DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Science Manuscript Template

General Instructions on using this template and submitting a manuscript to *Science*: Thank you for preparing a manuscript for submission to *Science*. Using this template, or following the guidelines below, will help us in processing your paper. Our goal is to be able to identify each section of your manuscript so that we can accurately record the title, authors, abstract, etc. and also be able to enrich it by including reference links and an accurate layout.

Please use the actual template, which starts on page 2. When you are ready to submit, please delete the text on this cover page.

You can submit your paper at https://cts.sciencemag.org
Additional instructions are available at http://www.sciencemag.org/site/feature/contribinfo/index.xhtml

If you are using LaTeX , please convert your paper into a Word docx file if possible. If this is not possible, please use our LaTeX template and upload a PDF version of your paper. Some conversion approaches are available here: http://www.tug.org/utilities/texconv/textopc.html

So that we can identify the parts of your paper, and even if you do not use our template, please begin each section with the specific key words listed below, some of which are followed by a colon. Please do not use paragraph breaks in the title, author list, or abstract.

Title:
Authors:
Affiliations:
One Sentence Summary:
Abstract:
Main Text:
References and Notes (followed by a numbered list)
Acknowledgements:
Fig. #: (Begin each figure caption with a label, "**Fig. 1**." for example, as a new paragraph.).
Table #: (Begin each table caption with a label "**Table 1.**", etc.) as a new paragraph.
Supplementary Materials: a list of the supplementary materials, followed by the actual text of the Supplementary Materials.

Several of these headings are optional, for example, not all papers will include a one-sentence summary, figures or tables, or supplementary material.

Please use the .docx format (all version after Word 2007).

More specific formatting instructions are provided in the actual template, which follows.

UNCLASSIFIED

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Title: Systemic polyarteritis nodosa as the cause of sudden-onset bilateral sensorineural hearing loss following Lassa virus infection

Authors: Kathleen A. Cashman[1*], Eric R. Wilkinson[1], Paul R. Facemire[2†], Todd M. Bell[2‡], Carl I. Shaia[2ĩ], Connie S. Schmaljohn[3*]

Affiliations:

[1]Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702.

[2]Pathology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702.

[3] Office of the Chief Scientists, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702.

*Correspondence to: E-mail: kathleen.a.cashman.ctr@mail.mil (K.A.C); connie.s.schmaljohn.civ@mail.mil (C.S.S.)

† Current Affiliation: Global Health Division, Office of the Surgeon General, Fall Church, VA

‡ Current Affiliation: National Center for Biodefense and Infectious Diseases, George Mason University, Fairfax, VA

ĩ 43rd Veterinary Detachment, Ft. Hood, TX 75644

Abstract: Lassa virus (LASV) causes a severe, often fatal hemorrhagic disease in endemic regions of Africa and sudden-onset sensorineural hearing loss is a consequence of infection in approximately 30% of recovering patients. The mechanism of hearing loss in LASV-infected patients has not been identified. Here, we show that a cynomolgus macaque experimentally infected with Lassa virus who survived the initial infection, developed bilateral sensorineural hearing loss, confirmed by brainstem auditory evoked response (BAER) analysis. Histological examination of tissues from infected primates revealed pathology consistent with systemic polyarterisis nodosa (PAN), an autoimmune disorder associated with sudden hearing loss. Serological analyses to include the presence of autoimmune disease markers support this diagnosis. Our findings point toward an autoimmune etiology for LASV-associated sudden-onset hearing loss.

One Sentence Summary: Systemic polyarteritis nodosa is the proposed underlying pathology responsible for sudden-onset sensorineural hearing loss in a primate after Lassa virus infection.

Main Text: Lassa fever (LF) is an acute viral hemorrhagic disease caused by infection with the rodent-borne arenavirus Lassa virus (LASV). LF is endemic to West Africa with hundreds of thousands of cases occurring annually. The overall case fatality rate is ~15-20% but can be much higher in some outbreaks (*1, 2*). Neurological sequelae are common in both mild and severe cases, most notably, unilateral or bilateral sudden-onset sensorineural hearing loss, which appears both in acute and convalescent patients and can be permanent (*3-13*). Although a clear cause-and-effect relationship between LF and deafness is evident, the underlying pathology has not yet been identified. Here we report a nonhuman primate (NHP) deafness model with pathological findings indicating that systemic polyarterisis nodosa (PAN), an autoimmune

vasculitis disorder that has been associated with sudden hearing loss, is likely to be the basis of LF-associated deafness.

PAN in humans is characterized by necrotizing inflammatory lesions of small and medium sized arteries in most organ systems characterized by segmental fibrinoid necrosis and profound infiltration of polymorphonuclear neutrophils and monocytes (*14*). Typically, active and healing stages of arteritis are present simultaneously in all three layers of the vessels and perivascular space in the lesions (*14*). The etiology of PAN is unclear, thus is often categorized as idiopathic vasculitis (*15-17*). Rapid onset sensorineural hearing loss is a well-described complication of PAN and is thought to be due to inflammation and occlusion of the anterior inferior cerebellar artery (AICA) and downstream vessels leading to cochlear hypoxia (*18-24*). Relevant to this work, PAN has also been implicated as a downstream consequence of viral infections (*15-17*). There are no definitive tests that can be used to diagnose PAN; thus clinical diagnosis is based on the presence of at least three correlative symptoms as delineated by the American College of Rheumatology (ACR) to include: 1) unexplained weight loss, 2) mottled reticular skin patterns the extremities or torso; 3) testicular pain/tenderness, 4) myalgias, or weakness; 5) mono- or polyneuropathy; 6) hypertension; 7) elevated BUN or creatinine; 8) antibodies to hepatitis B surface antigen in serum; 9) arteriographic abnormalities; or 10) biopsy of small or medium-sized artery containing polymorphonuclear cells (*25*). In addition to these criteria, the presence of antineutrophil cytoplasmic antibodies (ANCA), circulating immune complexes, elevated C-reactive protein and IL-6, or sudden-onset, unexplained sensorineural hearing loss support the clinical diagnosis.

Studies of LASV pathogenesis have been reported using several nonhuman primate (NHP) species including cynomolgus macaques, which develop severe and usually fatal disease with symptoms and pathology mirroring observations from fatal LF cases in humans (*26*). Most LASV-infected macaques succumb 11-18 days after infection during the hemorrhagic phase of disease, with those surviving typically displaying neurological symptoms including tremors, ataxia, and seizures, which can be fatal. To determine if macaques surviving LASV infection develop deafness, we experimentally infected four animals and observed them for 45 days. All four macaques developed severe disease, with two of them succumbing during the hemorrhagic phase of disease (Fig. 1A). The other two NHP developed chronic neurological symptoms characterized by reduced appetite, tremors, and ataxia, as reflected by morbidity scores based on these symptoms (Fig. 1B). All animals had detectable serum viremias by day 6, with those of the two survivors peaking 14 days after infection followed by virus clearance by day 28 (Fig. 1C). Blood chemistry and hematology findings were consistent with those from earlier LASV studies in cynomolgus macaques (Fig. S1-2). Antibody responses measured by ELISA and plaque reduction neutralization tests (PRNT) demonstrated that the two survivors developed robust IgG responses (Fig. 2A), which could explain the high levels of total protein despite low levels of albumin in their serum (Fig 2B, C), but had modest amounts of neutralizing antibodies (Fig. 2D). By day 28 both of the surviving NHP appeared to develop hearing loss based on subjective measurements of sound response and tuning fork tests. These NHPs were screened for sensorineural hearing loss at day 45 by measuring brainstem auditory evoked response using an analog audiometer (BAERCOM). The BAERCOM device, developed for use in canines and also used successfully in non-domestic animal species, measures the neural response to clicking sounds produced at varying decibel (dB) levels (*27*). One of the surviving NHPs demonstrated unilateral hearing loss at 75 dB, and the other NHP showed a normal response pattern at this dB level (Fig. 3A).

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Necropsy of the two macaques succumbing during the acute phase revealed gross pathological findings consistent with earlier reports of LASV infection of cynomolgus macaques to include prominent pathological changes in lungs, along with additional abnormalities noted in liver, spleen, heart, pancreas, lymph nodes and brain (Fig. S4). Surviving NHPs also showed these pathological changes and in addition, had severe gross lesions and histologic vasculitis lesions in most organ systems that resembled those commonly seen in PAN patients (Fig S3-4). Samples removed from the skulls of the macaques showed moderate lymphoplasmacytic to chronic-active perivascular inflammation of the inner ear adjacent to the cochlear nerve The inflammation occasionally surrounded smaller branches of the cochlear nerve, resembling pathological changes seen in humans diagnosed with PAN (Fig. 3B-E) (*21*). Confirmatory serological evidence for PAN was obtained by testing samples from the two survivors for the presence of immune complexes, anti-neutrophilic cytoplasmic antibodies (ANCA), and C-reactive protein, all of which are hallmarks of PAN in humans. We found that C-reactive protein was elevated in both NHP starting at day 14, reached a peak at day 28, then decreased but remained elevated through the end of the study (Fig. 4A). Sample volume limitations prevented us from including the animals that succumbed during the acute phase in this analysis. Animals that succumbed during the acute phase did not develop measureable circulating immune complexes (CICs) (Fig. 4B); however, the two survivors had highly elevated CICs by day 21, which persisted to the endpoint. Both surviving macaques developed ANCA by day 21, with one of the two animals showing highly elevated levels on days 28 and 45 (Fig 4C). Cytokine and chemokine levels were measure in these NHPs by a multiplex assay. Cytokines, chemokines or vascular growth factors that have either been shown to be elevated in patients with PAN or have been identified as potential therapeutic targets are present at elevated levels in the chronically ill animals (*28-32*).

In summary, the NHP in our study experienced seven of the 10 ACR criteria for diagnosis of PAN in humans, all four of the serological markers, and measureable deafness in one NHP following experimental infection with LASV. Our results strongly point toward PAN as the underlying cause of LF-associated acute, rapid-onset sensorineural hearing loss.

References and Notes:

1. C. A. Silva, M. Cocuzza, E. F. Borba, E. Bonfa, Cutting-Edge Issues in Autoimmune Orchitis. *Clin Rev Allergy Immunol*; published online EpubAug 13 (10.1007/s12016-011-8281-z).
2. L. M. Branco, J. N. Grove, M. L. Boisen, J. G. Shaffer, A. Goba, M. Fullah, M. Momoh, D. S. Grant, R. F. Garry, Emerging trends in Lassa fever: redefining the role of immunoglobulin M and inflammation in diagnosing acute infection. *Virology journal* **8**, 478 (2011)10.1186/1743-422X-8-478).
3. . pp. http://www.who.int/mediacentre/factsheets/fs179/en/index.html.
4. D. Cummins, Rats, fever and sudden deafness in Sierra Leone. *Tropical doctor* **22**, 83-84 (1992); published online EpubApr (
5. D. Cummins, J. B. McCormick, D. Bennett, J. A. Samba, B. Farrar, S. J. Machin, S. P. Fisher-Hoch, Acute sensorineural deafness in Lassa fever. *Jama* **264**, 2093-2096 (1990); published online EpubOct 24-31 (
6. T. S. Ibekwe, P. O. Okokhere, D. Asogun, F. F. Blackie, M. M. Nwegbu, K. W. Wahab, S. A. Omilabu, G. O. Akpede, Early-onset sensorineural hearing loss in Lassa fever. *Eur Arch Otorhinolaryngol* **268**, 197-201; published online EpubFeb (10.1007/s00405-010-1370-4).
7. B. S. Liao, F. M. Byl, K. K. Adour, Audiometric comparison of Lassa fever hearing loss and idiopathic sudden hearing loss: evidence for viral cause. *Otolaryngol Head Neck Surg* **106**, 226-229 (1992); published online EpubMar (
8. A. M. Macher, M. S. Wolfe, Historical Lassa fever reports and 30-year clinical update. *Emerging infectious diseases* **12**, 835-837 (2006); published online EpubMay (

9. J. B. McCormick, I. J. King, P. A. Webb, K. M. Johnson, R. O'Sullivan, E. S. Smith, S. Trippel, T. C. Tong, A case-control study of the clinical diagnosis and course of Lassa fever. *The Journal of infectious diseases* **155**, 445-455 (1987); published online EpubMar (
10. P. E. Mertens, R. Patton, J. J. Baum, T. P. Monath, Clinical presentation of Lassa fever cases during the hospital epidemic at Zorzor, Liberia, March-April 1972. *The American journal of tropical medicine and hygiene* **22**, 780-784 (1973); published online EpubNov (
11. P. O. Okokhere, T. S. Ibekwe, G. O. Akpede, Sensorineural hearing loss in Lassa fever: two case reports. *J Med Case Reports* **3**, 36 (2009)1752-1947-3-36 [pii]
10.1186/1752-1947-3-36).
12. L. P. Rybak, Deafness associated with Lassa fever. *JAMA* **264**, 2119 (1990); published online EpubOct 24-31 (
13. F. B. Simmons, Lassa fever and sudden hearing loss. *Otolaryngol Head Neck Surg* **109**, 559 (1993); published online EpubSep (
14. J. Churg, A. Churg, Idiopathic and secondary vasculitis: a review. *Mod Pathol* **2**, 144-160 (1989); published online EpubMar (
15. N. Mohan, G. Kerr, Infectious etiology of vasculitis: diagnosis and management. *Curr Rheumatol Rep* **5**, 136-141 (2003); published online EpubApr (
16. M. Lidar, N. Lipschitz, P. Langevitz, Y. Shoenfeld, The infectious etiology of vasculitis. *Autoimmunity* **42**, 432-438 (2009); published online EpubAug (10.1080/08916930802613210 [pii]).
17. T. Caldeira, C. Meireles, F. Cunha, C. Valbuena, J. Aparicio, A. Ribeiro, Systemic polyarteritis nodosa associated with acute Epstein-Barr virus infection. *Clin Rheumatol* **26**, 1733-1735 (2007); published online EpubOct (10.1007/s10067-006-0486-9).
18. T. Nakashima, S. Naganawa, M. Sone, M. Tominaga, H. Hayashi, H. Yamamoto, X. Liu, A. L. Nuttall, Disorders of cochlear blood flow. *Brain Res Brain Res Rev* **43**, 17-28 (2003); published online EpubSep (S0165017303001899 [pii]).
19. H. A. Jenkins, A. M. Pollak, U. Fisch, Polyarteritis nodosa as a cause of sudden deafness. A human temporal bone study. *Am J Otolaryngol* **2**, 99-107 (1981); published online EpubMay (
20. P. Gussen, Polyarteritis nodosa and deafness. A human temporal bone study. *Arch Otorhinolaryngol* **217**, 263-271 (1977); published online EpubAug 26 (
21. S. Joglekar, A. F. Deroee, N. Morita, S. Cureoglu, P. A. Schachern, M. Paparella, Polyarteritis nodosa: a human temporal bone study. *Am J Otolaryngol* **31**, 221-225; published online EpubJul-Aug (S0196-0709(09)00026-X [pii]
10.1016/j.amjoto.2009.02.006).
22. M. Wolf, J. Kronenberg, S. Engelberg, G. Leventon, Rapidly progressive hearing loss as a symptom of polyarteritis nodosa. *Am J Otolaryngol* **8**, 105-108 (1987); published online EpubMar-Apr (
23. J. C. Amor Dorado, P. Barreira Fernandez Mdel, S. Regueiro Villarin, M. A. Gonzalez-Gay, [Audiovestibular manifestations in systemic vasculitis]. *Acta Otorrinolaringol Esp* **60**, 432-442 (2009); published online EpubNov-Dec (S0001-6519(09)00064-8 [pii]
10.1016/j.otorri.2009.01.012).
24. S. Berrettini, C. Ferri, F. Ravecca, L. LaCivita, L. Bruschini, L. Riente, M. Mosca, S. Sellari-Franceschini, Progressive sensorineural hearing impairment in systemic vasculitides. *Semin Arthritis Rheum* **27**, 301-318 (1998); published online EpubApr (S0049-0172(98)80051-6 [pii]).
25. R. W. Lightfoot, Jr., B. A. Michel, D. A. Bloch, G. G. Hunder, N. J. Zvaifler, D. J. McShane, W. P. Arend, L. H. Calabrese, R. Y. Leavitt, J. T. Lie, et al., The American College of Rheumatology 1990 criteria for the classification of polyarteritis nodosa. *Arthritis Rheum* **33**, 1088-1093 (1990); published online EpubAug (
26. L. E. Hensley, M. A. Smith, J. B. Geisbert, E. A. Fritz, K. M. Daddario-DiCaprio, T. Larsen, T. W. Geisbert, Pathogenesis of Lassa fever in cynomolgus macaques. *Virology journal* **8**, 205 (2011)10.1186/1743-422X-8-205).
27. A. A. Webb, C. L. Cullen, L. A. Lamont, Brainstem auditory evoked responses and ophthalmic findings in llamas and alpacas in Eastern Canada. *Can Vet J* **47**, 74-77 (2006); published online EpubJan (
28. G. E. Grau, P. Roux-Lombard, C. Gysler, C. Lambert, P. H. Lambert, J. M. Dayer, L. Guillevin, Serum cytokine changes in systemic vasculitis. *Immunology* **68**, 196-198 (1989); published online EpubOct (
29. L. Guillevin, Virus-induced systemic vasculitides: new therapeutic approaches. *Clin Dev Immunol* **11**, 227-231 (2004); published online EpubSep-Dec (

30. D. Saadoun, I. Bieche, F. J. Authier, I. Laurendeau, F. Jambou, J. C. Piette, M. Vidaud, T. Maisonobe, P. Cacoub, Role of matrix metalloproteinases, proinflammatory cytokines, and oxidative stress-derived molecules in hepatitis C virus-associated mixed cryoglobulinemia vasculitis neuropathy. *Arthritis Rheum* **56**, 1315-1324 (2007); published online EpubApr (10.1002/art.22456).
31. D. Saadoun, I. Bieche, T. Maisonobe, T. Asselah, I. Laurendeau, J. C. Piette, M. Vidaud, P. Cacoub, Involvement of chemokines and type 1 cytokines in the pathogenesis of hepatitis C virus-associated mixed cryoglobulinemia vasculitis neuropathy. *Arthritis Rheum* **52**, 2917-2925 (2005); published online EpubSep (10.1002/art.21270).
32. K. Kikuchi, T. Hoashi, S. Kanazawa, K. Tamaki, Angiogenic cytokines in serum and cutaneous lesions of patients with polyarteritis nodosa. *J Am Acad Dermatol* **53**, 57-61 (2005); published online EpubJul (S0190962205006857 [pii]
10.1016/j.jaad.2005.02.018).

Acknowledgments: The authors would like to thank Heather Esham and Joshua Shamblin for assistance with blood sample collection. Additionally, we are grateful to the technical staff of the Pathology Division for assistance with processing, cutting and staining tissues for microscopic analysis. Thank you to LTC Robin Burke for helpful discussions about the BAERCOM analysis. This study was funded by the Military Infectious Disease Research Program (MIDRP). Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

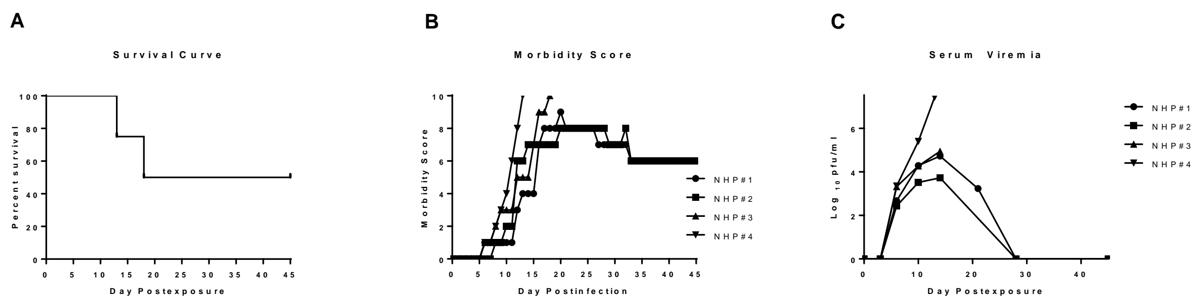

Fig. 1. Animal Study Outcomes. (A) The survival curve shows that two NHPs that succumbed to disease during the acute phase on days 13 and 17 postexposure. Two NHPs survived to the study endpoint 45 days postexposure. (B) A graphical representation of the disease signs experienced by NHPs during the course of the study. All NHPs started exhibiting disease signs approximately day 6 postexposure. Disease signs rose quickly in all NHPs, culminating in two NHPs succumbing during the acute phase. The two NHPs that survived to the study endpoint experienced severe disease signs that did not resolve, but that did not require euthanasia. Disease signs included loss of appetite, reduced activity, hunched posture, respiratory distress, and neurological deficits including trembling, muscle weakness, and ataxia. (C) Serum viremia was determined by standard plaque assay of samples collected at specific timepoints postexposure and calculated and expressed as Log_{10} pfu/ml.

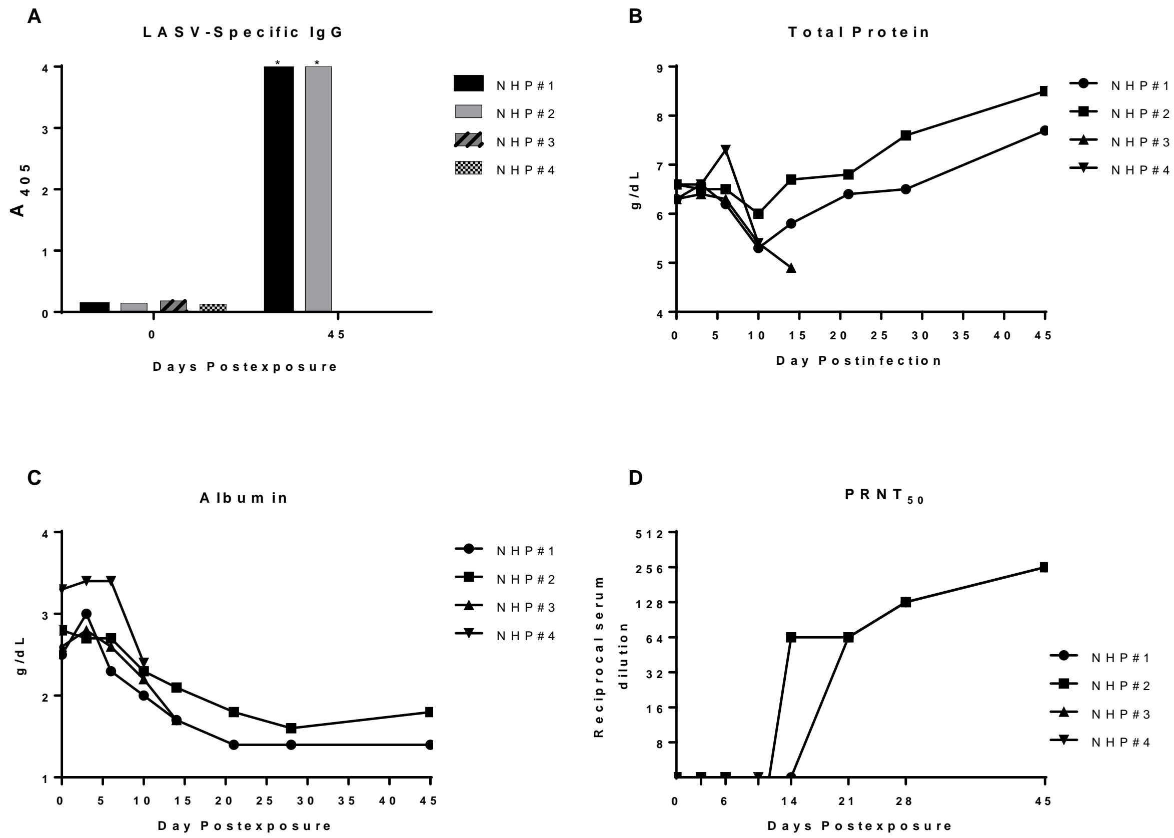

Fig. 2. You can place graphics above each equation as part of this file.

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

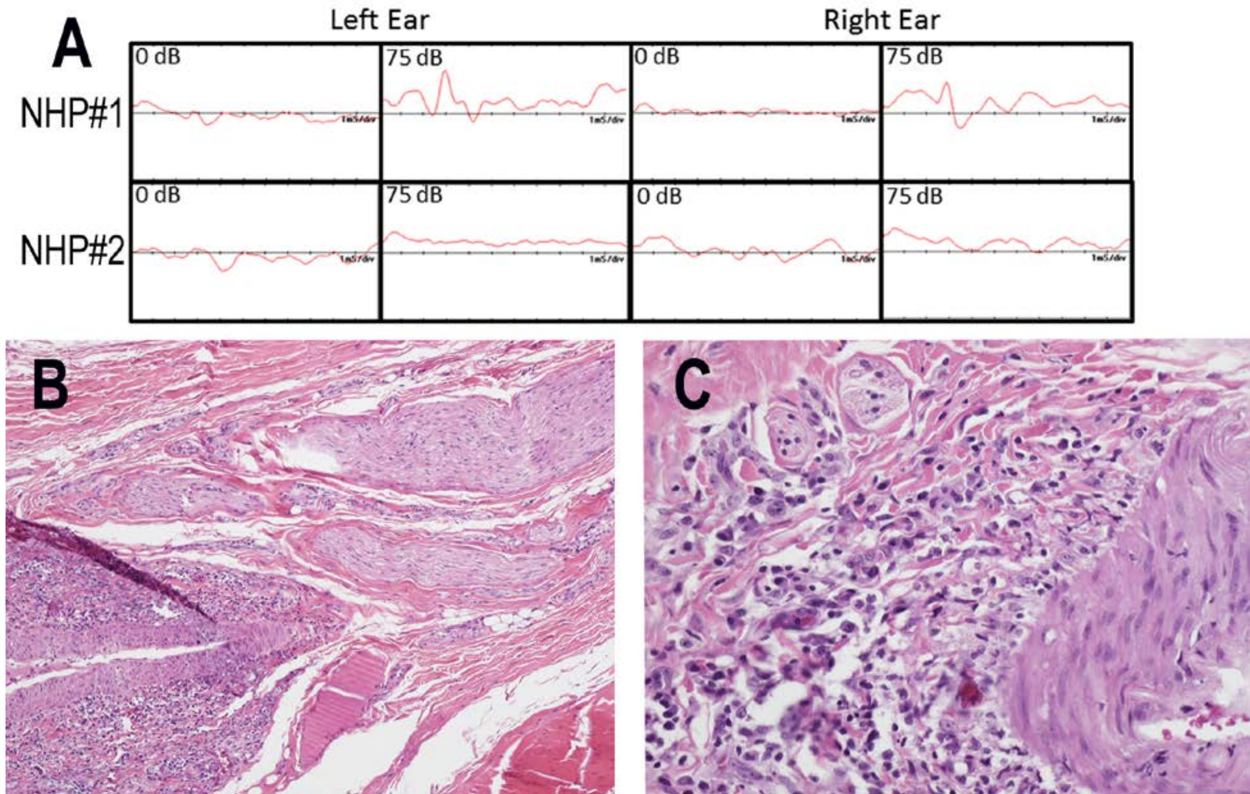

Fig 3. Evidence for sensorineural hearing loss in a surviving NHP after LASV exposure. A brainstem autitory evoked response device (BAERCOM) was used to assess the surviving NHPs for a hearing response on day 45 postexposure. Clear waveforms indicating a hearing response were observed in the male survivor (NHP#1) at 75 dB, whereas the female survivor (NHP#2) has the absence of a waveform for both the left and right ear at 75 dB (A). Histologic examination of the inner ear structures (B-C) also reveal evidence for deafness in the female surviving NHP at day 45 postexposure. Please help with arrows to the relevant areas and add to the text for the legend for B and C if necessary. Draw arrows on this picture then scan it back to me so I can add them in photoshop.

UNCLASSIFIED

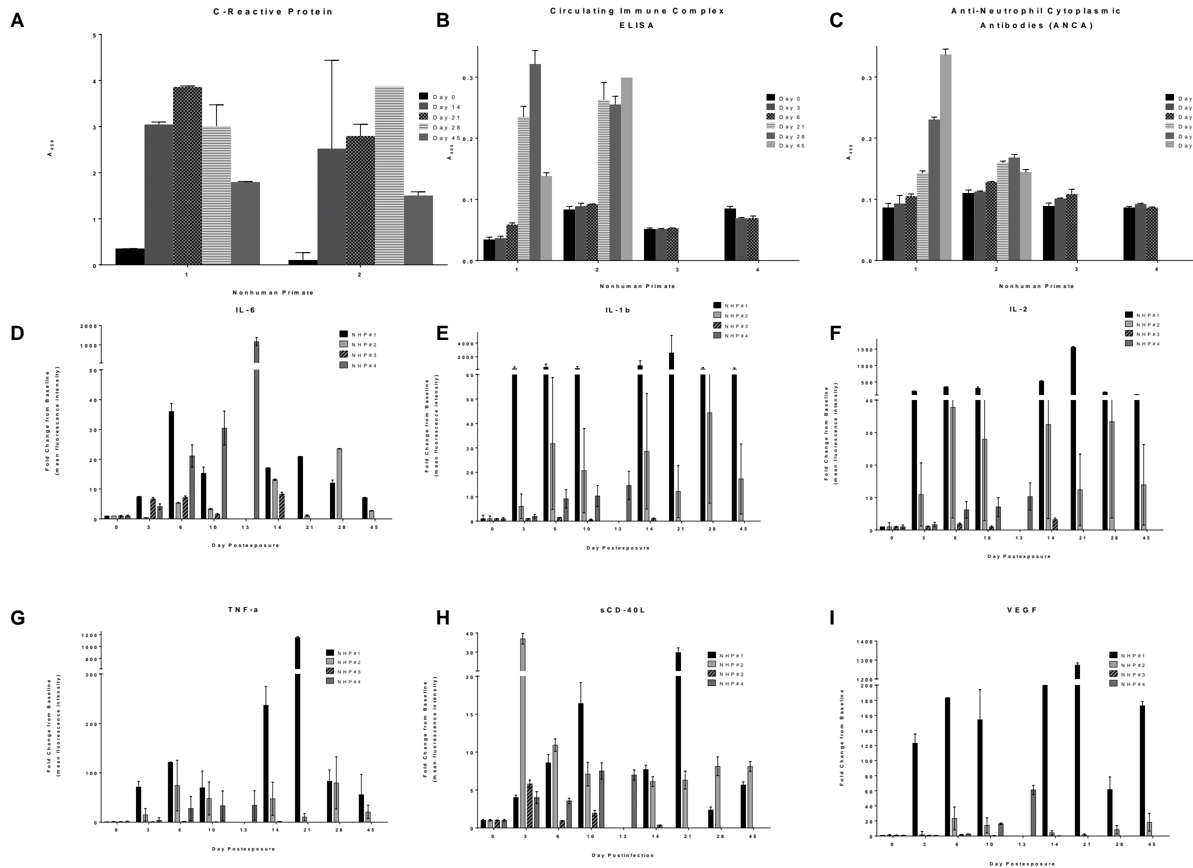

Fig. 4. C-reactive protein (A) remains high throughout the disease course for surviving primates despite being free of circulating virus in serum by day 28. (B) Surviving NHPs experienced a spike in CICs beginning on day 21 postexposure, and remaining very high for the NHP that became deaf, but decreasing for the other NHP. Both surviving NHPs had CIC levels significantly above baseline levels at the end of the study. (C) ANCA were present in NHPs above background levels starting at day 21 postexposure. One NHP experienced a large increase over baseline levels on days 28 and 45 postexposure. The other NHP had levels closer to but significantly above baseline. Selected cytokine level changes, shown as fold change from baseline (D-I).

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Supplementary Materials:

Materials and Methods

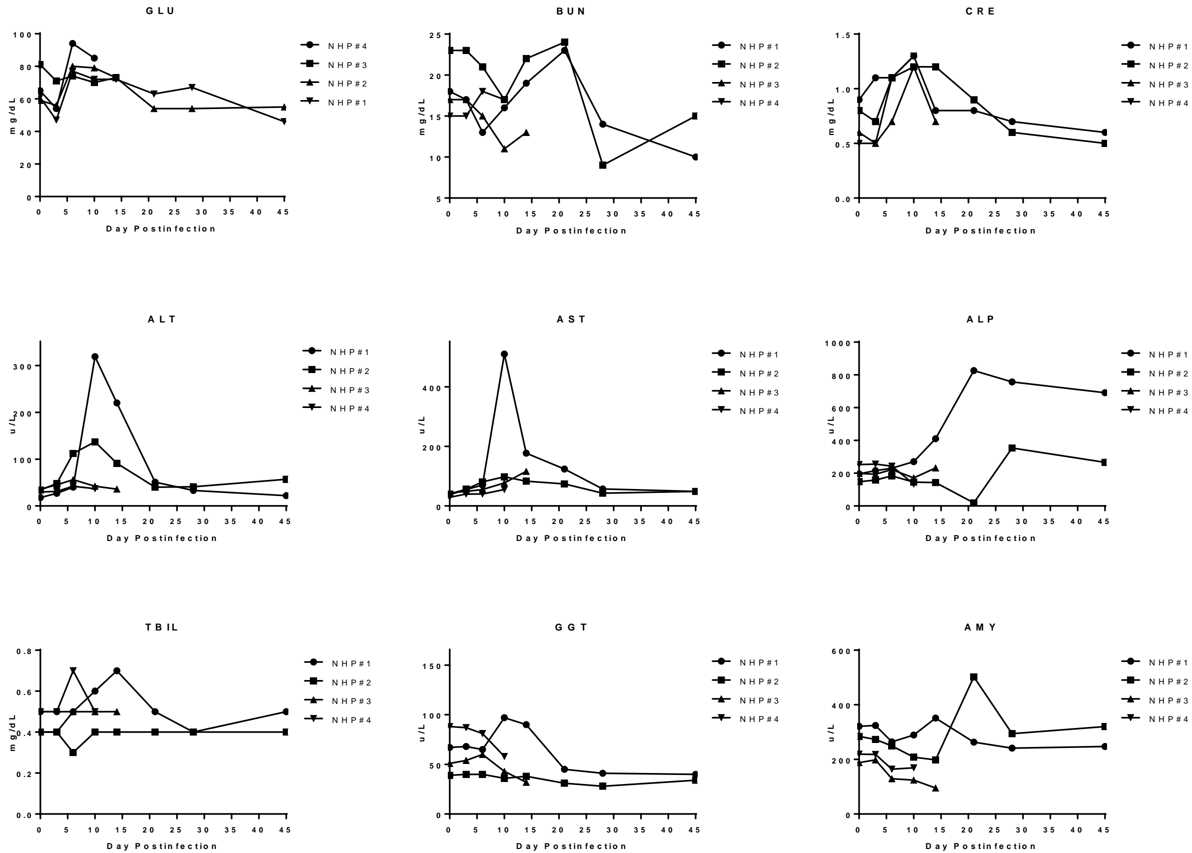

Fig. S1. Additional Blood Chemistry Analyses. Calling All pathologists! Pathophysiology writeup is welcome here. ☺

UNCLASSIFIED

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

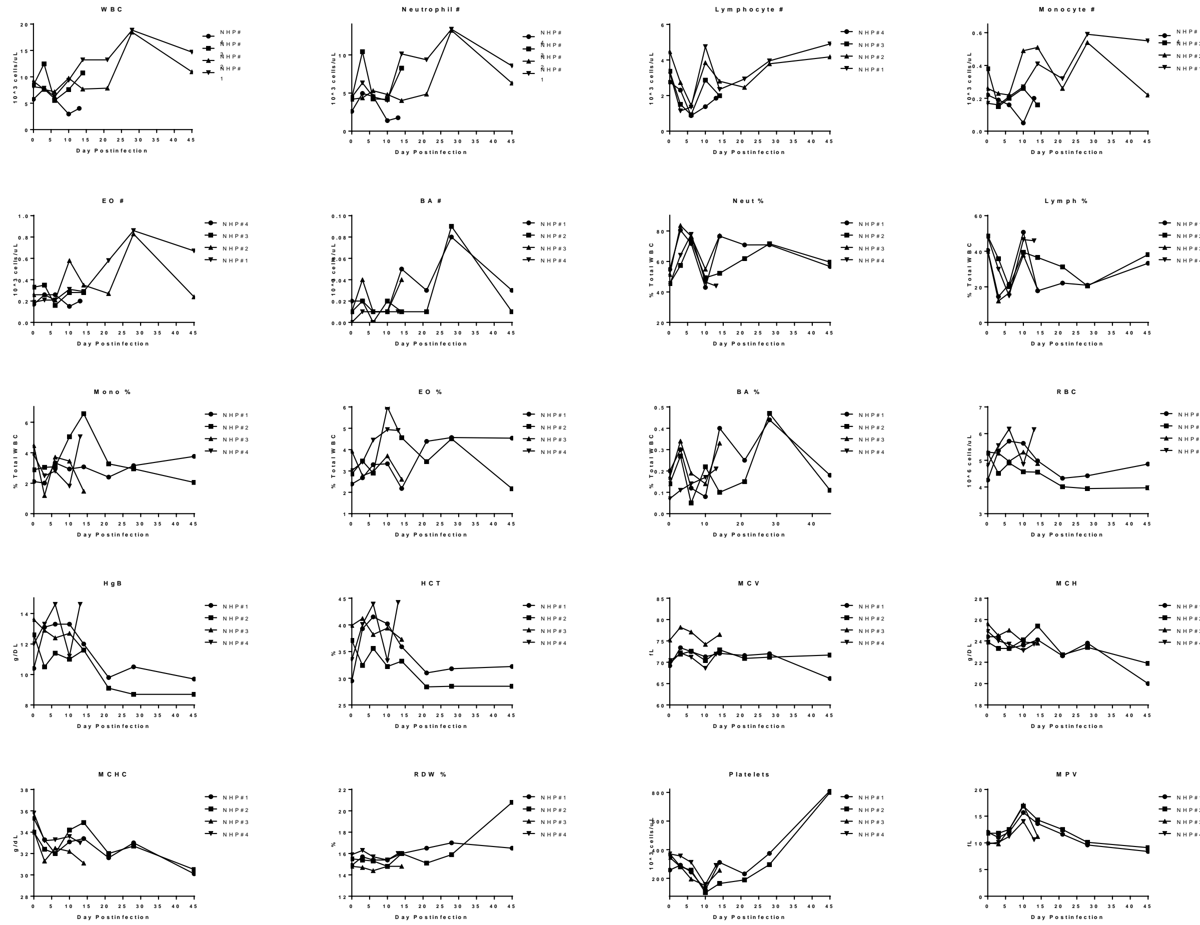

Fig. S2. Complete Blood Counts. Calling All pathologists! Pathophysiology writeup is welcome here. ☺

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

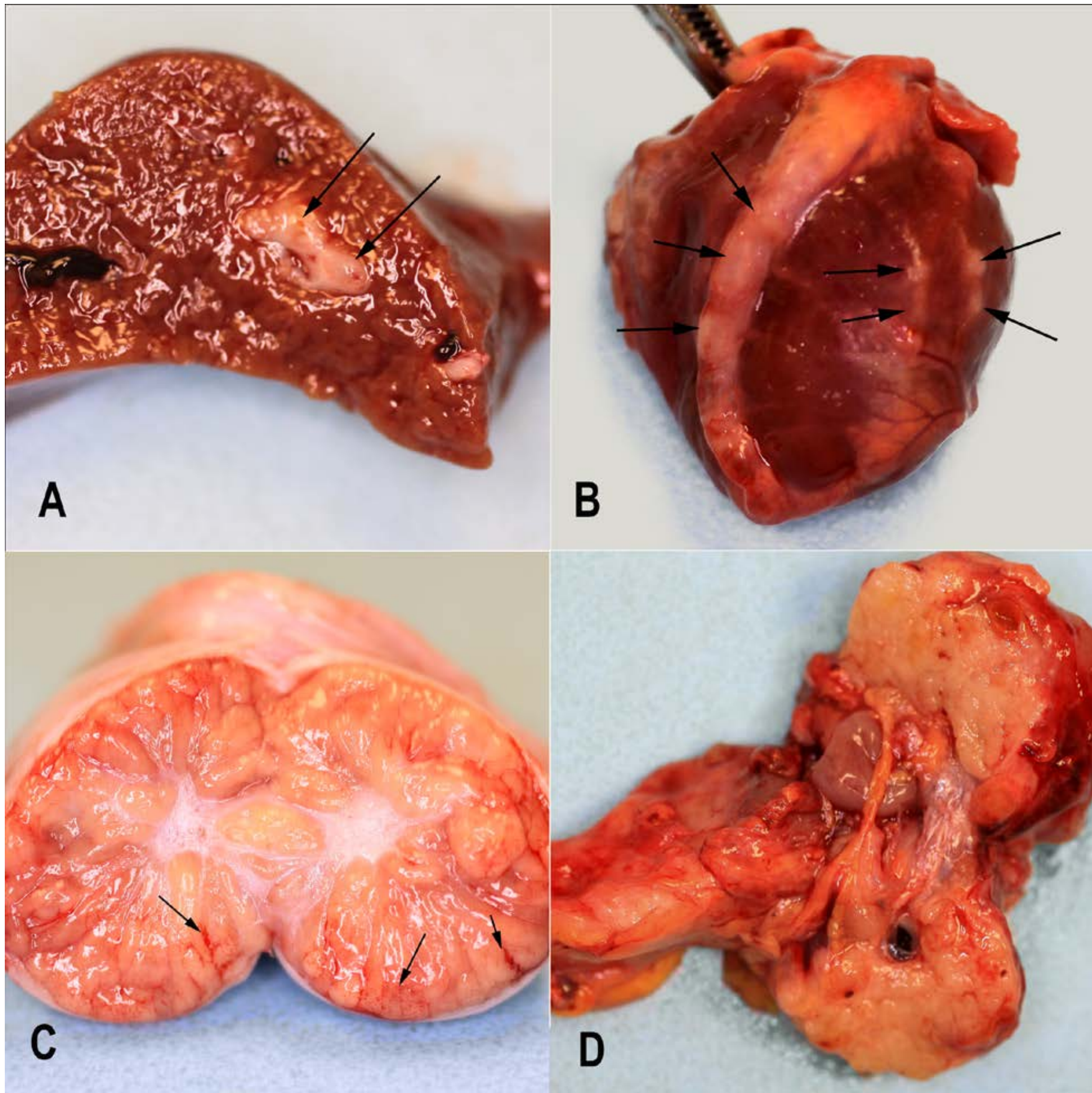

Fig. S3. Gross Pathology Findings. Moderate thickening of hepatic (A) and coronary (B) vessels that resembled athlerosclerosis in the female survivor. The coronary vessels exhibit the "string of pearls" appearance described for polyarteritis lesions. Testicular hemorrhage (C) and a white fibrous pancreatic mass (D) in the male survivor. This caption and description of lesions could probably use some sprucing up. Please check my placement of arrows and let me know if additional arrows should be included. Draw them on the figure and scan it back to me so I can draw them in photoshop.

UNCLASSIFIED

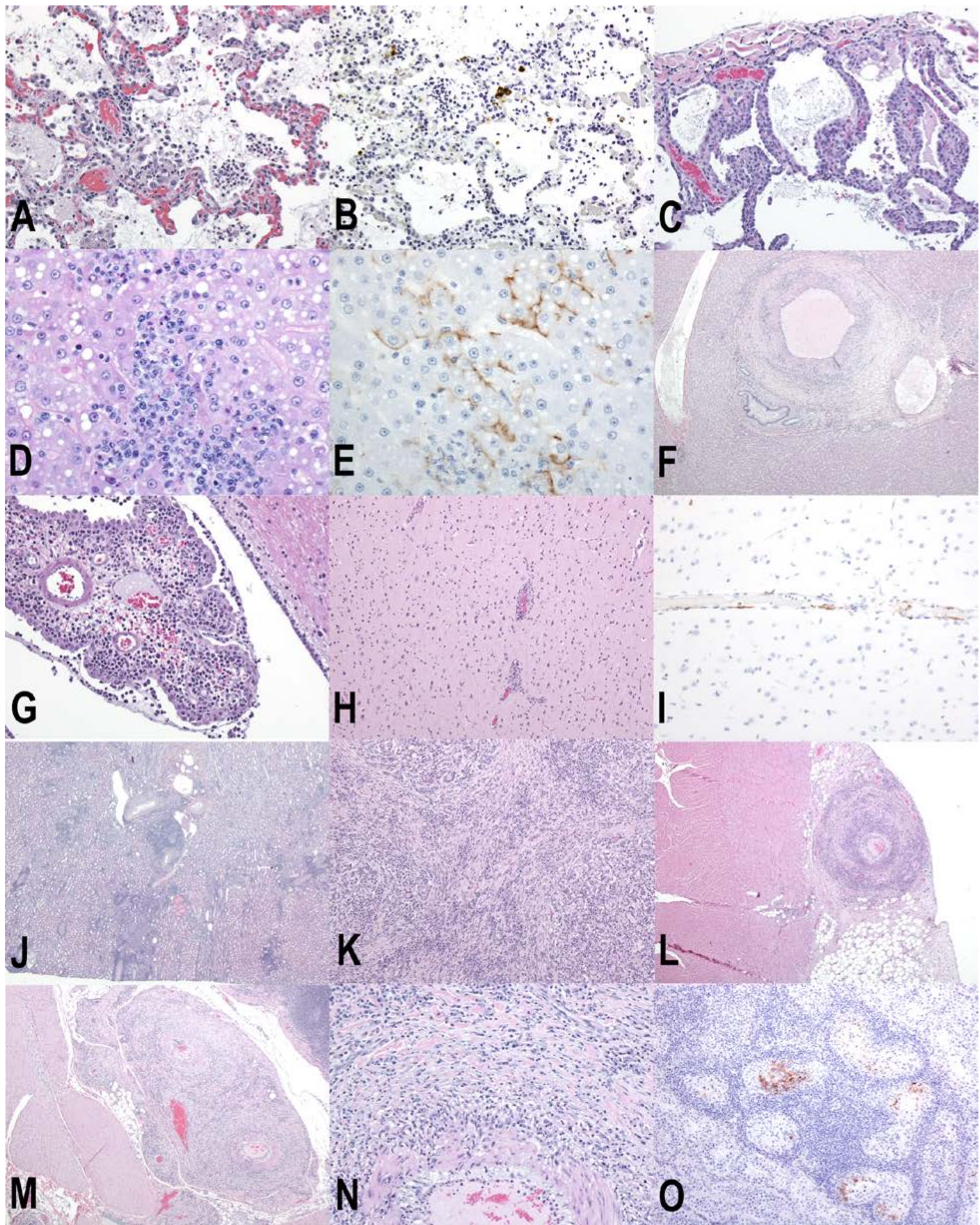

Fig S4. Histopathological Findings. Lungs (A) Lymphoplasmacytic interstitial pneumonia with type II pneumocyte hyperplasia in an 18 dpi animal, 20x. (B) Cytoplasmic immunoreactivity in low numbers of alveolar macrophages, pneumocytes and endothelial cells in an 18 dpi animal,

20x. (C) Interstitial fibrosis with type II pneumocyte hyperplasia in a surviving NHP, 20x. (D) Degeneration and necrosis with subacute inflammation, Councilman bodies and vacuolar change in the liver of a 12 dpi animal, 20x. (E) Positive apical to membranous hepatocyte and cytoplasmic endothelial immunoreactivity in a 12 dpi animal, 40x. (F) Necrotizing and proliferative vasculitis in the liver of a surviving NHP, 4x. (G) Lymphoplasmacytic choroid plexitis in the brain of a 12 dpi animal, 20x. (H, I) Meningoencephalitis, chronic-active perivascular multifocal gliosis and satellitosis with lack of corresponding immune reactivity in a surviving NHP. (J) Membranous glomerulonephritis and vasculitis in kidney of a surviving NHP, 20x. (K) Chronic-active pancreatitis with fibrosis and duct hyperplasia in a surviving NHP, 10x. (L) Necrotizing and proliferative arteritis with narrowed lumen in the coronary artery a surviving NHP, 10x. (M) Necrotizing and proliferative arteritis with narrowing of the lumen in the mesenteric artery of a surviving NHP, 4x. (N) Higher magnification of vascular changes noted in panel M, 20x. (O) Chronic-active orchitis in the testicles of the male survivor with positive cytoplasmic immunoreactivity in seminiferous tubule cells, 45 dpi, 10x. Is there more that could be added here since there are no restrictions on length for supplementary figures? Also, I would like to add arrows where appropriate. If you could draw them and scan back this figure I will do the arrows in Photoshop.

Materials and Methods:

Virus. Monolayers of Vero E-6 cells were infected at an MOI of 0.1 with a stock of LASV. Three days post-infection, supernatants were collected, consolidated and clarified by centrifugation. Virus-containing supernatant was titered by standard plaque assay.

Primate Study. A stock of LASV was diluted to a concentration of 1000 pfu/ml in sterile physiological saline. Macaques were infected with 1000 pfu LASV via a single intramuscular injection of 1 ml virus-containing solution in the quadriceps muscle group. Macaques were monitored daily for disease progression and were euthanized when moribund according to IACUC-approved euthanasia criteria. Anecdotal hearing response screening, including tuning fork tests and sound response tests were conducted with unanesthetized animals daily. Blood samples were collected at days 0, 3, 6, 10, 14, 28 and 45 postinfection. Blood samples were analyzed for CBC, blood chemistry and serum viremia. Hearing loss was assessed in anesthetized macaques using a BAERCOM device (UFI, Inc.) according to manufacturer's instructions. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.The BSL-4 facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Blood Chemistry and Complete Blood Count Analysis. Serum samples collected pre- and postinfection were diluted 1:3, then analyzed for glucose, blood urea nitrogen, creatinine, uric acid, calcium, albumin, total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), total bilirubin, gamma glutamyl transferase, and amylase.

Approximately 100 µl of serum was applied to a General Chemistry 13-panel rotor and evaluated in a Piccolo point-of-care blood chemistry analyzer (Abaxis). Values for each serum sample were recorded and compiled. For the CBC analysis, 75 µl of whole EDTA-treated blood was aliquoted and run on a Hemavet Instrument (Drew Scientific). Values for each serum sample were recorded and compiled.

Analysis of Viremia. Serum samples collected pre- and postinfection were assayed for viral titers via a standard plaque assay with some modifications.40 Briefly, Vero cells, seeded in 6-well cell culture plates, were adsorbed with gentle rotation at 37°C, 5% CO2 with 10-fold serial dilutions of serum for 1 h, then an overlay of 0.8% molecular grade agarose in EBME (basal medium Eagle with Earle's salts) fortified with 10% fetal bovine serum and 20 µg/ml gentamicin was applied to each well and allowed to solidify. After the overlay solidified, cells were incubated at 37ºC, 5% CO2 for 4 days, then stained with neutral red (Invitrogen, Carlsbad, CA). After an overnight incubation at 37ºC in the stain, plaques were counted and recorded.

Evaluation of Sensorineural Hearing Loss. Hearing screenings were performed using a brainstem auditory evoked response communication (BAERCOM) device (UFI, Inc.) according to manufacturer's instructions. Briefly, needle probes were placed subdermally into the skin above each ear in the area of the temporal bones. An additional probe was placed subdermally on the crown of the head. The probes were plugged into the BAERCOM device which was set to medium resolution recording. An earplug was placed into one ear at a time and audiometric readings were collected at 0 dB and 75 dB for each ear. The BAERCOM software produced graphs for each reading.

Measurement of Cytokine/Chemokine Production in Plasma. Plasma was obtained from EDTA-treated blood samples collected on days 0, 3, 6, 10, 14, 28 and 45 postinfection. Cytokine and chemokine levels were measured using a Milliplex MAP Non-Human Primate Cytokine Premixed 23-Plex Immunology Multiplex Assay Magnetic Bead Panel (Merck-Millipore). The assay was performed according to manufacturer's instructions and results were obtained on a Luminex FlexMap 3D Instrument. DATA PENDING

Immune Complex, Anti-Neutrophil Cytoplasmic Antibodies (ANCA) and C-Reactive Protein ELISAs. Serum samples collected before viral infection and at days 0, 3, 6, 10, 14, 21, 18 and 45 postinfection were screened using a Raji Cell Immune Complex ELISA (MicroVue CIC-Raji Cell Replacement EIA, Quidel Corp.), which measures C3d-bound CIC present in plasma or serum in a 96-well format. ANCA antibodies were assessed using an ANCA Screen IgG Test Kit (Diagnostic Automation Inc.) that measure levels of anti-Myeloperoxidase (MPO) and/or anti-Proteinase-3 (PR3) IgG antibody in serum. To measure circulating levels of C-Reactive protein in serum, the Monkey C-Reactive Protein ELISA (Life Diagnostics, Inc.) was utilized. All assays were performed according to manufacturer's instructions and results were obtained on a Molecular Diagnostics SpectraMax M5 multimode microplate reader.

Analysis of LASV-Specific Antibody Production. Serum samples collected before viral infection and at days 0, 3, 6, 10, 14, 21, 18 and 45 postinfection were analyzed for LASV-specific antibody production by ELISA (Pre-made LASV-Specific antibody capture ELISA kits were generously provided by Tulane University and Coregenix, Denver, CO). ELISA kits consisted

DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

of a combination of LASV antigens (Combo 5.5) or LASV GPC. Twofold serial dilutions, ranging from 1:8 to 1:1024 were made for each serum sample, then 100 µl of each dilution was added to the wells and incubated for 1 h at room temperature. Plates were then washed 5 times in the wash buffer provided with the kit. After the wash step, 100 µl of a 1:5000 dilution of human-specific anti-IgG HRP-conjugated secondary antibodies (KPL, Inc.) was added to each well and incubated for 30 minutes at room temperature. Following another 5 washes, 100 µl TMB substrate was added to each well and incubated at room temperature for 10 minutes before adding 100 µl stop solution. Plates were read in a microtiter spectrophotometer and absorbance values at A450 were recorded. Experiments using serum collected from LASV-infected animals were performed in biosafety level-4 conditions.

Pathologic analysis of tissues. Tissues were trimmed and processed according to standard protocol. Sections were trimmed at 5-6 µm thickness and stained with hematoxylin and eosin. Immunohistochemistry was performed on replicate tissue sections for both partial and full necropsies using an Envision kit. A monoclonal antibody specific for Lassa virus GP1 was used at a dilution of 1:15000. After deparaffinization and peroxidase blocking an antigen retrieval step was performed using a TRIS/EDTA buffer in a steamer for 30 minutes. Then sections were covered with primary antibody and incubated at room temperature for 30 minutes at room temperature. They were rinsed, and the peroxidase-labeled polymer (secondary antibody) was applied for 30 minutes. Slides were rinsed and a substrate-chromogen solution (DAB, Dako) was applied for five minutes. The slides were rinsed in distilled water and counterstained with hematoxylin for two minutes, dehydrated, cleared with xyless and then coverslipped. Slides were evaluated using a Nikon Eclipse 600 light microscope.

UNCLASSIFIED