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Rumi Habib April 8, 2019

The Role of The NS1 Protein in Zika Virus Pathogenesis

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Zika Virus (ZIKV) is an emerging single-stranded positive-sense RNA virus of the genus *Flavivirus*. Most cases of ZIKV infection are mild or subclinical, resulting in an acute disease with common symptoms including a rash, fever, and joint pain. However, during the 2015-2016 ZIKV outbreak in South and Central America, it was observed that ZIKV infection can result in complications such as thrombocytopenia, Guillain–Barré syndrome, and most significantly, infant microcephaly. These observations showed that ZIKV pathogenesis is still poorly understood, leading the World Health Organization to declare the outbreak an international public health emergency in 2015 and list ZIKV as a priority pathogen. The ZIKV genome is divided into 3 structural and 7 non-structural proteins. In cells infected with ZIKV, non-structural protein 1 (NS1) dimerizes and plays a role in the viral replication complex. However, it has also been shown to be secreted from infected cells in a hexameric form (sNS1). This secreted form of the protein has been shown to be highly immunogenic and is being investigated as a potential vaccine candidate for ZIKV. This means that the immune response to sNS1 should act as a strong selective pressure against the secretion of the protein by the virus, yet sNS1 secretion is a strongly conserved feature of flavivirus pathogenesis, suggesting that sNS1 plays an important role in ZIKV-host interactions. In Dengue virus infection (a closely related virus), sNS1 has been implicated in the vascular leakage seen during the course of the disease, and in the generation of autoreactive antibodies. We hypothesized that sNS1 plays a similar part in the vascular permeabilization seen during ZIKV pathogenesis such as in the uterus, the blood-brain barrier, and the blood-ocular

barrier. To test this hypothesis, we used a lentiviral vector to generate a cell line stably expressing ZIKV NS1. In addition, we injected mice with ZIKV NS1 to look for impacts on proteins involved with the coagulation pathway, and the generation of autoreactive antibodies.

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And to the members of the Skountzou Lab, for putting up with all the flaming microwaves.

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Introduction

The Impact of Zika Virus

Flaviviruses are an arthropod-borne genus of positive-sense single-stranded RNA viruses responsible for significant public health and economic burden. Members transmitted by mosquitoes of the species *Aedes* include pathogens such as the Zika virus (ZIKV), Dengue virus (DENV), and Yellow Fever virus. These viruses are spread worldwide, and the mosquito-mediated geographic distribution of flaviviruses has been shown to be highly dynamic[1]. Changes in the natural habitats of the *Aedes* mosquitoes, exacerbated by global warming, present a significant risk of flavivirus spread into previously non-endemic areas. As a recent example, the 2015-2016 ZIKV epidemic saw the virus spread from Brazil to almost all of the Americas, and into the United States, where it became a serious public health issue[2].

While mortality associated with ZIKV infection is rare, the recent epidemic in the western hemisphere saw a wave of infants born with microcephaly and adults with Guillain-Barré syndrome[2], prompting the WHO to declare the outbreak an international public health emergency and designate ZIKV a priority pathogen[3]. This resulted in considerable research on ZIKV pathogenesis, leading to several new discoveries regarding ZIKV pathology such as its ability to be sexually transmitted and viral neurotropism[2]. These discoveries highlighted our poor understanding of ZIKV biology, and the mechanisms by which ZIKV infection produces these symptoms is still unknown. To better understand the mechanisms of ZIKV pathogenesis, we decided to look at how NS1 antigenemia, a conserved feature of flavivirus pathogenesis, and the resulting host response contribute to ZIKV pathogenesis.

The sNS1 Protein

In infected cells, the ZIKV genome is translated as a single polyprotein, which is then cleaved into 3 structural and 7 non-structural proteins[4]. The first of these non-structural proteins, Non-Structural protein 1 (NS1), is a 50 kDa protein that exists within the cell as a dimer, where it plays an important role in viral RNA replication. Structural studies of NS1 have shown that the protein dimer has hydrophobic and hydrophilic faces, resulting in localization to intracellular membranes[5]. On the "inner" face of the dimer, a β-roll domain and an adjacent "greasy finger" loop form a hydrophobic surface for membrane interaction[6], while the dimer's "outer" face is polar and contains glycosylation sites[5]. NS1 is also actively reprocessed and secreted from infected cells as a hexamer (sNS1) at concentrations high enough that it has been used as a diagnostic marker for flavivirus infections[7,8]. In the sNS1 hexamer, three dimers assemble with the glycosylated, polar faces pointing outward and the hydrophobic faces pointing inward, interacting with lipid molecules to form a barrel-shaped protein structure with a central channel containing lipids[5,9]. The active secretion of a protein from infected cells, though not uncommon for large DNA viruses, is rare for an RNA virus. The secretion of sNS1 becomes even more perplexing in light of evidence that NS1 has been shown to be highly immunogenic[10,11]. Responses to NS1 have been shown to be protective against several flaviviruses[8] and are expected to be a strong selective pressure against the secretion of sNS1 by the infected cells. Paradoxically, despite what should be a strong selective pressure against the production of sNS1, it is still a highly evolutionarily conserved feature of flavivirus infection[12]. This implies an important role for the protein in the viral life cycle, a role that is poorly understood.

Studies with DENV and Japanese Encephalitis virus have shown a role for sNS1 in increasing the efficiency of virus transfer from infected individuals to mosquitoes[13]. Furthermore, levels of sNS1 antigenemia have been correlated with DENV disease severity, and sNS1 has been shown to interact with complement and coagulation pathway proteins, linking it with the vascular permeabilization seen during DENV hemorrhagic fever[12]. Similar patterns involving sNS1 also exist with other flaviviruses known to cause hemorrhagic symptoms[8], and together these observations indicate a potential role for sNS1 in producing significant physiological changes in the host during infection, suggesting that it is an important factor in flavivirus pathogenesis.

Unlike DENV, ZIKV sNS1, having only recently come into focus, is relatively poorly understood. However, given that ZIKV and DENV are closely phylogenetically related[14], the ZIKV sNS1 protein would be expected to resemble the DENV sNS1 protein in its effects during infection. Furthermore, a study found that a mutation in the ZIKV NS1 gene may have contributed to the emergence of the 2015-2016 epidemic strain, and the epidemic viruses have been shown to have several differences in pathogenesis and the host immune responses they elicit compared to older ZIKV strains[15]. These observations together suggest an important role for sNS1 in the pathogenesis of ZIKV.

Investigating the Role of sNS1 in ZIKV Pathogenesis

Given this gap in understanding, we investigated the role of sNS1 in ZIKV pathogenesis *in vivo*. We hypothesized that ZIKV sNS1 plays a role in altering the coagulation pathway by generating autoreactive antibodies, inducing vascular permeabilization similar to that seen in the pathogenesis of other flaviviruses. sNS1 has been associated with producing hemorrhagic symptoms during flavivirus infection[8,12,16] and it is possible that it is related to ZIKV's ability to cross the placenta and blood-brain barrier. The antibody response to DENV NS1 has been shown to alter clotting in mice injected with the protein, and changes in the levels of platelets have also been associated with vascular leak[16]. Although most studies regarding vascular permeabilization have looked at DENV, human case studies show that similar effects might be relevant in ZIKV pathogenesis, with reports of thrombocytopenia during ZIKV infection[17,18]. Furthermore, among the flaviviruses ZIKV is unique in that it is the only human flavivirus to show ocular and placental tropism[2]. Structural analyses have found regions of the ZIKV sNS1 hexamer that are distinct from other flaviviruses[5], and it is possible that these differences in the structure may be related to this distinct pathogenesis. Therefore, our objective was to identify the role of sNS1 in inducing abnormalities in the coagulation pathway via alterations of the levels of proteins in the pathway and the generation of autoreactive antibodies. In order to do this, we attempted to purify the NS1 protein from a cell line stably transduced using lentiviral vectors, and examine its effects *in-vivo* in a BALB/c mouse model.

Materials and Methods

Molecular Cloning

The Zika virus NS1 gene (strain PRVABC59) was synthesized *de novo* by Eurofins genomics (Louisville, KY, USA). A 6xHis polyhistidine tag for later metal affinity purification of the protein preceded by a Human Rhinovirus 3C (HRV 3C) Protease cleavage site for removal of the 6xHis tag was added to the 3' end of the NS1 sequence, BamHI and NotI restriction sites at the 5' and 3' ends of the synthesized gene, respectively (Figure 1). The plasmids to make 2nd generation lentiviral vectors (from Dr. Oskar Laur at the Emory Integrated Genomics Core and Dr. John Shires at the NIH Tetramer Core) consisted of a packaging plasmid (pCMVR8.74), an envelope plasmid (pMD2.G), and a transfer plasmid (pLEX-MCS).

The transfer plasmid and recombinant genes were digested simultaneously with 10U of NotI and 20U of BamHI restriction enzymes (New England Biolabs, Ipswitch, MA) at 37˚C for 1 hour in NEBuffer 3.1 according to the manufacturer's instructions, and purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ligation reactions were carried out for 10 minutes at room temperate with an approximately 7:1 insert-to-vector ratio. The ligated plasmid was transformed into TOP10 *E. coli* (Invitrogen, Carlsbad, CA) using a 30-second heat shock at 42˚C, and the bacteria were cultured on ampicillin plates overnight at 37˚C. Transformed bacteria were cultured on selective Luria broth (LB) agar plates containing 100 µg/ml Ampicillin (VWR, Radnor, PA) overnight at 37˚C. A single colony was used to inoculate liquid LB cultures containing ampicillin (100 µg/ml) and grown overnight at 37˚C. Plasmids were isolated via QiaPrep Spin Miniprep kits (Qiagen, Hilden, Germany)

according to the manufacturer's instructions. The successful insertion and sequence fidelity of the inserted gene was verified via Sanger sequencing (Eurofins genomics, Louisville, KY, USA). The overnight culture was then used to inoculate liquid LB media with ampicillin selection (100µg/ml) at a 1:100 ratio and grown overnight at 37˚C with shaking at 220 rpm. Plasmid was isolated via Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The gene for eGFP was amplified from a different lentiviral transfer plasmid (EF.PGK.GFP) via PCR, using the forward primer 5' ATC GAG GAT CCG CCA CCA TGG TGA GCA AG 3' and the reverse primer 5' TCG ATG CGG CCG CTT ACT TGT ACA GCT CGT C3'. BamHI and NotI restriction sites (underlined) were introduced at 5' and 3' ends, respectively. EF.PGK.GFP was a gift from Linzhao Cheng[19] (Addgene plasmid # 17618 ; http://n2t.net/addgene:17618 ; RRID:Addgene_17618). The eGFP gene was then cloned into the pLEX-MCS transfer plasmid (pLex-MCS-eGFP) using the same method described above for the NS1 gene. The eGFP transfer plasmid was used to make lentiviral vectors for eGFP, which was then used to optimize and troubleshoot the transfection and transduction steps. The same lentiviral packaging and envelope plasmids were used to generate both the NS1 and eGFP lentiviral vectors.

Generation of a Stable Cell Line

HEK 293T cells were grown in 60mm dishes and simultaneously transfected with all three plasmids (envelope, packaging, and either the NS1 or eGFP transfer plasmid) using calcium phosphate transfection. The cell supernatant containing lentiviral vectors was filtered in 45µm syringe filters, and transferred to Vero cells in 100mm dishes daily for 3 days following transfection, after which the transfected cells were discarded. HEK 293T

cells were grown in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin, and Vero cells were grown in DMEM supplemented with 10% FBS, 1% HEPES, and 1% Penicillin/Streptomycin. Following the transduction of Vero cells, the cells were observed for fluorescence to confirm the presence of eGFP, and following confirmation, 10µg/ml puromycin was added to the cell culture medium to select for transduced cells. After generating a cell line stably expressing eGFP, we generated a cell line transduced with NS1 using the same procedure (Figure 2).

ELISA for NS1 Secretion

The secretion of NS1 was analyzed by sandwich ELISA. Nunc Maxisorb plates were coated with 500ng anti-ZIKV NS1 IgG1 (ThermoFisher, Monoclonal, EA88) and blocked with 2% BSA in PBS-T for 1 hour at 37˚C. Undiluted supernatants from cells transduced with NS1, eGFP, as well as ZIKV NS1 (1µg/ml, obtained through BEI Resources, NIAID, NIH: Nonstructural Protein 1 (NS1) with N-terminal Histidine Tag from Zika Virus, Recombinant from Baculovirus, NR-50872) were added to the wells after the blocking step and incubated for 1.5 hours at 37˚C. At the end of the incubation, polyclonal mouse sera from mice injected with either NS1 or PBS were added for an additional 1.5 hours at 37˚C. Goat anti-mouse IgG2a conjugated to HRP in 1:1000 dilution (1µg/ml) was added at the final step of the ELISA. The wells were washed 3 times with PBS-T between incubation steps. Plates were developed using o-phenylenediamine (OPD) dissolved in citrate buffer and the reaction was terminated with 1M H_3PO_4. Absorbance was measured at a wavelength of 490 nm in a Bio-Rad xMark plate reader.

Western Blot for NS1 Secretion

Cell supernatants along with NS1 obtained from BEI were denatured in SDS sample buffer at 95˚C for 10 minutes, resolved on an 8% polyacrylamide gel at 100V for 1.5 hours, and transferred to a nitrocellulose membrane using a semi-dry transfer at 15V for 1 hour. They were blocked with blocking buffer (5% milk in 1x TBS with 0.1% tween20) for 1 hour at room temperature, and incubated with a mouse anti-6xHis tag antibody (1µg/ml in blocking buffer) overnight at 4˚C. The membrane was then washed in TBST (1x TBS with 0.1% tween20) and incubated with a goat anti-mouse IgG antibody conjugated to Horseradish Peroxiadase (HRP). The membrane was developed using Amersham ECL Western blotting reagents, and chemiluminescence was imaged with an exposure time of 60 seconds using a ChemiDoc imager (BioRad, Hercules, CA).

Mouse Antibody Responses

BALB/c mice (Charles River Laboratories, Wilmington, MA) were housed at Emory University's Division of Animal Resources, and experiments were conducted in accordance with Emory University's Institutional Animal Care and Use Committee (IACUC). BALB/c mice were injected with 12.5µg of recombinant NS1 (BEI Resources, NIH, Bethesda, MD) adjuvanted with 20µl AddaVax (InvivoGen, San Diego, CA), and blood samples were collected at 12 and 35 days post-injection via submandibular bleed. Serum from blood samples was obtained after 2 rounds of centrifugation at 13,300rpm.

Anti-NS1 antibody levels were determined by ELISA. Plates were coated with 100ng of recombinant NS1 (BEI Resources, NIH, Bethesda, MD), and incubated with serum diluted 1:100 (day 12) or 1:200 (day 35) in PBST (1xPBS + 0.1% tween20). To generate standard

curves, plates were coated with 100µg/well of IgH+L (SouthernBiotech, AL), and incubated with 1:2 serially diluted mouse anti-IgG1 or IgG2a (SouthernBiotech, AL) starting at a concentration of 1µg/ml. The standards and samples were then incubated with HRP-conjugated goat anti-mouse IgG1 or IgG2a antibodies diluted 1:2000 (0.5µg/ml) (SouthernBiotech, AL). Plates were developed and absorbance was measured as described above for the capture ELISA.

Anti-plasminogen Antibody ELISA

Mice were bled at 49 days post-NS1 injection into 1.5 ml centrifuge tubes containing 15 µl of citrate buffer (composition of citrate buffer). 1.5 ml tubes were then centrifuged at 2000 rpm for 15 minutes. Plates were coated with mouse plasminogen (280ng/well, Haematologic Technologies Inc), and an ELISA for total IgG was performed as described above.

Results

Generation of Stably Transduced Cell Line

To obtain sufficient amounts of NS1 in a secreted hexameric form, we first attempted to generate Vero cell lines stably transduced with the NS1 sequence containing an N-terminal polyhistidine tag. As there is mixed evidence regarding the ability of insect cells to secrete sNS1, we chose to use Vero cells to ensure proper mammalian glycosylation and post-translational modification[20]. Additionally, Flamand et al. have previously demonstrated the production of soluble hexameric NS1 in Vero cells[21]. During the process of developing a cell line stably transduced with NS1, the pLex-MCS-eGFP plasmid and resulting eGFP lentiviral vectors were used as a positive control because of their ease of visualization. Fluorescence microscopy was used to verify the transformation of HEK 293T cells with the pLex-MCS-eGFP vector, and fluorescence was observed starting 1 day post-transfection (Figure 3A-B). The number of fluorescent cells and intensity of fluorescence increased each day post-transfection until the cells were discarded on day 3. This showed the successful transformation of cells with the vector. An increase in the number of fluorescent cells over successive days was indicative of either the production of lentiviral particles (due to the transduction of neighboring HEK 293T cells) or simply a delay between transfection with the plasmids and the production of eGFP at levels detectable by microscopy. A comparison with brightfield images showed modest to low transfection efficiencies (Figure 3C-D).

Upon successful transfection of HEK 293T cells, we next attempted to transduce Vero cells to produce a stably transduced cell line. Vero cells were transduced over 3 days with filtered HEK 293T cell supernatant, and eGFP expression was visualized via fluorescence

microscopy. A comparison to brightfield images showed very low transduction efficiency, suggesting low titers of lentiviral particles in the HEK 293T cell supernatant. Once transduced cells were observed, puromycin was added to culture media to select for stably transduced cells. In the case of eGFP transduced cells, non-fluorescent cells could be visualized detaching from culture plates as a sign of cell death (Figure 4F-J). Puromycin is an aminonucleoside analog antibiotic that inhibits protein synthesis by causing premature chain termination, and is selective for both eukaryotic and prokaryotic cells. A puromycin resistance gene was included on the lentiviral transfer plasmid, allowing the resistant, transduced cells to expand, while killing cells that lacked the gene. Although NS1 expression could not be directly visualized, puromycin resistance was observed in the case of NS1-transduced cells as well.

Confirmation of NS1 Secretion

Following the generation of a puromycin-resistant cell line, we proceeded to verify that the cell line was secreting the desired protein. This was first done by a sandwich ELISA of the supernatant of cells transduced with NS1 (Figure 5A). Raw optical densities (OD) from the sandwich ELISA compared using a paired t test demonstrated a significant difference in the presence of NS1 ($p=0.0443$) between the cell culture supernatant of NS1 and eGFP transduced cells. The measured OD of recombinant NS1 obtained from BEI Resources added to the wells at a concentration of 1µg/ml suggests that the NS1 concentration was the limiting factor in the formation of the NS1-antibody complex (Figure 5B). In addition, it showed that NS1 was present at concentrations far lower than 1 µg/ml in the supernatant of the NS1-transduced cells.

Furthermore, a western blot was used to verify that the expressed protein was present at the correct size (Figure 6). The NS1 monomer, depending on the extent of glycosylation migrates with an apparent molecular weight of 45-55 kDa. Western blot analysis of NS1-transduced cell supernatant showed a band between 45-50 kDa when probed with an anti-6xHis tag antibody, consistent with secretion of NS1. The NS1 obtained from BEI migrated slightly faster than the NS1 expressed in Vero cells, consistent with the protein being expressed in a baculovirus expression system, as lower levels of glycosylation are typically observed in proteins expressed from insect cell lines.

Mouse Antibody Responses to NS1

Prior to evaluating the anti-NS1 antibody response, we first established the immunogenicity of our NS1 vaccination. After collecting sera from mice at days 12 and 35 post vaccination, we measured the NS1 specific IgG response using an ELISA. ELISA data showed antibody responses to NS1 injection 12 and 35 days post-injection, with anti-NS1 IgG concentrations increasing at the later time point. Antibody concentrations increased from approximately 5.5 µg/ml to 11 µg/ml for IgG1 (Figure 7A) and from approximately 2.5 to 4.5 µg/ml for IgG2a (Figure 7B). Furthermore, no significant levels of anti-NS1 IgG were detected for mock-injected mice. This showed that the mice successfully generated an anti-NS1 antibody response.

Generation of Anti-plasminogen Antibodies

Following the confirmation of an anti-NS1 immune response, we looked for the presence of autoreactive anti-plasminogen antibodies via ELISA, as has been previously reported for DENV infection[22]. A total IgG ELISA for autoreactive anti-plasminogen antibodies showed no significant antibody levels, and no significant difference between NS1-injected and mock-injected mice (Figure 8). In conclusion, vaccination with NS1 did not induce any detectable anti-plasminogen IgG.

Discussion

Since the exact function of NS1 protein in ZIKV pathogenesis is still unclear, our objective was to elucidate its possible role in the induction of clotting abnormalities and vascular permeabilization seen during ZIKV infection[16,17]. To achieve this goal, we attempted to purify NS1 from a stably transduced cell line and investigate the physiological impact of the NS1 protein *in-vivo* with respect to the levels of coagulation factors and the generation of autoreactive antibodies. Here, we report the successful generation of an NS1 transduced Vero cell line. Vero cells transduced with NS1 lentiviruses were resistant to selection via puromycin, their supernatant produced signal over background via ELISA, and a reactive band of the expected size was observed when assayed via Western blot. Furthermore, following the detection of the NS1 band in a western blot using an anti-6xHis tag antibody, we demonstrated that the 6xHis tag was successfully expressed enabling us to harvest the protein from cell culture via metal affinity chromatography for use in downstream experiments. In parallel, we characterized the antibody response to NS1 in mice using commercially obtained NS1, so far concluding that intramuscular injection with NS1 does not generate autoreactive antibodies to plasminogen.

Plasminogen is an important proenzyme in coagulation pathways, where it is converted to plasmin, a serine protease that dissolves fibrin clots. Autoantibodies to plasminogen enhance its activation, resulting in reduced clotting[22]. Plasminogen was chosen for our studies because it is a protein shared by both the intrinsic and extrinsic coagulation pathways, is present in both humans and mice, has a large array of reagents available, and autoreactive antibodies to plasminogen have previously been documented as a result of DENV NS1 antigenemia[22].

It is unclear whether the NS1 obtained from BEI is dimeric or hexameric in structure, hindering conclusions regarding the interactions of the protein with coagulation factors in mice. However, the conserved presence of the same B cell epitopes as those of hexameric sNS1 allow us to draw conclusions regarding the generation of binding antibodies to these shared epitopes. Although we did not observe any autoreactive antibodies to mouse plasminogen using the ZIKV NS1 protein, this finding contrasts with the closely related DENV NS1, where autoantibodies are produced against human plasminogen[22]. It is possible that this discrepancy may be the result of our use of mouse rather than human plasminogen, though the genes for human and mouse plasminogen share 91.25% sequence homology. Alternatively, our data may simply represent a difference in the pathogenesis of the two viruses.

During the course of this project, another group successfully showed that intravenously injecting sNS1 from various flaviviruses resulted in tissue-specific vascular permeabilization that reflected the clinical complications seen during infection with those viruses, supporting the hypothesis that NS1 plays a role in the vascular permeabilization seen during ZIKV infection[23]. The study reported the effects upon injection of NS1 at levels far higher than those seen during natural flavivirus infection, and showed that such effects were the result of the disruption of endothelial barriers by the sNS1 protein. However, the effects of the antibody response to NS1 during ZIKV infection and whether ZIKV NS1 generates autoreactive antibodies similar to those seen during DENV infection remain to be elucidated. Furthermore, liver pathology as seen during DENV infection[24] is still poorly understood in the context of ZIKV, an important consideration given that the liver is the site where most of the proteins in the coagulation pathways are produced.

Although NS1 antigenemia has been correlated with severe disease outcomes for DENV[25], it should be noted that symptoms such as neurological complications and hemorrhaging as a result of flavivirus infection only represent a small fraction of infections despite the consistent secretion of sNS1, and that such disease manifestations do not provide the viruses with any known gain in fitness. This suggests that sNS1 plays a far more fundamental role in the flavivirus life cycle beyond its interactions with vasculature, and more studies are therefore necessary to determine its importance in terms of viral fitness. An example of such an answer came from Liu et al who reported that sNS1 increased the rate of DENV acquisition by mosquitoes in an *in-vitro* blood feeding system[13]. However, in an *in-vivo* model of mice infected with DENV-2, passive immunization with NS1 antibodies counteracted this effect, suggesting a complex uncharacterized temporal interaction between sNS1 secretion, degree of antigenemia, the resulting immune response, and the timing of viral uptake during the mosquito blood meal. The mechanism of this enhancement was found to be mediated by sNS1 suppression of mosquito immune responses in the midgut, and this phenomenon has yet to be explored in the context of ZIKV infection. This highlights a relatively understudied area of flavivirus research, virus-mosquito interactions, suggesting that part of the answer to the question of sNS1's role in terms of viral fitness may be elucidated there.

While transmission to its mosquito vector is an important part of the ZIKV life cycle, there is mixed evidence regarding whether infected mosquito cells produce NS1 in its hexameric form[20], which led us to focus on the role of the protein in the mammalian host for this project. The future directions of this project include confirming that sNS1 is being secreted in its hexameric form via native gel electrophoresis, after which production of

NS1 will be scaled up for *in-vivo* studies of its role in pathogenesis in a BALB/c mouse model. Preliminary work from our lab has demonstrated that ZIKV infection disrupts the levels of coagulation factors including plasminogen and Factor III in mice. sNS1 production and purification will allow for the comparison of sNS1 administration with ZIKV infection, allowing us to determine whether the change in the levels of coagulation factors is the result of sNS1 antigenemia or some other aspect of ZIKV pathogenesis. Furthermore, the serum from mice injected with commercially obtained NS1 will be further examined for the presence of autoreactive antibodies to other coagulation factors including Factor VII (belonging to the external coagulation pathway), Factor Xa, and Serpin C1 (which are shared by both internal and external pathways). In conclusion, this project has so far demonstrated the successful transduction of a cell line with ZIKV NS1. Furthermore, the preliminary lack of anti-plasminogen specific IgG antibodies suggests that the ZIKV NS1 protein's role in pathogenesis may be different from other flaviviruses, helping to explain the unique pathogenesis of ZIKV.

Figures

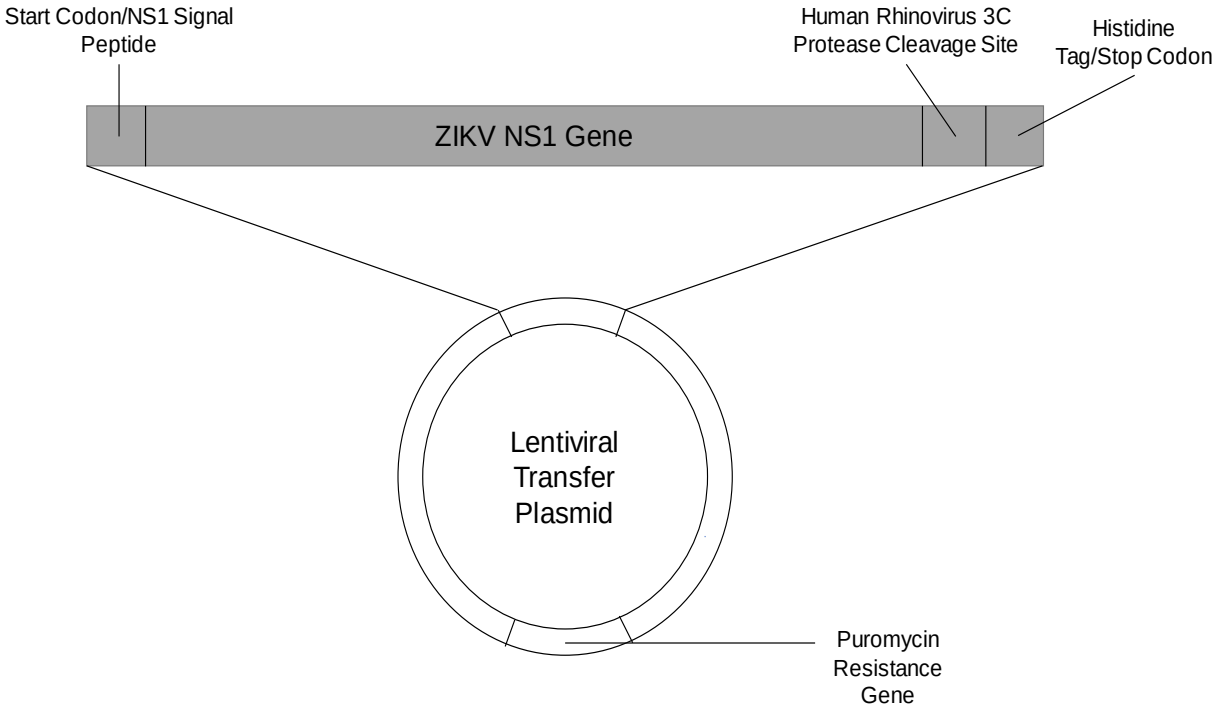

Figure 1: **The NS1 insert.** Diagram showing the ZIKV NS1 gene with a 6xHis tag and an HRV 3C protease cleavage site cloned into the lentiviral transfer plasmid. The transfer plasmid included a puromycin resistance gene for the selection of transduced cells.

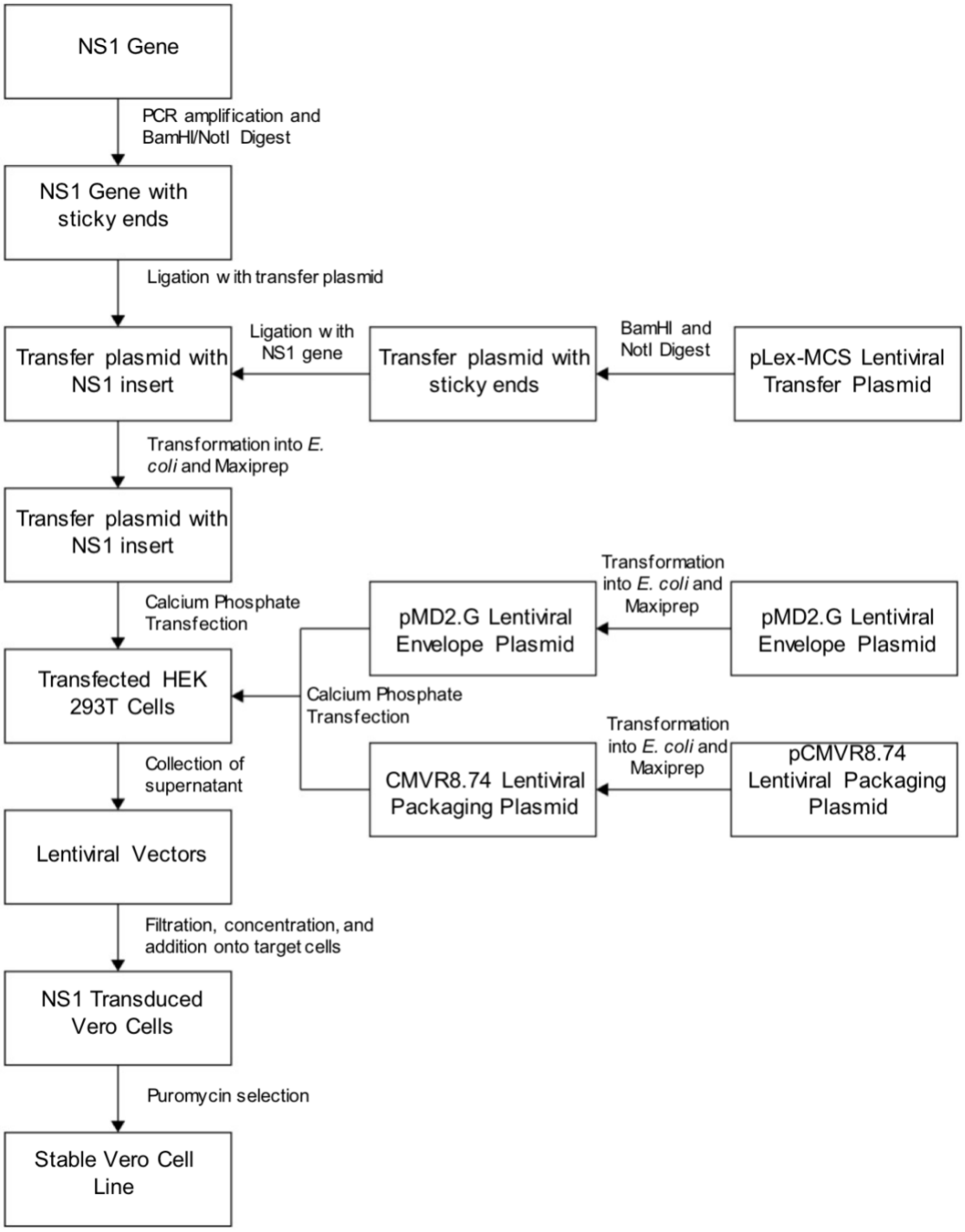

Figure 2: Cloning strategy. An overview of the process of generating a stably transduced cell line. An identical process was used to generate the eGFP-transduced cell line.

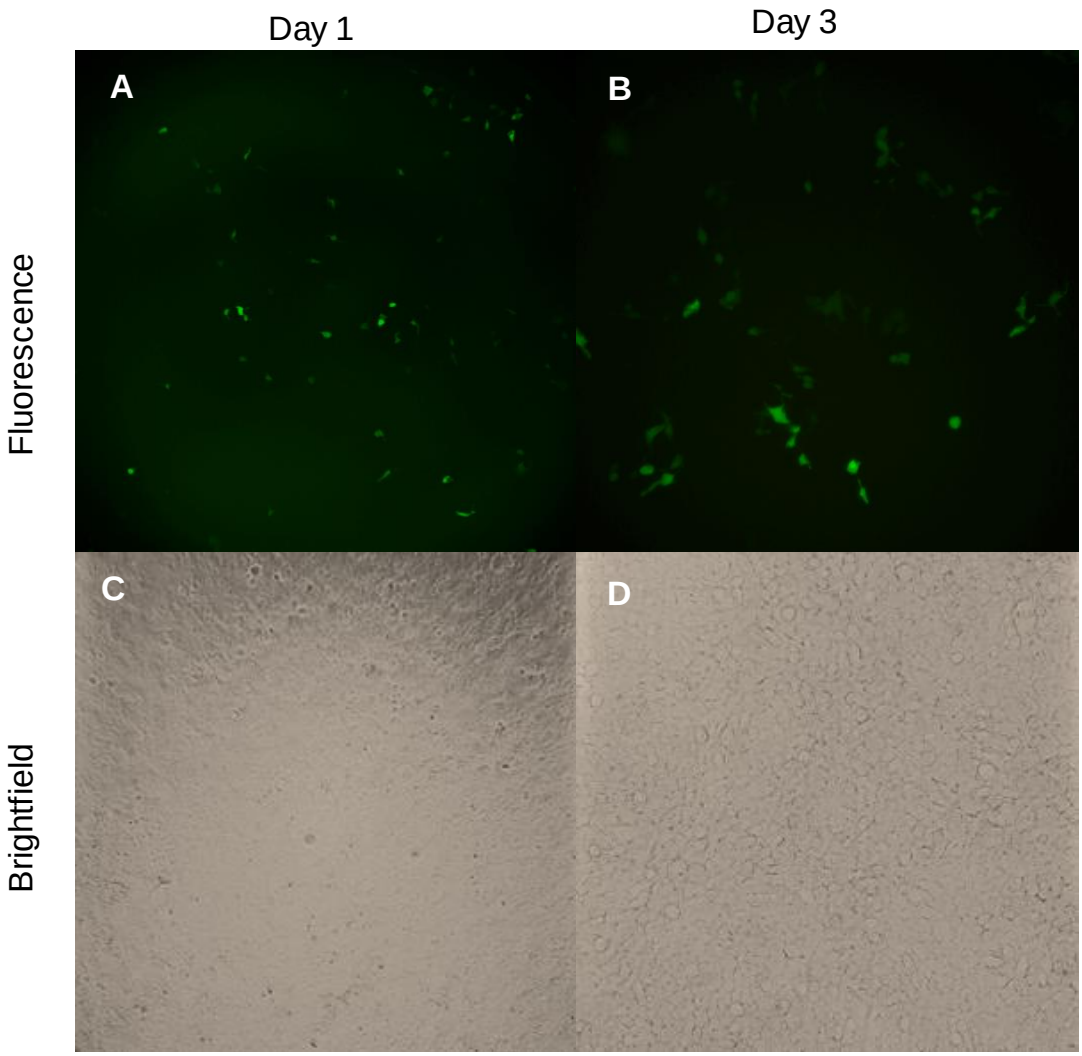

Figure 3: HEK 293T cells transfected with the pLex-MCS-eGFP transfer plasmid.

(A-B) Fluorescence microscopy was used to examine whether the eGFP transfer plasmid was successfully transfected into HEK 293T cells. Green fluorescence showed the successful transfection and expression of recombinant eGFP by the cells. (C-D) A comparison to brightfield images of total cells showed modest transfection efficiency.

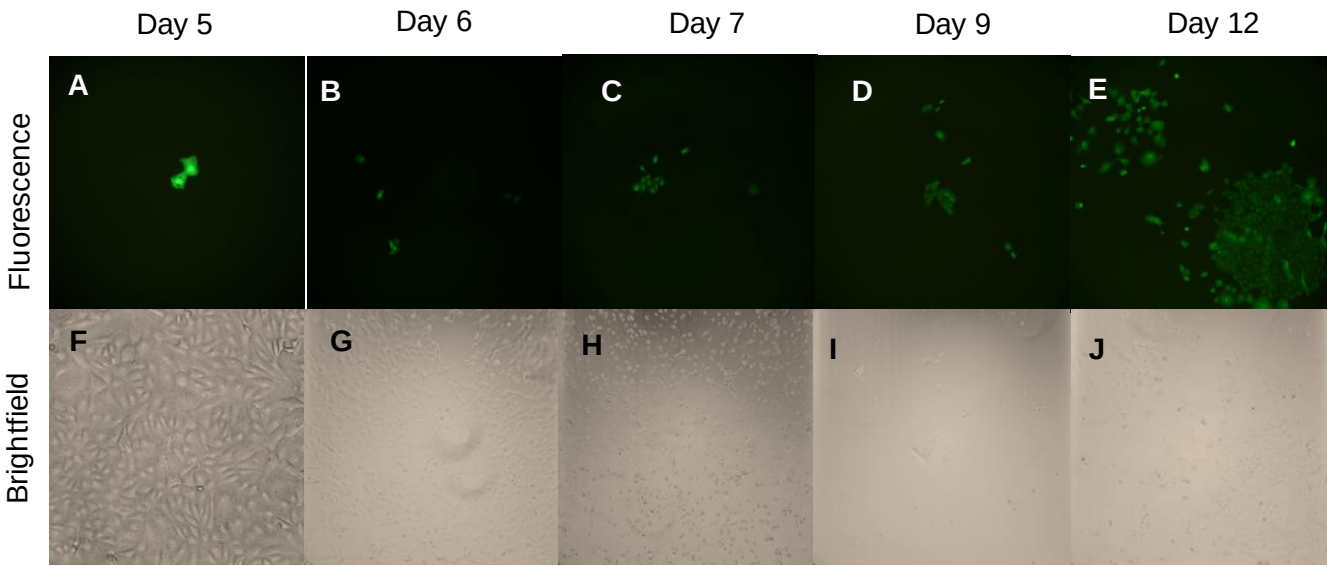

Figure 4: Vero cells transduced with eGFP lentiviral vectors.

Vero cells were transduced with lentiviral vectors contained in the cell supernatant of transfected HEK 293T cells. (A-E) Fluorescence microscopy was used to determine whether cells were successfully transduced, and to track the expansion of transduced cells. (F-J) Puromycin was introduced at day 6, and non-transduced cells could be seen dying while the transduced cells continued to expand.

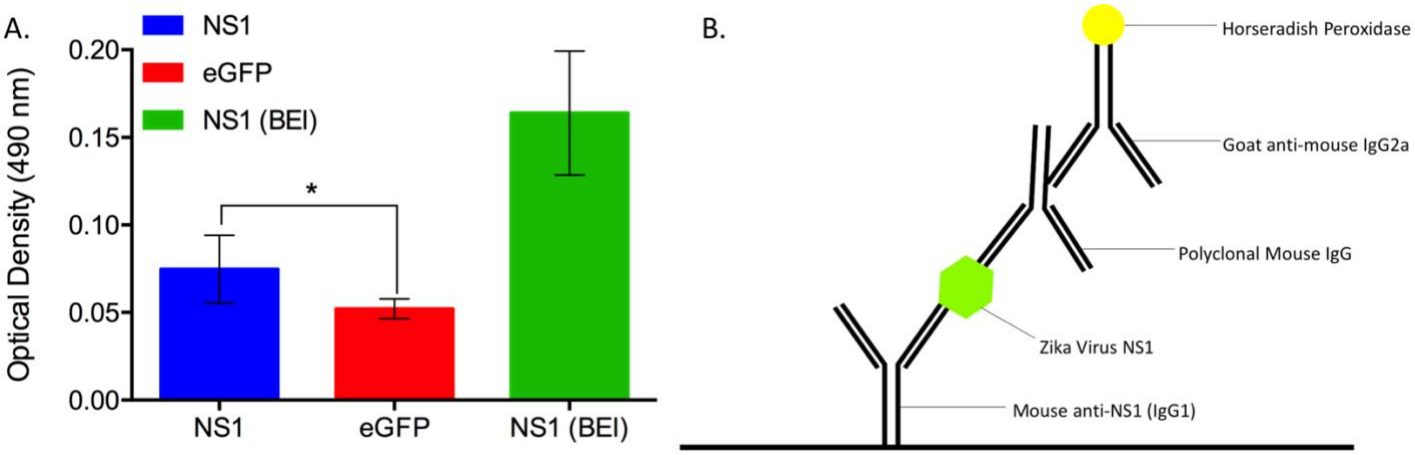

Figure 5: Sandwich ELISA for the presence of NS1.

(A) ELISA data suggesting the presence of NS1 in the transduced Vero cell supernatant. Raw optical density data was compared using a two-tailed paired t-test, the difference was found to be significant ($p<0.05$). (B) A diagram outlining the antibody-NS1 complex used to detect NS1. An anti-IgG2a secondary antibody was used to prevent binding of the secondary antibody to the IgG1 capture antibody used to coat the plate.

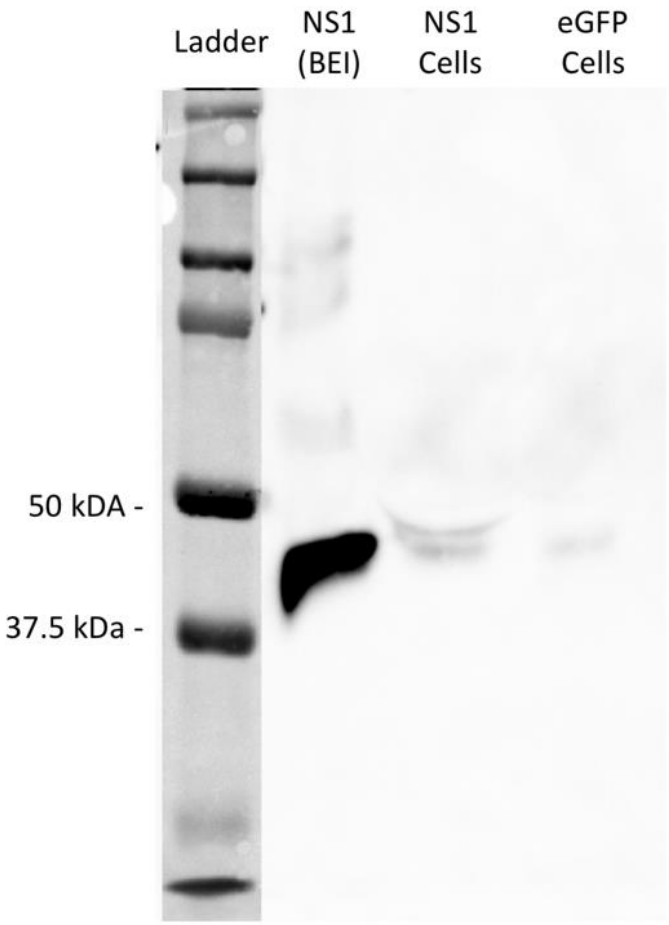

Figure 6: Western blot for the presence of NS1.

A band at the 45-50 kDa mark in the supernatant from NS1-transduced cells confirmed the presence of NS1 in the cell supernatant. NS1 from BEI was used as a positive control, and supernatant from eGFP transduced cells as a negative control.

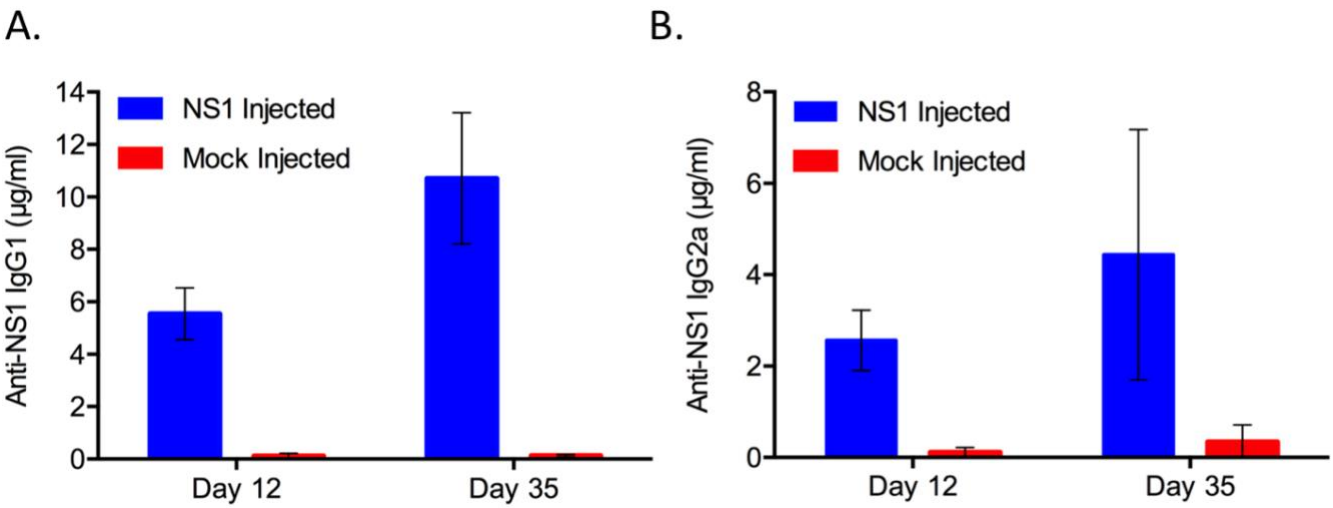

Figure 7: Mouse anti-NS1 antibody concentrations. ELISA data showing the mean concentrations of mouse anti-NS1 (A) IgG1 and (B) IgG2a at days 12 and 35 post-injection. Antibody concentrations were seen to increase from day 12 to day 35, and were significantly higher than mock-injected mice, verifying the presence of anti-NS1 antibodies in mouse serum.

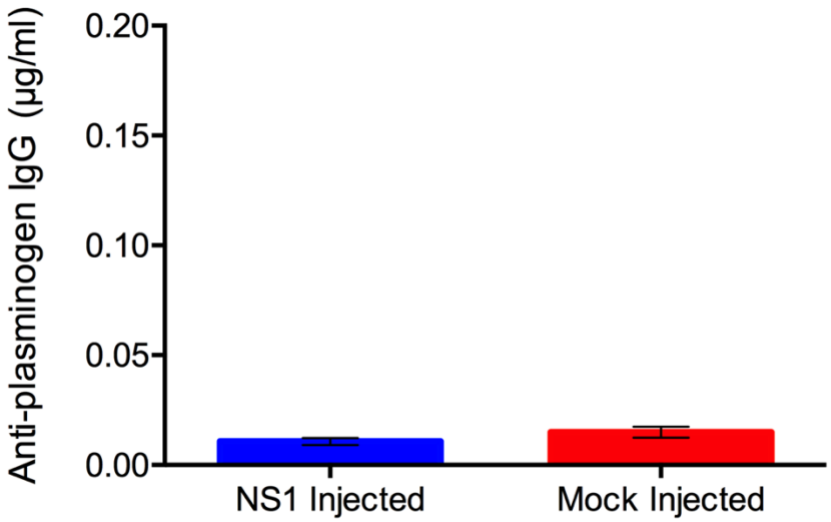

Figure 8: Mouse anti-plasminogen titers. ELISA data showing the levels of total mouse anti-plasminogen IgG antibodies at day 49 post-injection. No significant anti-plasminogen antibody titer was detected in either group, nor was a significant difference detected between NS1 and mock-injected mice.

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