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Effective screening of SARS-CoV-2 neutralizing antibodies in patient serum using lentivirus particles pseudotyped with SARS-CoV-2 spike glycoprotein

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Pseudotyped particles have significant importance and use in virology as tools for studying the biology of highly pathogenic viruses in a lower biosafety environment. The biological, chemical, and serological studies of the recently emerged SARS-CoV-2 will be greatly aided by the development and optimization of a suitable pseudotyping system. Here, we pseudotyped the SARS-CoV-2 Spike glycoprotein (SPG) on a traditional retroviral (MMLV) as well as a third generation lentiviral (pLV) vector and tested the transduction efficiency in several mammalian cell lines expressing SARS-CoV-2 receptor hACE2. While MMLV pseudotyped the vesicular stomatitis virus G glycoprotein (VSV-G) efficiently, it could not pseudotype the full-length SPG. In contrast, pLV pseudotyped both glycoproteins efficiently; however, much higher titers of pLV-G particles were produced. Among all the tested mammalian cells, 293Ts expressing hACE2 were most efficiently transduced using the pLV-S system. The pLV-S particles were efficiently neutralized by diluted serum (>:640) from recently recovered COVID-19 patients who showed high SARS-CoV-2 specific IgM and IgG levels. In summary, pLV-S pseudotyped virus provides a valid screening tool for the presence of anti SARS-CoV-2 specific neutralizing antibodies in convalescent patient serum.

The recent emergence of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2) in Wuhan, China in late 2019 and its subsequent spread to the rest of the world has created a pandemic situation unprecedented in modern history^{1–4}. While initial challenges included diagnosis and proper containment of the infection, contemporary efforts are directed towards the measurement of antiviral total and neutralizing antibodies in recovered and symptomatic patients as well as in non-symptomatic vulnerable population^{5–7}. Studying the development of herd immunity, effectiveness of various upcoming vaccine candidates as well as establishing social parameters for re-opening of the world economy all depend on our ability to accurately measure neutralizing antibodies and establish the kinetics of their persistence in serum. Convalescent plasma therapy has shown early therapeutic successes in severe COVID-19 patients⁸. Thus, the determination of neutralizing antibody titers in convalescent serum should provide significant assistance in clinical decision making regarding transfusion for therapeutic and possibly prophylactic indications.

Due to the highly transmissible and pathogenic nature of SARS-CoV-2, handling of live virus requires a biosafety level 3 (BSL3) containment⁹. Thus, only facilities equipped with BSL-3 can safely study neutralizing responses using live virus. In order to extend this capability to other BSL-2 laboratories that are more widely available, a safer way is needed to measure both total antiviral antibody levels based on ELISAs and neutralizing antibody responses in a practical, reproducible surrogate assay that effectively replaces the need for the live SARS-CoV-2. To address this issue, we report the development of a high titer pseudotype virus that can be easily produced and successfully employed to screen patient serum for neutralizing antibodies in a lower biosafety level laboratory. Since the backbone of this virus consists of a non-replicating lentivirus, it poses no risk of infection to the personnel involved and the technology can readily be scaled up to the levels where it can be employed for

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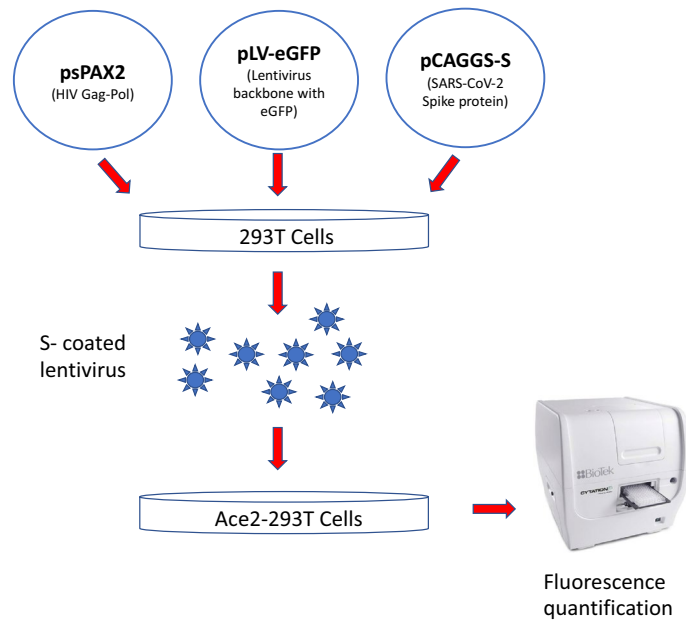


Figure 1. A schematic of the production of lentivirus particles pseudotyped with SARS-CoV-2 Spike (S) protein. See materials and methods for details on plasmids and cell types used.

screening of large sample numbers. These pseudotyped particles (pLV-S) can also be used for immunological, pharmacological and biochemical studies as it represents SPG in its native conformation.

Results

SARS-CoV-2 Spike protein can be efficiently pseudotyped on a lentiviral vector. We used a traditional retroviral pseudotyping system based on Murine Molony Leukemic Virus (MMLV) vector^{10,11} and a lentiviral vector (pLV)^{12,13} in parallel to compare the pseudotyping efficiencies in these two related but different systems. The pseudovirus particles were packaged in 293T cells by transient expression of HIV packaging protein (Fig. 1). The pLV vector pseudotyped both VSV-G (pLV-G) and SARS-CoV-2 spike glycoprotein (pLV-S) efficiently, whereas MMLV vector could only pseudotype VSV-G (Fig. 2). The success of pseudotyping was assessed by the expression of green fluorescent protein (GFP) since both MMLV and pLV vectors integrate the gene encoding for GFP. The ultrastructural analysis of pLV-S virus showed spike like projection on the surface (Fig. S1) similar to the spikes seen in a typical coronavirus.

The efficiency of SARS-CoV-2 SPG pseudotyped lentivirus transduction depends on cell type. We compared the efficiency of pLV-S transduction in 293T and VeroE6 cells since both of these cells are known to express the putative S receptor hACE2 on their surface in sufficient quantities to allow for virus entry¹⁴. The 293T cells were transduced much more efficiently with pLV-G than pLV-S, whereas Vero E6 cells showed a low level of transduction for both viruses (Fig. 3). This came as a surprise since Vero E6 cells have been conventionally used to grow SARS-CoV and SARS-CoV-2 viruses. We compared the differences in number of GFP+ cells produced on different cells types when infected with pLV-S. The 293T cells expressing hACE2 produced the maximum number of GFP+ cells whereas Vero cells had the minimum numbers (Fig. 3E). Interestingly, the ATCC 293T cells gave decent number of GFP+ cells. We surmised whether these differences could be due to the levels of ACE2 expression in these cells. When probed in a western blot, the 293T cells (ATCC # CRL3216) and Vero E6 (ATCC # CRL1586) cells had very little ACE2 expression compared to ACE2 overexpressing 293Ts (BEI NR52511) cells (Fig. 3F) directly correlating with the number of GFP+ cells produced on these cells as a result of pLV-S transduction.

Serum from COVID-19 convalescent patient effectively neutralizes pLV-S. We tested the impact of patient serum on pLV-S transduction. Serial dilutions (1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280) of serum were made in the serum-free cell culture medium and incubated with equal amounts of pLV-S virus, then plated on 293T cells. Fluorescence images captured at 48 h post infection showed significant reduction in pLV transduction with the serum from patient N304 but not from N308 (Fig. 4). Patient N304 tested positive in a RT-PCR assay for SARS-CoV-2, suffered a severe COVID-19 pneumonia and eventually recovered. Patient N308 suffered mild symptoms consistent with an upper respiratory infection but tested negative for SARS-CoV-2 infection by RT-PCR. We also used this neutralization assay to test the serum from several other patients that had varying levels of SARS-CoV-2 (Spike receptor binding domain—specific) IgM and IgG levels in ELISA. The IC₅₀ values in these assays were calculated in Graphpad Prism as described before¹⁵ (Table 1). As a control, the VSV-G glycoprotein coated lentivirus (pLV-G) was not neutralized when using 1:40 dilution of convalescent patient serum

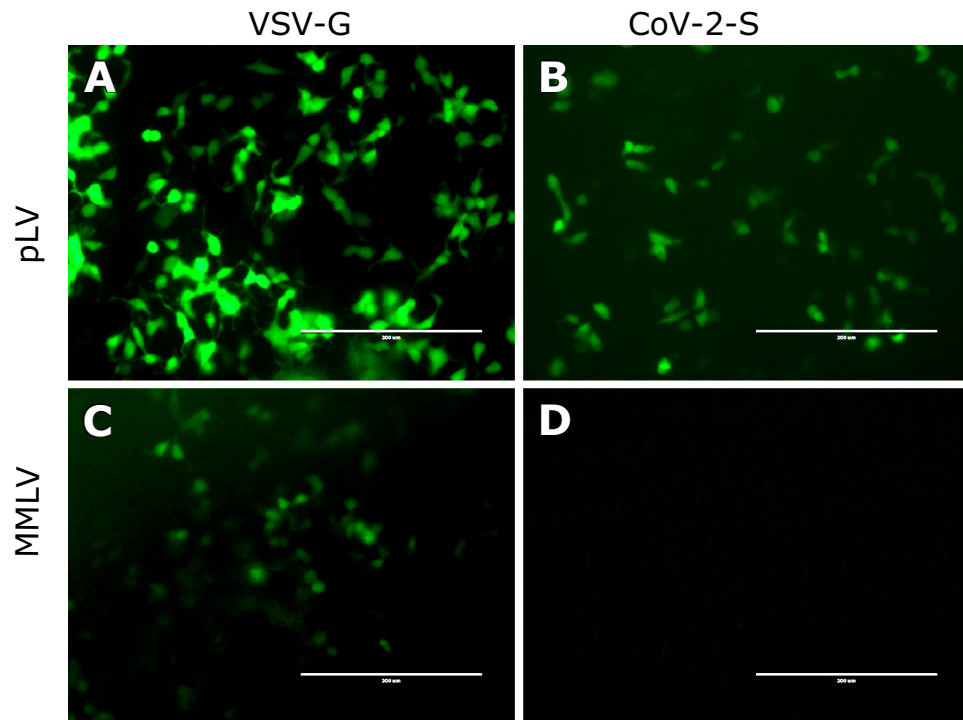


Figure 2. Transduction of HEK293T cells with lentiviral vector (pLV) pseudotyped with VSV-G (A) or CoV-2-S protein (B), and retroviral vector (MMLV) pseudotyped with VSV-G (C) or CoV-2-S (D). Both lentiviral and retroviral backbones incorporate enhanced green fluorescent protein (eGFP) that is expressed upon integration into target cells. The fluorescence was recorded at 48 h post transduction. Magnification 20X. Scale bar: 200 μm.

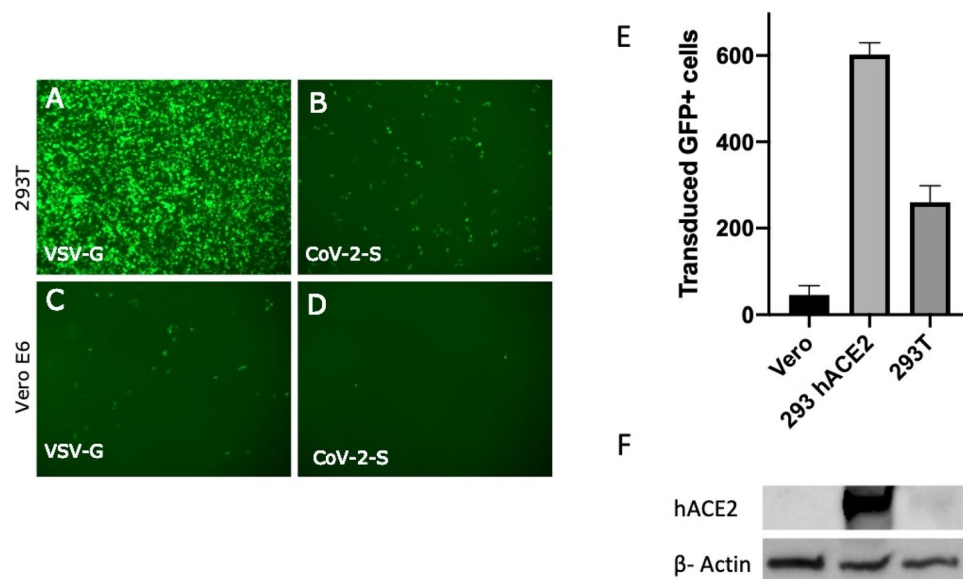


Figure 3. (A) Transduction of pLV pseudotyped with VSV-G (A,C) or CoV-2 Spike glycoprotein (B,D) in HEK293T (A,B) or Vero E6 (C,D) cells. The lentiviral backbone incorporates enhanced green fluorescent protein (eGFP) that is expressed upon integration into target cells. The fluorescence was recorded at 48 h post transduction. Magnification 4X. (E) Transduction efficiency of pLV pseudotyped with CoV-2 Spike glycoprotein in Vero E6, hACE2-HEK293T and 293T cells. The fluorescence was recorded at 48 h post transduction. The experiments were done in triplicates and standard error of mean was plotted as error bars. (F) Whole cell lysates from Vero E6, hACE2-293T and 293T cells were run on SDS-PAGE and probed with anti ACE2 antibody. Beta-actin was used as a loading control.

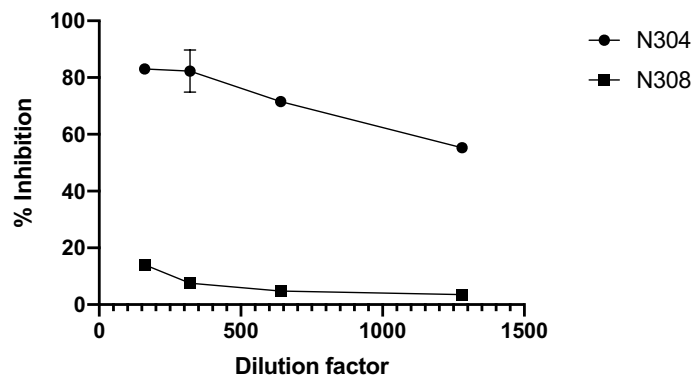


Figure 4. Neutralization of SARS-CoV-2 S glycoprotein pseudotyped pLV (pLV-S) using diluted patient serum. The serum was obtained from a convalescent patient (N304) or a mildly symptomatic individual (N308) at 30 days after onset of symptoms. Patient N304 tested positive for SARS-CoV-2 in a RT-PCR diagnostic test and N308 tested negative. Relative inhibition of pseudovirions at serial dilutions of patient serum compared to mock-serum control is shown. The fluorescence was recorded at 48 h post transduction. The titers were performed in triplicates and standard error of mean was plotted as error bars. Some error bars are within the symbols.

	IgG	IgM	Neut. Log IC ₅₀
N304	5590	39,928	4.93
N305	> 50,000	> 50,000	2.13
N307	8753	49,355	2.27
N308	1332	363	1.23
N309	4512	36,716	2.17
N310	56	4633	2.12

Table 1. The receptor binding domain (RBD)—specific IgM and IgG titers were measured in an ELISA using the recombinant RBD of the SARS-CoV-2 Spike protein as antigen. Log IC₅₀ values were calculated in the pLV-S neutralization assay using Graphpad Prism as the dilution which decreases the GFP + cell numbers by 50% of the control (human serum from the pre-COVID-19 healthy individual) numbers. Serum samples from obtained from de-identified patients N304, N305, N307, N308, N309 and N310.

from three different patients (Fig. S2). Since these assays were conducted on ATCC 293T cells expressing low levels of ACE2 (Fig. 3E), we also tested the same patient serum on ACE2 overexpressing cells to assess whether overexpression of ACE2 can impact the inhibition curve. Similar inhibition patterns were observed on these two cell types (Fig. S3).

Discussion

Here we report the development of a lentiviral pseudotyping system for SARS-CoV-2 and its efficacy in detecting neutralizing antibody titers in convalescent patient serum. While a few pseudotyping systems for SARS-CoV-2 are in development, this report provides initial evidence that lentiviral pseudotyped SARS-CoV-2 can be used to determine neutralizing antibody titers in patient samples.

We first tried a traditional retroviral pseudotyping system based on MMLV. We have used this system earlier for creating cell lines stably expressing viral proteins of interest^{16,17}. While the control VSV-G protein was pseudotyped successfully, we could not pseudotype SARS-CoV-2 SPG onto this backbone. Since retroviral vectors require actively dividing cells for successful transduction, we tested 293T cells at different levels of confluency but were unable to transduce them. The relative efficiency of VSV-G to transduce similar cells indicate that inherent qualities or complexity of SPG structure may make it more difficult to pseudotype it in this system.

Next, we utilized a third generation lentiviral system to pseudotype VSV-G and SPG. This system was able to pseudotype both glycoproteins although, as expected, efficiency was much better for VSV-G. The transduction efficiency would logically be guided by the level of SPG receptor expression on cells, therefore, we tested various mammalian cell lines that were available to us and are known express hACE2. None of the cells transduced as efficiently as hACE2-293Ts; however, 293Ts produced decent amount of GFP + cells (Fig. 2). Since the differences between hACE2-293Ts and 293Ts were only about threefold, we decided to use 293Ts for patient serum screening.

Serum samples were obtained from patients at University of Mississippi Medical Center. The first patient (N304) tested positive for SARS-CoV-2 in a RT-PCR assay and was convalescent at the time of sample collection. The second patient (N308) was mildly symptomatic and tested negative for SARS-CoV-2 (N308) in the same RT-PCR assay. Both serums were collected thirty days after onset of symptoms while patients were totally

asymptomatic. Antibody testing in a SARS-CoV-2 SPG receptor binding domain ELISA revealed higher levels of RBD-specific IgM and RBD-specific IgG for N304 but lower levels for N308 (Table 1). Serial dilutions of serum N304 effectively neutralized pLV-S up to 1:1280 dilution whereas N308 did not neutralize the virus at higher than 1:80 dilution (Fig. 3).

Thus, the pseudotyped virus (pLV-S) can be effectively used for screening of patient serum for the presence of neutralizing antibodies associated with acquired immunity to subsequent infection upon re-exposure. Determining functional immunity has potential social implications as the world community begins to recover from the tremendous social impact of the pandemic. Information regarding the development of herd immunity is important in this effort and can be gained from the results of this assay as well. These pseudoparticles can also be utilized for screening of inhibitors of SPG-hACE2 binding and resultant virus entry. They can also be utilized for screening of potential vaccine candidates as they represent SPG on their surface in its native confirmation.

Methods

Cells. 293T (ATCC # CRL3216), Vero E6 (ATCC # CRL1586) and ACE2-293Ts (BEI NR52511) were cultured in DMEM (Corning Inc) supplemented with 10% fetal bovine serum (FBS, Fisher Scientific) at 37 °C with 5% CO₂.

ELISA. RBD-specific antibodies were measured generally according to the protocol developed in the Kramer Lab¹⁸. Briefly, 384-well MaxiSorp plates (Thermo Fisher Scientific) were coated with purified recombinant RBD at concentration of three µg per ml in PBS. The coating volume and reaction volumes were 25 µl per well. Plates were incubated overnight at 4 °C, washed 3 × with PBS-Tween20, and blocked with PBS containing 3% dry milk for one hour at room temperature. Blocking buffer was removed, and samples (one log dilutions in blocking buffer, 5 × 10¹–5 × 10⁴) were added to the wells. Plates were incubated for two hours at room temperature before washing three times. Horse radish peroxidase conjugated to anti-human IgG FC (Southern Biotech) or anti-human IgM (Southern Biotech) was diluted 1:2000 in PBS containing 1% dry milk, added to the wells, and incubated at room temperature for 1 h. Plates were washed five times and developed with tetramethyl-benzidine (Southern Biotech). After 30 min, development was stopped by adding 25 µl of 2 N H₂SO₄ to each well. Absorbance was measured at 450 nm. The end point dilution titer is the serum dilution that results in an absorbance of 0.2 absorbance units over background.

Generation of pseudotype particles. HEK293T cells (2 × 10⁶) were plated in a 100-mm tissue culture dish and transfected the next day when they were about 75% confluent with a combination of the following plasmids: 9 µg of *pLV-eGFP* (a gift from Pantelis Tsoulfas (Addgene plasmid # 36083; <https://n2t.net/addgene:36083>; RRID:Addgene_36083), 9 µg of *psPAX2* (a gift from Didier Trono (Addgene plasmid # 12260; <https://n2t.net/addgene:12260>; RRID:Addgene_12260), and 3 µg of *pCAGGS-S* (*SARS-CoV-2*) (Catalog No. NR-52310; BEI Resources) or *VSV-G* (a gift from Tannishtha Reya (Addgene plasmid # 14888; <https://n2t.net/addgene:14888>; RRID:Addgene_14888) as control. Polyethylenimine (PEI) reagent (Millipore Sigma, #408727) was used for transfection following manufacturer's protocols. Next day, the cells were checked for transfection efficiency under a fluorescent microscope, indicated by GFP fluorescence. The supernatants from cell culture at 24 h were harvested and stored at 4 °C and more (10 ml) complete media (DMEM + 10% FBS) was added to the plates. The supernatant from cell culture at 48 h was harvested and combined with the 24 h supernatant for each sample. The combined supernatants were spun in a tabletop centrifuge for 5 min at 2000×g to pellet the residual cells and then passed through a 0.45 micron syringe filter. Aliquots were frozen at – 80 °C. New 293T cells plated in 12 well tissue culture dish were infected with the harvested virus (supernatant) with a dilution range of 10² to 10⁷. Virus titers were calculated by counting the GFP positive cells in the dilution with 20–100 GFP positive cells. MMLV pseudovirions were made in a similar way. HEK293T cells were transfected with the following plasmids: 10 µg pLNCX-GFP, 1.2 µg pCMV-tat-HIV, 6 µg pJK3, and 3 µg of pL-VSV-G (control) or pCAGGS-S (*SARS-CoV-2*). Supernatants were recovered in a fashion similar to above.

Antibodies and immunoblot analyses. Monoclonal anti-Ace2 antibody (AF933; R&D Systems, Minneapolis, MN) was used to detect the Ace2 protein in cell lysates. Peroxidase-labeled anti-mouse IgG (Vector Laboratories, Burlingame, CA) was used as the secondary antibody. Blots were detected using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom).

Microscopy. Images were acquired on an inverted Evos-FL microscope (Thermo Fisher Scientific, Waltham, MA) using 4X or 20X objective in the GFP channel. Samples for transmission electron microscopy (TEM) were prepared by fixing the pseudovirus aliquots in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature. Samples were then washed with the same buffer and postfixed with buffered 1.0% osmium tetroxide at room temperature for 1 h. Following several washes with 0.1 M cacodylate buffer, samples were dehydrated with ethanol, infiltrated, and embedded in Eponate 12 resin (Ted Pella Inc., Redding, CA). Ultrathin sections (60 to 70 nm) of resin were cut and counterstained using uranyl acetate and lead citrate. Examination of ultrathin sections was carried out on a Hitachi H-7500 TEM operated at 75 kV, and images were captured using a Gatan BioScan (Pleasanton, CA) charge-coupled device camera. The images were acquired and analyzed with the Digital Micrograph (Pleasanton, CA) software.

Patient serum screening. Samples were obtained either from discarded clinical samples or from individuals recruited to participate in this study. Written informed consent was obtained from the individuals recruited

for this study. The study was reviewed and approved by the Institutional Review Board of the University of Mississippi Medical Center which follows the national and international guidelines consistent with the principles established by the Declaration of Helsinki. Serial dilutions of the patient serum samples (1:40; 80; 160; 320; 640 and 1280) were made in DMEM with end volume of 60 μ l each. Sixty μ l of the supernatant stock (diluted to give 200–500 GFP + cells/well) was mixed with the serum and incubated for 1 h at 37 °C. Stock + serum dilutions were laid over 293T cells plated in 96 well tissue culture dishes and incubated at 37 °C and 5% CO₂ for 2 h. Medium was replaced with complete medium (DMEM + 10% FBS) and incubated for another 48 h. The assay was read and analyzed using Lionheart FX automated fluorescent microscope (BioTek Instruments, Inc., Winooski, VT, USA).

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Author contributions

R.T., J.T.B. and G.D.M. wrote the main manuscript text. S.J.S., D.M., P.S., M.G.M, J.T.B. and R.T. performed the experiments. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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