell MJ, Vogel P, Kondig JP, Kell WK, Smith JF, et al. Comparative neurovirulence of attenuated and non-attenuated strains of Venezuelan equine encephalitis virus in mice. *Am J Trop Med Hyg*. January 1; 2001 64(1):49–55. [PubMed: 11425162]

4. Yang M, Baranov E, Moossa AR, Penman S, Hoffman RM. Visualizing gene expression by whole-body fluorescence imaging. *Proceedings of the National Academy of Sciences of the United States of America*. October 24; 2000 97(22):12278–82. [PubMed: 11050247]
5. Contag CH, Contag PR, Mullins JI, Spilman SD, Stevenson DK, Benaron DA. Photonic detection of bacterial pathogens in living hosts. *Molecular Microbiology*. 1995; 18(4):593–603. [PubMed: 8817482]
6. Wu JC, Sundaresan G, Iyer M, Gambhir SS. Noninvasive Optical Imaging of Firefly Luciferase Reporter Gene Expression in Skeletal Muscles of Living Mice. *Mol Ther*. 2001; 4(4):297–306. [PubMed: 11592831]
7. Contag PR, Olomu IN, Stevenson DK, Contag CH. Bioluminescent indicators in living mammals. *Nat Med*. 1998; 4(2):245–7. [PubMed: 9461201]
8. Luker GD, Prior JL, Song J, Pica CM, Leib DA. Bioluminescence Imaging Reveals Systemic Dissemination of Herpes Simplex Virus Type 1 in the Absence of Interferon Receptors. *J Virol*. October 15; 2003 77(20):11082–93. [PubMed: 14512556]
9. Contag CH, Jenkins D, Contag PR, RSN. Use of reporter genes for optical measurements of neoplastic disease in vivo. *Neoplasia*. 2000; 2(41)
10. Cook SH, Griffin DE. Luciferase Imaging of a Neurotropic Viral Infection in Intact Animals. *J Virol*. May 1; 2003 77(9):5333–8. [PubMed: 12692235]
11. Osorio JE, Iams KP, Meteyer CU, Rocke TE. Comparison of Monkeypox Viruses Pathogenesis in Mice by In Vivo Imaging. *PLoS ONE*. 2009; 4(8):e6592. [PubMed: 19668372]
12. Weaver SC, Ferro C, Barrera R, Boshell J, Navarro J-C. VENEZUELAN EQUINE ENCEPHALITIS. *Annual Review of Entomology*. 2004; 49(1):141–74.
13. Kinney RM, Chang GJ, Tsuchiya KR, Sneider JM, Roehrig JT, Woodward TM, et al. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'noncoding region and the E2 envelope glycoprotein. *Journnal of Virology*. 1993; 67(3)
14. Johnson BJB, Kinney RM, Kost CL, Trent DW. Molecular Determinants of Alphavirus Neurovirulence: Nucleotide and Deduced Protein Sequence Changes during Attenuation of Venezuelan Equine Encephalitis Virus. *J Gen Virol*. September 1; 1986 67(9):1951–60. [PubMed: 3755750]
15. Hart MK, Pratt W, Panelo F, Tammariello R, Dertzbaugh M. Venezuelan equine encephalitis virus vaccines induce mucosal IgA responses and protection from airborne infection in BALB/c, but not C3H/HeN mice. *Vaccine*. 1997; 15(4):363–9. [PubMed: 9141206]
16. Kuehne RW, Pannier WL, Stephen EL. Indirect mouse model for the evaluation of potential antiviral compounds: results with Venezuelan equine encephalomyelitis virus. *Antimicrob Agents Chemother*. 1977; 11(4)
17. Lukaszewski RA, Brooks TJG. Pegylated Alpha Interferon Is an Effective Treatment for Virulent Venezuelan Equine Encephalitis Virus and Has Profound Effects on the Host Immune Response to Infection. *J Virol*. June 1; 2000 74(11):5006–15. [PubMed: 10799574]
18. Sambrook, JRD. *Molecular cloning: a laboratory manual*. 3. New York: Cold Spring Harbor Laboratory Press; 2001.
19. Volkova E, Gorchakov R, Frolov I. The efficient packaging of Venezuelan equine encephalitis virus-specific RNAs into viral particles is determined by nsP1–3 synthesis. *Virology*. 2006; 344(2):315–27. [PubMed: 16239019]
20. Seregin AV, Yun NE, Poussard AL, Peng B-H, Smith JK, Smith JN, et al. TC83 replicon vectored vaccine provides protection against Junin virus in guinea pigs. *Vaccine*. 28(30):4713–8. [PubMed: 20452431]
21. Kinney RM, Johnson BJB, Welch JB, Tsuchiya KR, Trent DW. The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. *Virology*. 1989; 170(1):19–30. [PubMed: 2524126]
22. Strayer DR, Carter WA, Brodsky I, Cheney P, Peterson D, Salvato P, et al. A Controlled Clinical Trial with a Specifically Configured RNA Drug, Poly(I). POLY(C12U), in Chronic Fatigue Syndrome Clinical Infectious Diseases. January 1; 1994 18(Supplement 1):S88–S95.
23. Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, et al. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell

- targeted vaccine. *Proceedings of the National Academy of Sciences*. February 19; 2008 105(7): 2574–9.
24. Nicodemus CF, Wang L, Lucas J, Varghese B, Berek JS. Toll-like receptor-3 as a target to enhance bioactivity of cancer immunotherapy. *American Journal of Obstetrics and Gynecology*. 202(6): 608.e1–e8. [PubMed: 20080226]
 25. Charles PC, Walters E, Margolis F, Johnston RE. Mechanism of Neuroinvasion of Venezuelan Equine Encephalitis Virus in the Mouse. *Virology*. 1995; 208(2):662–71. [PubMed: 7747437]
 26. Jackson AC, Rossiter JP. Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice. *Acta Neuropathologica*. 1997; 93:349–353. [PubMed: 9113200]
 27. Vogel PAD, Kell W, Ibrahim MS, Downs MB, Pratt WD, Davis KJ. Venezuelan equine encephalitis in BALB/c mice: kinetic analysis of central nervous system infection following aerosol or subcutaneous inoculation. *Arch Pathol Lab Med*. 1996; 120:164–72. [PubMed: 8712896]

Figure 1. TC83-Luciferase and clinical development

(A) Schematic presentation of the genome of the TC83-Luciferase virus with the inserted firefly luciferase gene under the control of a second sub-genomic promoter. (B) Analysis of weight loss following IN infection with TC83-Luciferase, no significant clinical change is identified until 6 days post infection ($p < .05$). (C) Clinical temperature data analysis shows no significant difference between infected and uninfected mice throughout the study. (D) Histology and immunohistochemistry specific to TC83 from brain sections taken at 6 days post infection show high levels of virus antibody present in the brain along with signs of encephalitis in the tissue.

Figure 2. Imaging of TC83-Luciferase infection in ICR mice

IVIS of TC83-Luciferase following IN infection of ICR mice from days 1 to 5 post infection given IP luciferin (A1–5) or IN luciferin (B1–5).). Histology (C1,3) and immunohistochemistry (C2,4) slides of the brain at day 6 post infection showed signs of encephalitis and viral antigen. The mice receiving IP luciferin had a maximum signal by day 3 post-infection with a decreasing but detectable signal until 8 days post infection (D). Mice receiving IN luciferin peaked at 2 days post infection and lost their bioluminescent signal by 4 days post infection (D). Titration of brain tissue taken from mice (E).

Figure 3. IP Ampligen treatment prevents brain infection

Brain tissue histology and immunochemistry at 6 days post infection showing the difference between IP therapeutic and prophylactic treatments of the TLR3 agonist Ampligen. IVIS imaging of ICR mice for the first five days of infection (A1–5) in mice receiving Ampligen at –4 and +24 hours show no bioluminescent signal throughout the study. Histology (A6,8) and immunohistochemical slides (A7,9) from brain tissue taken at +6 DPI from mice (bottom) show no signs of disease or detectable antigen. Mice receiving IP Ampligen at +24 hours post infection (B1–5) resulted in decreased bioluminescent signal and minimal disease development (B6,8) while immunohistochemistry (B7,9) detected virus antigen in the brain unlike the mice receiving prophylactic Ampligen. Positive control slides and IVIS imaging can be found in Figure 2 for comparison.

Figure 4. IVIS identification of vaccine protection

Mice were vaccinated at 21 days before infection with subcutaneous inoculation of TC83. IVIS pictures of mice receiving IP luciferin identified no detectable signal of virus up to 5 days post IN challenge (A1–5) as compared to non-vaccinated mice (C1–5 taken from Figure 2A). Histology and immunohistochemistry identified no visible signs of disease (B1,3) or virus antigen (B2,4). A graphical comparison of all IVIS image strengths identify IP luciferin injection of untreated mice results in the highest signal detected with a significant reduction based upon treatment or IN luciferin injection (D).

Figure 5. Three-Dimensional localization of bioluminescent signal in the CNS

A computer generated 3-dimensional image of bioluminescent signal from a mouse infected with TC83-Luciferase at 1 dpi presenting two localized points of infection, a larger signal visualized within the nasal region and a small signal deeper within the CNS.