ADP-ribosylhydrolase activity of Chikungunya virus macrodomain is critical for virus replication and virulence

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Chikungunya virus (CHIKV), an Old World alphavirus, is transmitted to humans by infected mosquitoes and causes acute rash and arthritis, occasionally complicated by neurologic disease and chronic arthritis. One determinant of alphavirus virulence is non-structural protein 3 (nsP3) that contains a highly conserved MacroD-type macrodomain at the N terminus, but the roles of nsP3 and the macrodomain in virulence have not been defined. Macrodomain is a conserved protein fold found in several plus-strand RNA viruses that binds to the small molecule ADP-ribose. Prototype MacroD-type macrodomains also hydrolyze derivative linkages on the distal ribose ring. Here, we demonstrated that the CHIKV nsP3 macrodomain is able to hydrolyze ADP-ribose groups from mono(ADP-ribosyl)ated proteins. Using mass spectrometry, we unambiguously defined its substrate specificity as mono(ADP-ribosyl)ated aspartate and glutamate but not lysine residues. Mutant viruses lacking hydrolase activity were unable to replicate in mammalian BHK-21 cells or mosquito Aedes albopictus cells and rapidly reverted catalytically inactivating mutations. Mutants with reduced enzymatic activity had slower replication in mammalian neuronal cells and reduced virulence in 2-day-old mice. Therefore, nsP3 mono(ADP-ribosyl)hydrolase activity is critical for CHIKV replication in both vertebrate hosts and insect vectors, and for virulence in mice.

Significance

Viral outbreaks can be sudden and result in devastating social and economic consequences. Chikungunya virus (CHIKV) outbreaks of rash, arthritis, and neurologic disease have occurred in Africa, Asia, Europe, and the Americas. There are no licensed vaccines to prevent or drugs to treat CHIKV infection, and the mechanisms of disease generation are poorly understood. Here, we identify a macrodomain-containing CHIKV protein with enzymatic ADP-ribosylhydrolase activity that is critical for virulence in mammals and define the substrate specificity of this enzyme. Given the conservation of viral macrodomains in all alphaviruses and coronaviruses, our study may guide the design of small molecule chemical compounds that block hydrolase activity for treatment of a range of virus infections.


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RNA viruses, including alphaviruses, coronaviruses, and hepatitis E virus (16–18). Study of viral macrodomain function has provided conflicting results with respect to their importance for virus replication. In general, viruses with mutations in the ADP-ribose–binding site of the macrodomain replicate well in most tissue culture cells, but often exhibit attenuated replication in differentiated cells and decreased virulence in vivo (10, 19–23). For instance, mutations of conserved residues in the ADP-ribose–binding region of the Sindbis virus macrodomain impair replication and RNA synthesis in neurons and attenuate neurovirulence in mice (10). Similarly, mutation of the macrodomain of coronavirus nsP3 at a conserved site attenuates virulence and replication in mice and affects induction of and sensitivity to IFN and inflammatory cytokines (19, 20, 22, 23).

Viral macrodomains are of the MacroD subfamily, which includes archaeal Af1521 and human MacroD2. Recently, our group and others have found that some MacroD-type macrodomains bind and hydrolyze ADP-ribose monomers conjugated to the side chains of protein residues, a posttranslational modification known as ADP-ribosylation (23–27). ADP-ribose groups can be attached singly as mono(ADP-ribose) (MAR) or in branched or linear polymeric chains as poly(ADP-ribose) (PAR) by the enzymatically active members of a family of 17 ADP-ribosyltransferases, commonly known as poly(ADP-ribose) polymerases (PARPs) (28–30). ADP-ribosylation involves the transfer of ADP-ribose from NAD+ onto a range of amino acids, including aspartate (D), glutamate (E), and lysine (K) (30–32). Until recently, it has not been possible to identify the site of ADP-ribosylation by mass spectrometry (MS) (reviewed in refs. 31 and 33). Sequence alignment of the CHIKV macrodomain with the known human MAR hydrolase MacroD2 revealed that the residues required for catalysis are conserved (refs. 25 and 26 and SI Appendix, Fig. S1). In this study, we determined that the CHIKV macrodomain possesses MAR hydrolase activity and unambiguously identified its substrate specificity by MS. Furthermore, using recombinant viruses with targeted mutations in the macrodomain, we demonstrate that hydrolase function is important for viral replication in neuronal cells in vitro and for neurovirulence in mice.

Results

CHIKV nsP3 Macrodomain Removes Mono(ADP-Ribose) from Modified Aspartate and Glutamate, but Not Lysine Residues. To determine whether the macrodomain of CHIKV nsP3 (nsP3MD) possesses MAR hydrolase activity, we subcloned nsP3MD from the CHIKV vaccine strain 181/Clone 25 (181/25) into a bacterial expression plasmid and purified recombinant nsP3MD into a bacterial expression plasmid and purified recombinant nsP3MD from NAD+ to generate recombinant PARP10 in vitro characterization. We incubated the catalytic domain of PARP-10 (PARP10CD) in vitro with either nsP3MD or buffer alone resulted in minimal loss of 32P signal. We further characterized nsP3MD by monitoring the time dependence of this decrease in 32P signal (Fig. 1B). nsP3MD kinetics were slower than MacroD2 under the tested reaction conditions; yet, both enzymes decreased the signal from 32P-MARylated PARP10CD by up to 40% within 10 min. Of note, ~10% of the 32P signal remained even after the demodification was allowed to proceed for 2 h, suggesting that certain ADP-ribosylated sites were more resistant to the hydrolase activity of MacroD2 and nsP3 than others. The reaction products at the 1-h time point were further analyzed by thin-layer chromatography (TLC) and autoradiography, showing the 32P-labeled product released from modified PARP10CD had the same retention factor as pure ADP-ribose (SI Appendix, Fig. S2). Therefore, CHIKV nsP3MD possesses enzymatic activity for hydrolysis of ADP-ribose groups from MARylated substrates.

Biochemical and mutagenesis data have inferred that human and archaeal MAR hydrolases remove ADP-ribose groups from acidic residues (24–26), but the specificity of viral MAR hydrolases has not been examined. To identify the amino acid residues demodified by nsP3MD, we first determined the sites of modification on MARylated PARP10CD by using a phosphodiesterase-based MS approach that can identify different amino acid ADP-ribosylation sites (24, 34–36). In brief, MARylated PARP10CD was incubated with a phosphodiesterase that cleaves the pyrophosphate of ADP-ribose to leave a phosphoribose tag at the original site of modification. Proteins were then digested and phosphoribosylated peptides were enriched by immobilized metal phosphate of ADP-ribose to leave a phosphoribose tag at the original site of modification. Proteins were then digested and phosphoribosylated peptides were enriched by immobilized metal affinity chromatography and sequenced by liquid chromatography (LC)–MS/MS to identify sites of modification. This approach identified six highly confident phosphoribosylated sites on PARP10CD based on our criteria of >99% site localization probability and high fragment coverage of the modification site in MS/MS spectra, at D, E, or K residues (SI Appendix, Fig. S3 and Table S1). To test whether nsP3MD was capable of removing MAR from these modified residues, we incubated PARP10CD with either nsP3MD or buffer alone, followed by treatment with phosphodiesterase and proteases, and subjected these samples to LC–MS/MS analyses. To quantify the differences in intensity of modified peptides under these different conditions, we extracted ion chromatograms from three technical replicates for individual phosphoribosylated PARP10CD peptides (SI Appendix, Fig. S4) and quantified the resulting peaks (Fig. 1C). There was a significant reduction in the ion intensity of peptides with phosphoribosylated D and E residues in samples incubated with nsP3MD. However, no reduction of the PARP10 peptide modified at K916 residue was observed (Fig. 1C). Together, these data show that
Category 2

NDB, no detectable binding.

(\text{in a mutant divided by the number of plaques in WT. CHIKV 181/25 normalized to nsP3 activity of nsP3 Ribose Binding.} McPherson et al. CHIKV strain

\text{CHIKV, we generated and biochemically characterized nsP3 mutants relative to nsP3} \text{mutants that are defective in ADP-ribose-binding and/or MAR hydrolase activity. (A) Active site of CHIKV nsP3\textsuperscript{MD} bound to ADP-ribose (37) highlighting residues targeted in this study. (B) Quantitative representation of MAR hydrolase activity of nsP3\textsuperscript{MD} mutants relative to nsP3\textsuperscript{MD} WT. Assays were performed in triplicate, buffer control was subtracted, and obtained values were normalized to activity levels of nsP3\textsuperscript{MD} WT. Representative raw data shown in SI Appendix, Fig. S5. (C) Quantitative representation of ADP-ribose affinity of nsP3\textsuperscript{MD} mutants normalized to nsP3\textsuperscript{MD} WT. Raw data shown in Table 1 and SI Appendix, Fig. S7.}

\text{nsP3\textsuperscript{MD} is capable of removing MAR from modified aspartate and glutamate, but not lysine residues on PARP10\textsuperscript{CD}. Generating nsP3\textsuperscript{MD} Mutants Defective in MAR Hydrolysis and ADP-Ribose Binding. To determine whether the MAR hydrolase activity of nsP3\textsuperscript{MD} plays a role in the replication and/or virulence of CHIKV, we generated and biochemically characterized nsP3\textsuperscript{MD} mutants before incorporating the mutations into recombinant}

\begin{table}[h]
\caption{Compiled quantitation data from in vitro, cell culture, and in vivo experiments on nsP3 WT and mutants of CHIKV 181/25 strain}
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\text{CHIKV strain} & \text{ADP-ribose} & \text{Hydrolase activity, \%} & \text{Virus} & \text{Sequencing} & \text{Replication} & \text{Mortality in} & \text{Mean days of death} \\
& \text{K_D, \(\mu\text{M}\)} & & \text{progeny} & & \text{(NSC-34, \%)} & \text{mice, \%} & \\
\hline
\text{WT} & 22.9 \pm 3.7 & 100 & Yes & & 100 & 100 & 2.5 \\
\text{Category 1} & & & & & & & \\
D10A & NDB & 16.1 \pm 2.3 & No & Reverted to WT & N/A & N/A & N/A \\
G32A & 21.0 \pm 3.7 & 75.3 \pm 9.3 & Yes & & 7 & 58 & 6.9 \\
G32S & 42.9 \pm 17.7 & 48.0 \pm 16.0 & Yes & & 4 & 4 & Undefined \\
G32E & NDB & 3.8 \pm 9.1 & No & Reverted to WT & N/A & N/A & N/A \\
T111A & 71.4 \pm 11.5 & 64.1 \pm 4.0 & Yes & & <1 & 44 & 5.3 \\
G112E & NDB & 3.7 \pm 2.5 & No & Reverted to WT & N/A & N/A & N/A \\
R144A & 64.9 \pm 14.9 & 96.2 \pm 2.3 & No & Reverted to WT & N/A & N/A & N/A \\
\text{Category 2} & & & & & & & \\
Y114A & 4.84 \pm 0.69 & 40.6 \pm 4.2 & Yes & & 16 (Delayed) & 75 & 5.3 \\
G32E V113R Y114N & 6.46 \pm 1.25 & 15.0 \pm 7.2 & No & Reverted to either G32A V113R & N/A & N/A & N/A \\
\hline
\end{tabular}
\end{table}

\text{Hydrolase activity is calculated as a percentage of nsP3 WT activity after subtraction of buffer control. Replication rate is defined as the number of plaques in a mutant divided by the number of plaques in WT. CHIKV 181/25\textsuperscript{MD} and mutant derivatives were used in mouse experiments. N/A, not available; NDB, no detectable binding.}

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zymatic activity, respectively (Figs. 2 A and B). Quantitative representation of MAR hydrolase activity of nsP3MD mutants relative to nsP3 WT. Assays were performed in triplicate, buffer control was subtracted, and obtained values were normalized to activity levels of nsP3 WT. Representative raw data shown in SI Appendix, Fig. S9. (C) Quantitative representation of ADP-ribose affinity of nsP3 mutants normalized to nsP3 WT. Raw data shown in Table 1 and SI Appendix, Fig. S9.

To unambiguously determine the importance of the MAR hydrolase activity of nsP3MD in CHIKV replication and virulence, we sought to generate nsP3 mutants with impaired hydrolase activity that still bound to free ADP-ribose (category 2 mutants). Studies of other macrodomains have shown that mutations in V113 and Y114 disrupt various hydrolase activities without affecting ADP-ribose binding (26, 42). Therefore, we generated nsP3MD G32E V113R Y114N and nsP3MD Y114A and showed that they have higher affinity for ADP-ribose than the WT protein while possessing ADP-ribose binding and showed that they have higher affinity for ADP-ribose than the parent virus (Fig. 4 A and Table 1). Viruses with single mutations at G32 to either A or S replicated with similar kinetics, but with lower virus yields that correlated with the loss of MAR hydrolase activity and ADP-ribose binding in vitro, implicating one or both of these biochemical characteristics of nsP3 as important for viral replication. Virus yield was further lowered with the T111A mutant, which has an intermediate hydrolase activity between G32A and G32S mutant but a much lower affinity to ADP-ribose (Fig. 4 A and Table 1). These data indicate that limiting ADP-ribose binding, which is likely required for hydrolase activity, further reduces the replication of these enzymatically impaired mutants. Replication of the Y114A mutant, which has a stronger affinity for ADP-ribose than WT but 40% of hydrolase activity, was distinct in that it displayed delayed replication kinetics and a lower yield compared with the parent virus (Fig. 4 A and Table 1). These data show that for virus replication even enhanced levels of nsP3 ADP-ribose binding do not compensate for impaired MAR hydrolase activity.

Mutations Affecting nsP3MD MAR Hydrolase Activity and ADP-Ribose Binding Attenuate CHIKV Virulence in Mice. Next, we assessed the importance of the MAR hydrolase activity and ADP-ribose binding of nsP3 in vivo. First, we reversed attenuating mutations I12T and R82G in the E2 glycoprotein of CHIKV 181/25 to generate a recombinant CHIKV strain virulent for newborn mice (CHIKV 181/25E2 I12T R82G) (45). nsP3 mutations were then introduced into this more virulent background and 1,000 pfu of the resulting viruses were used for intracranial infection of 2-d-old mice (Fig. 4B). Mice infected with CHIKV 181/25E2 I12T R82G all died 2–4 d after infection (mean day of death; MDD = 2.5). All viruses with mutated nsP3 macrodomains had reduced virulence. Mutants G32A and G32S had mortalities of 58% (MDD = 6.9, P < 0.0001) and 4% (P < 0.0001), respectively (Fig. 4B and Table 1). Notably, T111A had intermediate virulence between

![Fig. 3.](attachment:image)

**Characterization of CHIKV 181/25 nsP3 G32E V113R Y114N mutant revertants.** (A) Sequencing chromatogram of nsP3 G32E V113R Y114N containing plasmid clones and virus particles produced at P0 from BHK-21 cells. (B) Quantitative representation of MAR hydrolase activity of nsP3MD mutants relative to nsP3 WT. Assays were performed in triplicate, buffer control was subtracted, and obtained values were normalized to activity levels of nsP3 WT. Representative raw data shown in SI Appendix, Fig. S9. (C) Quantitative representation of ADP-ribose affinity of nsP3 mutants normalized to nsP3 WT. Raw data shown in Table 1 and SI Appendix, Fig. S9.

nsP3MD MAR Hydrolase Activity and ADP-Ribose Binding Are Critical for CHIKV Replication in Cell Culture. Characterized mutations were introduced into a plasmid containing the full-length cDNA of the RNA genome of CHIKV 181/25 (43). The plasmid was in vitro transcribed, and the resulting RNA was transfected into BHK-21 cells for virus production. Sequencing of the RNA of the resulting viral particles revealed that viruses produced from transfection of CHIKV 181/25 RNA with D10A, G32E, and G112E had reverted the mutated residue back to the original residue (Table 1 and SI Appendix, Fig. S8). Furthermore, G32E reversion occurred with both codons for glutamate (GAA and GAG) and in two different cell lines, mammalian BHK-21 and mosquito Aedes albopictus C6/36, both lacking an IFN response (SI Appendix, Fig. S8) (44). Intriguingly, CHIKV 181/25 did not tolerate the R144A mutation despite the presence of near WT levels of MAR hydrolase activity and the ability to bind ADP-ribose in vitro (Fig. 2 B and C, Table 1, and SI Appendix, Fig. S5), suggesting that macromdomain properties other than ADP-ribose binding and hydrolase activity may be important for CHIKV replication. Notably, R144 is not conserved in all alphaviruses, suggesting a function potentially specific to CHIKV (SI Appendix, Fig. S1). Interestingly, viruses produced from the G32E V113R Y114N cDNA mutated to a mixture of ∼80% G32A V113R Y114N and ∼20% V113R Y114N (Fig. 3 A). Biochemical characterization of these nsP3MD mutants showed that their enzymatic activity was partially restored, and their affinity for ADP-ribose was increased ∼70- to 300-fold compared with WT (Fig. 3 B and C and SI Appendix, Fig. S9). These data suggest that CHIKV replication is incompatible with nsP3MD mutations that severely attenuate MAR hydrolase activity, even when ADP-ribose binding is restored with the additional mutations V113R Y114N. Together, these results indicate that ADP-ribose hydrolase activity in nsP3MD is critical for CHIKV replication.

To determine the importance of nsP3 MAR hydrolase activity for CHIKV replication, we infected mouse neuronal NSC-34 cells with the viable recombinant nsP3MD mutant viruses at a multiplicity of infection (MOI) of 10 and measured virus production by plaque assay in Vero cells. All of the assayed mutants replicated less well than the parent virus (Fig. 4A and Table 1). Viruses with single mutations at G32 to either A or S replicated with similar kinetics, but with lower virus yields that correlated with the loss of MAR hydrolase activity and ADP-ribose binding in vitro, implicating one or both of these biochemical characteristics of nsP3 as important for viral replication. Virus yield was further lowered with the T111A mutant, which has an intermediate hydrolase activity between G32A and G32S mutant but a much lower affinity to ADP-ribose (Fig. 4A and Table 1). These data indicate that limiting ADP-ribose binding, which is likely required for hydrolase activity, further reduces the replication of these enzymatically impaired mutants. Replication of the Y114A mutant, which has a stronger affinity for ADP-ribose than WT but 40% of hydrolase activity, was distinct in that it displayed delayed replication kinetics and a lower yield compared with the parent virus (Fig. 4A and Table 1). These data show that for virus replication even enhanced levels of nsP3 ADP-ribose binding do not compensate for impaired MAR hydrolase activity.

![Fig. 4.](attachment:image)
G32A and G32S with 44% mortality (MDOD = 5.3, P < 0.0001; Fig. 4B and Table 1). Compared with the parent, the Y114A mutant had less hydrolase activity but ~fivefold more ADP-ribose–binding ability and was the most virulent of the mutants (75%, MDOD = 5.3, P < 0.0001; Fig. 4B and Table 1). These results indicate that MAR hydrolase activity is critical for CHIKV virulence in vivo. However, comparison between two mutants, Y114A and G32S, that have comparable hydrolase activity but differ in ADP-ribose–binding ability (Fig. 2 B and C and Table 1) indicated that the virulence of mutant CHIKV can be increased with stronger than WT ADP-ribose–binding ability of nsP3.

Discussion

Using the macrodomain from the CHIKV nsP3 protein, we demonstrated (i) the substrate specificity of a viral macrodomain and (ii) that the macrodomain MAR hydrolase activity is critical for virus replication and virulence. Using MS, we unambiguously determined that CHIKV nsP3MD is able to remove ADP-ribose groups from modified aspartate and glutamate, but not lysine residues (Fig. 1). Previously, mutagenesis and biochemical data deduced that MacroD2 can hydrolyze MARylated acidic residues such as glutamate and aspartate (25, 26), and our proteomics data inferred that archael macrodomain A1521 specifically removes ADP-ribose groups from modified glutamates (24). Here, we definitively showed that a macrodomain is able to distinguish and remove different classes of ADP-ribosylation from a single protein. Given that nsP3MD is critical for virulence of CHIKV, such substrate specificity of its MAR hydrolase activity indicates that a certain class of ADP-ribosylation is critical for host–virus interactions.

Next, we took a structure-function approach to determine the importance of the MAR hydrolase activity of nsP3MD. Mutations in the nsP3 ADP-ribose–binding site reduced its MAR hydrolase activity while simultaneously reducing its affinity for free ADP-ribose (category 1 mutants). To delineate the importance of these two properties of nsP3 in cells and in vivo, we generated mutants with additional mutations at V113 and Y114 that retained ADP-ribose binding but lacked enzymatic activity (category 2 mutants) (Fig. 2 and Table 1). Intriguingly, we were unable to produce viable CHIKV of either category with the catalytically inactive D10A, G32E, G112E, and G32E V113R Y114N mutations. For G32E-based mutants, there was rapid catalytically inactivating D10A, G32E, G112E, and G32E V113R

Materials and Methods

PARP10 Demodification Assay. For each reaction, 1 μg of PARP10 was automodified with 0.5 μCi [3H]NAD+ for 30 min at 30 °C in automodification buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl2, 10 mM β-mercaptoethanol). Excess [3H]NAD+ was removed by desalting by gravity flow in a Micro Bio-spin Column (Bio-Rad) into demodification buffer (25 mM Tris-HCl pH 7.0, 200 mM NaCl, 10 mM β-mercaptoethanol). MARylated PARP10 was incubated with equimolar amounts of macrodomain proteins for 1 h at 37 °C unless otherwise indicated. Reactions were stopped with SDS/PAGE Running Buffer, and samples were subjected to SDS/PAGE on a 14% (wt/vol) Tris-Glycine gel (Invitrogen). Total protein levels were analyzed by SimplyBlue Safe Stain (Invitrogen), and [3H]signal was visualized by autoradiography. TLC analysis of reaction products was performed as described (27).

Isothermal Titrations of Calorimetry. The binding of ADP-ribose to nsP3MD was studied by isothermal titration calorimetry (ITC) using a VP-ITC instrument (MicroCal). Proteins were dialyzed into ITC buffer (20 mM Hepes pH 7.0, 100 mM NaCl) overnight at 4 °C. Dialyzed protein and ADP-ribose were respectively diluted to 40 μM and 1 mM in the dialyzed ITC buffer. Protein solution (1.4 mL) was loaded into the sample cell and titrated with 24 10-M μL injections after an initial 2-μL injection of ligand. The heat evolved at 25 °C after each injection was obtained by integration of the calorimetric signal. The data were analyzed on Origin 5.0 software and fitted by using a one binding-site model.

PARP10 Site Identification. Purified PARP10 was automodified with 1 mM NAD+ for 2 h at 37 °C in automodification buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl2, 10 mM β-mercaptoethanol). Excess NAD+ was removed by gel filtration using a Superose 12 10/300 GL column (Pharmacia). For each sample, 35 μg of PARP10 was incubated with or without 35 μg of nsP3 for 1 h at 37 °C followed by treatment with 35 μg of hsNudT16a for 2 h at 37 °C (34). Samples were then denatured, reduced, and alkylated in denaturing buffer (100 mM Tris-HCl pH 7.0 at 37 °C, 1.5 M guanidine-HCl, 1 mM CaCl2, 5 mM TCEP, 10 mM CAM) at 37 °C for 10 min. Denatured proteins were digested with 2 μg of LysC for 3 h at 37 °C, followed by 2 μg of trypsin overnight at 37 °C. Peptides were desalted, phosphoenriched, and analyzed by LC-MS/MS, and MaxQuant searches were performed as described (36).
**Cell Culture and Viral Replication Studies.** The murine neuronal NSC-34 cell line, a kind gift from Neil Cashman, University of British Columbia, Vancouver, BC, Canada (49), was grown in DMEM supplemented with heat-inactivated 10% (vol/vol) FBS, 100 μg/mL streptomycin, and 1% (vol/vol) FBS as above. A. albopictus clone C6/36 cells were grown in DMEM/10% (vol/vol) FBS as above at 28 °C in a 5% CO2 incubator. Mouse kidney 21 (BHK-21) and Vero cells were grown in DMEM/10% (vol/vol) FBS as above.

**Animals and Infection.** Timed pregnant CD-1 mice were purchased from Charles River Laboratories and maintained in an animal BSL2 facility. At postnatal day 2, mouse pups were infected intracranially with 1,000 pfu of virus in 10 μL of PBS while under isoflurane anesthesia. Pups were monitored 24 h post infection. Animals were monitored twice daily for 10 d. Survival was analyzed by using Kaplan–Meier survival curves and log rank test. All experiments were performed according to a protocol approved by the Johns Hopkins University Institutional Animal Care and Use Committee and followed guidelines of the National Institutes of Health and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

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