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Autoimmune-associated systemic vasculitis as the cause of sudden-onset bilateral sensorineural hearing loss following Lassa virus exposure in a cynomolgus macaque deafness model

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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 causal mechanism of hearing loss in LASV-infected patients has not been identified. To develop a model for LASV deafness, we experimentally infected four cynomolgus macaque nonhuman primates (NHPs) with LASV. All four NHPs developed typical signs and symptoms of Lassa fever and two succumbed during the acute phase of disease. Two NHPs survived to the study endpoint, 45 days post-exposure. Both of these survivors remained chronically ill and apparent hearing loss was observed using daily subjective measurements, including response to auditory stimulation and tuning fork tests. Bilateral sensorineural hearing loss at 75 dB was confirmed for one of the survivors by brainstem auditory evoked response (BAER) analysis. Histologic examination of inner ear structures and other tissues revealed the presence of severe vascular lesions consistent with autoimmune-associated systemic vasculitides. These autoimmune disorders have been associated with sudden hearing loss. Other vascular-specific damage was also observed to be present in many of the sampled tissues. Serological analyses revealed the presence of autoimmune disease markers supporting this diagnosis. Our findings point toward an autoimmune etiology for Lassa fever-associated sudden-onset hearing loss.

Significance Statement

Lassa virus is one of the most common causes of viral hemorrhagic fever. A frequent, but as yet unexplained consequence of infection with Lassa virus is acute, sudden onset sensorineural hearing loss in one or both ears. Deafness is observed in approximately 30% of surviving Lassa fever patients, an attack rate that is approximately 300% higher than mumps virus infection, which is the most common cause of virally-induced deafness. Here, we provide evidence from Lassa virus-infected cynomolgus macaques implicating autoimmune-associated vasculitis syndrome underlying the pathology of Lassa fever-associated deafness. This information could change the way Lassa fever patients are medically managed in the clinic in order to prevent deafness in survivors.

Author contributions

K.A.C. designed the study; K.A.C. and E.R.W. performed all research activities; C.I.S. performed necropsies; P.R.F., T.M.B. and C.I.S. performed pathologic analyses. K.A.C., analyzed data; E.R.W., P.R.F., and C.I.S. provided editorial comments; and K.A.C. and C.S.S. wrote the paper.

Introduction

Lassa fever (LF) is an acute viral hemorrhagic disease endemic to West Africa caused by infection with the rodent-borne arenavirus Lassa virus (LASV). The disease is characterized by fever, malaise, exudative pharyngitis that progresses to include mucosal bleeding, severe hemorrhagic conjunctivitis, peripheral and facial edema and coagulopathy. LF is fatal in approximately 15-20% of cases, with the case fatality rate much higher in some outbreaks (1, 2). Neurological sequelae, including memory loss, ataxia, and neuromuscular pain, are common in both mild and severe cases. Most notably, permanent unilateral or bilateral sudden-onset sensorineural hearing loss is observed in an unusually high percentage of LF survivors. The incidence rate of deafness associated with LF greatly exceeds that of any other viral disease known to cause postnatal deafness. For example, the most commonly occurring viral disease known to cause deafness is mumps, in which deafness occurs in less than 0.1% of cases (approximately 300 times less than that of LF) (3). LF-associated deafness is a considerable cause of disability in the endemic regions of West Africa (Nigeria, Liberia, Guinea and Sierra Leone) (4). Since most confirmed cases of LF-associated hearing loss are identified during the convalescent phase in recovering patients and followed LASV seroconversion, it has been speculated that hearing loss may be the result of an immune response to infection (5). More recent studies; however, assert that sudden onset sensorineural hearing loss can also occur during the acute phase of disease, indicating that direct damage due to viral infection may also contribute to hearing loss (6). In a prospective study, five of 37 LF patients experienced hearing loss during the acute phase of infection (6) and four of those five patients died from LF. Although the sample size was small in this study, sensorineural hearing loss occurring during the acute phase of disease was more often associated with a fatal outcome (6). Although a clear cause-and-effect relationship between LF and deafness is evident, the underlying pathology has not yet been identified.

Several nonhuman primate (NHP) species have been used to study the pathogenesis of LASV, including cynomolgus macaques, which develop severe and usually fatal disease with signs and pathology mirroring observations from fatal LF cases in humans (7). Typically, NHPs develop an acute disease approximately 10 days after infection, which is characterized by fever, malaise, loss of appetite, and reduced activity. Severe disease signs that require euthanasia often occur approximately 11-18 days after exposure. NHPs that survive this acute phase may go on to develop signs characteristic of neurologic damage, including tremors, ataxia, and seizures, which can be fatal in some NHPs. A recent publication describes the subjective determination of sensorineural hearing loss in a STAT1 knockout mouse model following Lassa virus exposure (8). Anecdotal evidence has suggested that NHPs, like humans, can become deaf after surviving LASV infection; however, to date neither an objective measurement of deafness in primates, nor the underlying cause of the deafness in macaques or in humans has been described.

Here we report for the first time the use of an auditory measurement of LASV-associated sensorineural hearing loss in cynomolgus macaques. Moreover, we describe pathological findings indicating that an autoimmune-associated systemic vasculitis disorder is likely to be the basis of LF-associated deafness.

Results and Discussion

LASV infection of cynomolgus macaques. To determine if macaques surviving LASV infection develop deafness, we experimentally infected four cynomolgus macaques and observed them for 45 days. A daily score was assigned to each macaque corresponding to the severity of disease signs observed. A score of 0 indicated that the macaque was well and showed no disease signs and a score of 10 indicated that the macaque was severely ill and required euthanasia. All four macaques developed signs of LASV disease by 6 days post-exposure (dpe), and two macaques escalated to meet established euthanasia criteria by 13 and 17 dpe, respectively (Figure 1A). The morbidity scores of these macaques reflect the time course of disease, which became severe approximately 9 dpe in all four, increasing steadily in severity for the two that succumbed, but stabilizing in the two survivivors approximately 16 dpe (Fig. 1B). The two macaques that survived the acute phase of disease improved slightly approximately by 28 dpe, but continued to demonstrate signs of disease including hunched posture, reduced appetite, tremors, and ataxia that persisted throughout the end of the study (Fig. 1B). Blood samples collected on study days 0, 3, 6, 10, 14, 21, 28 and 45 post-exposure were tested for infectious LASV by standard plaque assay and for neutralizing antibody responses by plaque reduction neutralization test (PRNT). All four macaques had detectable serum viremias by 6 dpe. One of the two macaques who succumbed had a very high viral titer at the terminal blood collection (up to approximately 1×10^7 pfu/ml). Serum viremia levels peaked in the survivors at 14 dpe, followed by clearance of detectable virus in serum by 28 dpe(Fig. 1C). Viral clearance correlated with a rise in neutralizing antibodies in both survivors (Fig. 1C).

Pathological findings in NHPs consistent with autoimmune-associated vasculitis in humans. Necropsy of the two macaques euthanized during the acute phase of disease, as well as the two survivors, revealed gross pathologic findings consistent with earlier reports of LASV infection of macaques, which included changes in the lungs, liver, spleen, heart, pancreas, lymph nodes, kidneys and brain (9, 10). Additionally, the survivors displayed severe gross lesions similar to those reported for human autoimmune-associated vasculitis, specifically polyarteritis nodosa (PAN) and microscopic polyarteritis (MPA) patients (Fig. 2). The surviving macaques experienced marked thickening of blood vessels in numerous organ systems including the liver, pancreas and heart (Fig. 2A, B, D). The coronary vessels exhibited a segmental vascular dilation and stenosis having a “string of beads” or “rosary sign” appearance as previously described for vasculitis lesions that are often visualized in living subjects radiographically and are diagnostic for PAN and other vasculitis syndromes (Fig. 2B) (11) . The male survivor experienced severe testicular edema during the in-life portion of the study. At necropsy, gross examination of the testicles revealed evidence of interstitial hemorrhage (Fig. 2C), consistent with autopsy findings in male patients with autoimmune vasculitis (12, 13). This finding is complicated by viral staining of testicular tissue at the end of the study (Fig. 3I) when the blood was free of virus by plaque assay (Fig. 1C), and all other tissues were negative for antigen when examined by immunohistochemistry. Viral persistence in the semen for up to 3 months in human survivors following recovery from acute LASV infection is a known phenomenon (14, 15), so it was not surprising that the surviving male macaque harbored virus in testicular tissue at 45 days post-exposure. Pancreatic lesions are rare in autoimmune vasculitis, but have been described,

specifically, for PAN (16, 17). The male survivor had a white, fibrous and nodular pancreatic mass consistent with these descriptions (Fig. 2D). None of the gross lesions observed in the surviving macaques on this study are typically described for acute LASV disease in primates, thus we propose that they are associated with the development of an autoimmune-associated vasculitis following viral clearance from the blood.

In addition to the gross findings, we also observed vascular lesions histologically in most organ systems similar to histologic findings reported in human cases of autoimmune-associated vasculitis (Fig. 3A-I). Some autoimmune-associated vasculitides, including PAN, MPA, and cryoglobulinemia in humans, are characterized by necrotizing vasculitis in most organ systems more specifically described as segmental fibrinoid necrosis and profound infiltration of polymorphonuclear neutrophils and monocytes (18, 19). Histopathology performed on tissue samples from the LASV survivors in this study demonstrate similar types of lesions and infiltrates (Figs. 3A, D-H). Proliferative vasculitis was identified in the liver of both survivivors (Fig 3A-B). Examination of the lung from the survivors revealed interstitial fibrosis with type II pneumocyte hyperplasia but no evidence of proliferative vasculitis. Pneumocyte hyperplasia is likely due to the primary LASV infection and unrelated to the post-exposure autoimmune syndrome experienced by this macaque. An inconsistency with the case definition for PAN in humans is the fact that we identified membranous glomerulonephritis characterized by expansion of the glomeruli with eosinophilic material, lymphoplasmacytic inflammation, degenerate neutrophils, tubular degeneration, necrosis and regeneration and eosinophilic proteinaceous casts present in both survivors. (Fig. 3D). The proliferative vasculitis identified in the kidneys is similar to that described in the liver and pancreas, with no corresponding immunoreactivity to LASV. It is unknown if this glomerulonephritis occurred due to the initial exposure to LASV and was resolving, or if it was an active process mediated by the autoimmune host response.

Rapid onset sensorineural hearing loss is a well-described complication of PAN and other autoimmune-associated vasculitides and is believed to be the result of inflammation and occlusion of the anterior inferior cerebellar artery (AICA) and downstream vessels resulting in cochlear hypoxia (20-26). Consistent with pathologic changes seen in humans diagnosed with PAN, tissue samples of the inner ear adjacent to the cochlear nerve of the two survivors display moderate sub-acute to chronic-active perivascular inflammation which multifocally surrounded smaller branches of the cochlear nerve (Fig. 4B-C) (23).

Auditory Response Measurements in NHPs Surviving LASV Infection. By 28 dpe both survivors appeared to develop hearing loss based on subjective measurements of sound response and tuning fork tests. Screening for sensorineural hearing loss was conducted 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 such as elephant seals (personal communication with UFI, Inc. personnel) and alpaca, measures the neural response to clicking sounds produced at varying decibel (dB) levels (27). The BAERCOM device clearly indicated that one of the two survivors had an absence of waveforms for both the left and right ears at 75 dB, confirming bilateral hearing loss at 75 dB (Fig. 4A). We were unable to reliably determine an auditory response below 75 dB in control animals, thus we predetermined that 75 dB would be our experimental cutoff value for

determination of deafness in cynomolgus macaques. Though we believe both survivors experienced hearing loss by 45 dpe based on subjective measurements of sound response, the male survivor showed a waveform on the BAERCOM device that signified an auditory response at 75 dB.

Serological Profiles of NHPs that survived LASV exposure. There are no definitive tests to diagnose systemic vasculitides; 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 on 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- or large-sized vessels containing polymorphonuclear cells (28). In addition to these criteria, circulating immune complexes (CICs), elevated C-reactive protein, elevated proinflammatory cytokines, or sudden-onset, unexplained sensorineural hearing loss support a clinical diagnosis of systemic vasculitides. Further refinements of the clinical diagnosis criteria for vasculitides were established at the second International Chapel Hill Consensus Conference on the nomenclature of Systemic Vasculitides in 2012 (29, 30). As a result of this conference, the presence of antineutrophil cytoplasmic antibodies (ANCA) distinguishes PAN from microscopic polyarteritis (MPA).

To obtain confirmatory serological evidence consistent with an aberrant autoimmune response in the survivors, we tested blood samples for immune markers indicative of a systemic autoimmune-associated vasculitis diagnosis. Sample volume limitations and the absence of samples beyond the acute phase prevented us from performing all tests on samples from the two macaques euthanized due to severe LASV disease. We found that both survivors had elevated levels of C-reactive protein in the blood starting at 14 dpe and peaking at 28 dpe despite the absence of measureable virus in the blood at that time (Fig. 5A). After 28 dpe, C-reactive protein levels decreased slightly, but remaining elevated through the end of the study. Neither macaque that succumbed during the acute phase developed measureable circulating immune complexes (CICs) (Fig. 5B); however, the two survivors developed highly elevated CICs by 21 dpe, which persisted to the study endpoint. According to the Chapel Hill Consensus Conference, immune complex presence in tissues and in serum suggests a diagnosis of cryoglobulinemic vasculitis (CV), distinguishing CV from both PAN and MPA. However, patients with CV have vasculitis in small vessels only; whereas, the macaques in this study had vasculitis lesions in small and medium sized vessels. As such, the presence of high levels of CIC observed in these macaques defies the established human categorization. It should be noted; however, that it is unknown if human LASV survivors also have such overlapping PAN and MPA markers.

Both survivors also developed ANCA by 21 dpe, with one demonstrating highly elevated levels on 28 and 45 dpe (Fig. 5C). It is difficult to differentiate between the known vasculitides due to overlapping findings, but based upon the 2012 Chapel Hill Consensus Conference, the presence of ANCA in both survivors, albeit at differing levels above baseline, and the presence of necrotizing glomerulonephritis suggests that the syndrome is similar to MPA, despite the absence of vasculitis in the lungs (Fig. 3D). Cytokines, chemokines and vascular growth factors

that have either been elevated in patients with PAN, MPA and other similar vasculitides, or have been identified as potential therapeutic targets for autoimmune vasculitis were measured and present at elevated levels in the chronically ill macaques. Proinflammatory mediators IL-1b and TNF-α (Fig. 6) became elevated in the surviving NHPs by 3 dpe. IL-1b became highly elevated in each of the survivors at different times after 14 dpe, and remained mildly elevated in in both survivors at 45dpe; whereas, TNF-α decreased slightly after peaking at 21 dpe in one macaque, then became differentially elevated in the two survivors at the study endpoint. Interleukin 6 (IL-6), a pro-inflammatory cytokine implicated as a factor in autoimmune disease, became highly elevated from baseline levels in the survivors as early as 3 dpe in the male, and remained consistently above baseline until end of study. IL-6 in the female survivor became highly elevated by 14 dpe, peaking at 28 dpe and returning to slightly above baseline by the end of study. Cytokines, IL-1b, TNF-α and IL-6, have all been discussed as potential therapeutic targets for PAN, MPA and other autoimmune vasculitides including granulomatosis with polyangiitis (GPA), formerly known as Wegener's Granulomatosis and eosinophilic granulomatosis with polyangiitis (EGPA), formerly known as Churg-Strauss syndrome (31-36). Other cytokines and chemokines reported to be involved in systemic autoimmune responses include IL-15, IL-5 and sCD40L. The survivors experienced very high elevations in IL-15 starting as early as 3 dpe, and continuing through 14 and 21 dpe, returning to levels similar to baseline by the end of study (Fig. 6). The normal function of IL-15 is to promote T cell proliferation, but also to prevent death of these cells by apoptosis. A potential role for dysregulated expression of IL-15 in autoimmunity is to promote the proliferation and survival of self-reactive lymphocytes (37-40), and IL-15 has been considered as a potential therapeutic target for autoimmune diseases such as rheumatoid arthritis and GPA (36, 41, 42). Interestingly, elevated levels of IL-5, a cytokine that functions to stimulate release of eosinophils into the bloodstream, as well as eosinophilia, were identified in the two survivors at 21 and 28 dpe, after viral clearance (Fig 6 and Supplementary Figure 1). Eosinophils were also noted histologically as a component of the vasculitis in tissues of survivors collected at necropsy (Fig. 3). Another marker of autoimmunity elevated in the survivors was sCD40L, cytokine and costimulatory molecule with a multitude of important functions in the natural response to infection. Expression of sCD40L is an early marker of T cell activation, and pairing with CD40 cell surface markers on B cells, macrophages and dendritic cells leads to an enhancement of antigen-specific immune responses. One of the ways in which this task is accomplished is through CD40 and sCD40L complex-mediated bridging of activated T cells and B cells to promote antigen-specific B cell differentiation, isotype switching from IgM to IgG, and memory B cell formation (43, 44). A proposed role for dysregulated and prolonged expression of sCD40L in autoimmunity is activation of self-reactive lymphocytes and prevention of their deletion, which leads to proliferation of autoantibodies (45). We were able to measure highly elevated levels of sCD40L in serum of the female survivor as early as 3 dpe. The male survivor developed elevated levels by 6 dpe and peaked at 21 dpe. Levels of sCD40L remained above baseline for both survivors throughout the end of the study.

Conclusions

The surviving macaques in our study displayed seven of the 10 ACR criteria for diagnosis of autoimmune-associated systemic vasculitis in humans. Additionally, they had elevation of all four of the serological markers which are hallmarks of PAN and many characteristics common to MPA and other autoimmune-associated vasculitides such as the presence of immune complexes in serum, measureable deafness, and histologic lesions commonly observed in systemic vasculitis patients. Our results do not specifically describe or indicate a specific subcategory of systemic vasculitis; however, these results strongly suggest that an ANCA-positive autoimmune-associated vasculitis-like syndrome serves as the underlying cause of rapid-onset sensorineural hearing loss in cases of Lassa fever.

Materials and Methods

Primate Exposure. Four adult cynomolgus macaques were infected with 1000 plaque forming units of LASV, Josiah strain (9) in sterile physiological saline via a single injection of 1 ml of the virus-containing solution into the quadriceps muscle group. Macaques were monitored daily for disease progression and were euthanized when moribund according to IACUC-approved euthanasia criteria.

Blood Sample Collection and Analyses for Viremia, Chemistry and Hematology. Blood samples were collected before infection and on days 0, 3, 6, 10, 14, 28 and 45 post-exposure from anesthetized macaques. Experiments using samples collected from LASV-infected animals were performed in biosafety level-4 conditions. Viremia was measured by standard plaque assay, as previously described (46, 47). Briefly, Vero cells, seeded in 6-well cell culture plates were incubated with 10-fold serial dilutions of each serum for 1 h at 37°C, 5% CO2, after which 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. Cells were next incubated at 37°C, 5% CO2 for 4 days, and a solution of neutral red (custom blended for USAMRIID by Invitrogen, Carlsbad, CA) was added. After an overnight incubation at 37°C in the stain, plaques were counted and recorded.

Chemistry and hematology assessments included measurements of blood glucose, 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 each serum sample was applied to a General Chemistry 13-panel rotor and evaluated in a Piccolo point-of-care blood chemistry analyzer (Abaxis). For the CBC analysis, 75 µl of whole EDTA-treated blood was aliquoted and run on a Hemavet Instrument (Drew Scientific). Results for CBC and chemistry analyses are included as supplementary figures.

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 to obtain a baseline measurement, and 75 dB for each ear. The BAERCOM software produced graphs for each reading.

Measurement of Immune Markers.
A Raji Cell Immune Complex ELISA (MicroVue CIC-Raji Cell Replacement EIA, Quidel Corp.), was used to measure C3d-bound CIC present in plasma or sera. 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. Cytokine and chemokine assays were performed on plasma samples 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.

Pathologic Analysis of Tissues. Necropsies were performed on each macaque immediately following euthanasia in the USAMRIID biosafety level 4 laboratory. Tissues from major organ systems were collected, immersion fixed in 10% neutral buffered formalin and held in biocontainment for a minimum of 21 days. Histopathology samples were routinely processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Immunohistochemistry was performed on replicate tissue sections for both partial and full necropsies using an Envision kit (Dako). A monoclonal antibody specific for Lassa virus GP1 (46) 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. Tissue slides were covered with primary antibody, incubated at room temperature for 30 minutes and rinsed. The secondary antibody, a peroxidase-labeled polymer, was applied for 30 minutes and the slides rinsed again. Substrate-chromogen solution (DAB, Dako) was applied for five minutes, the slides were rinsed in distilled water, counterstained with hematoxylin for two minutes, dehydrated, cleared with xyless and then coverslipped. Slides were examined with a Nikon Eclipse 600 light microscope.

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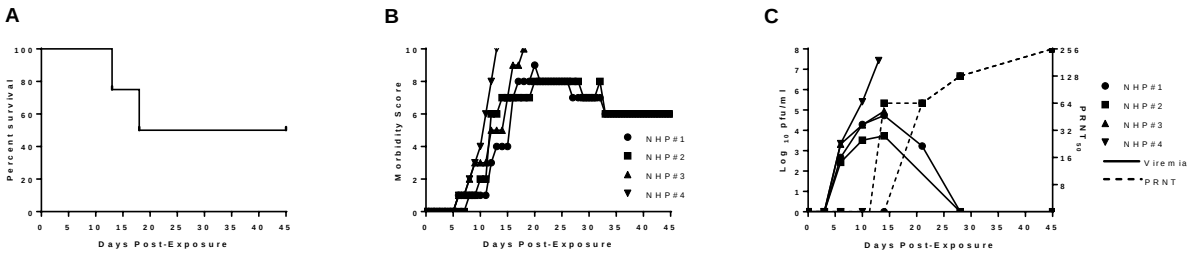


Fig. 1. Cynomolgus macaque study outcomes. *(A)* NHPs were infected by IM injection of 1000 pfu of LASV at day 0 and were observed daily for 45 days. All four NHPs exhibited signs of disease approximately 6 days post-infection with two NHPs reaching the euthanasia criteria on days 13 or 17. *(B)* Morbidity scores were assigned to the NHPs each day with 0 indicating no observable disease signs and 10 indicating severe disease signs requiring euthanasia. The two NHPs that survived to the study endpoint experienced severe disease signs, which did not resolve but did not require euthanasia. Signs of disease included loss of appetite, reduced activity, hunched posture, labored breathing, and neurological deficits including trembling, muscle weakness, and ataxia. *(C)* Serum viremia was determined by plaque assay is expressed Log_{10} pfu/ml. Development of neutralizing antibodies was measured by plaque reduction neutralization test (PRNT). Titers are expressed as the reciprocal serum dilution required to reduce the number of plaques by 50% ($PRNT_{50}$).

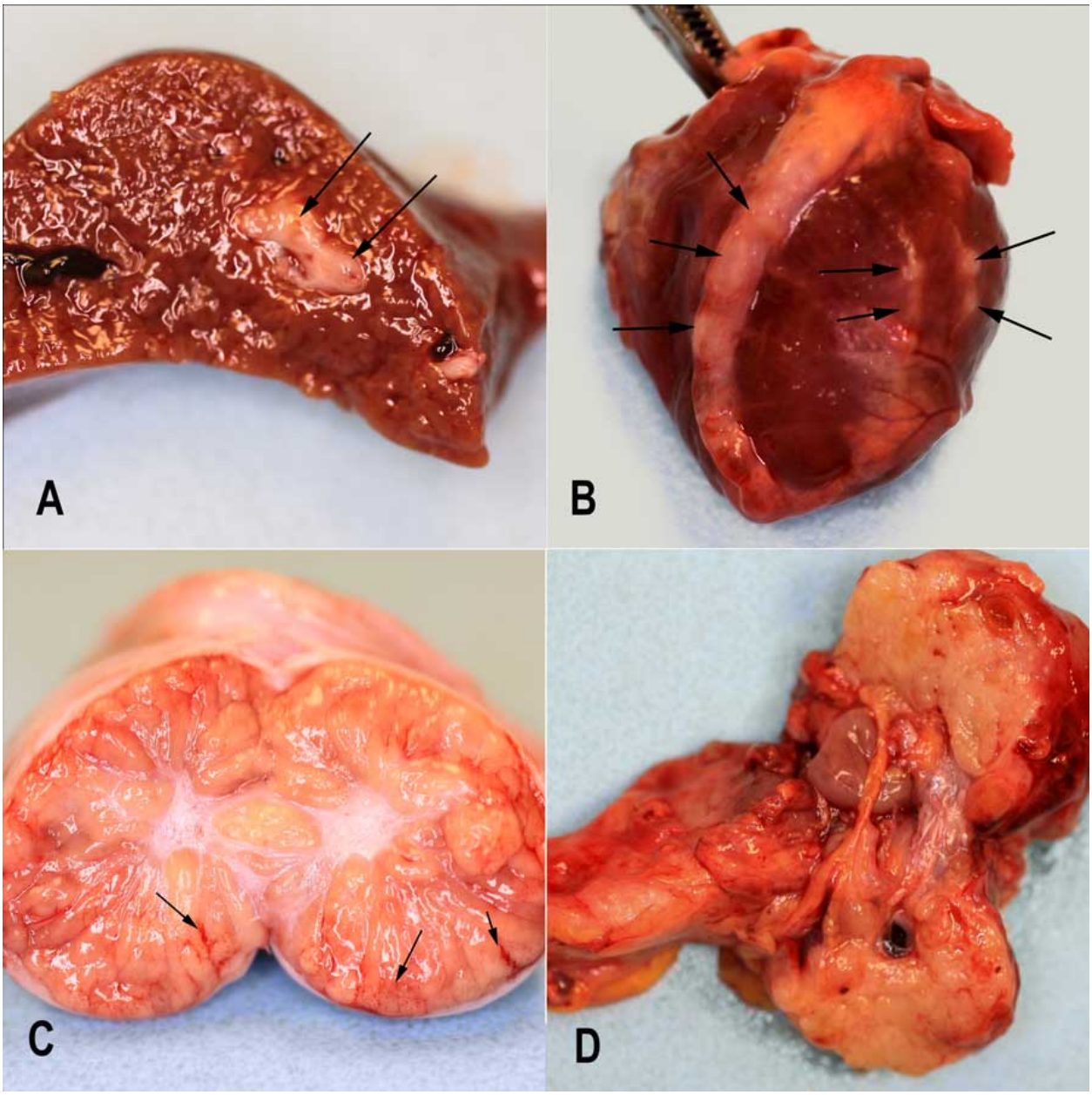


Fig. 2. Gross pathology findings from the two macaques surviving LASVinfection. *(A)* Marked thickening of hepatic vessels in the female survivor. *(B)* The coronary vessels exhibiting similar incremental vascular dilation and stenosis having the "string of beads" or "rosary sign" appearance described for polyarteritis/vasculitis lesions (11). *(C)* Multiple foci of interstitial testicular hemorrhage in the male survivor. *(D)* A white, fibrous and nodular pancreatic mass in the male survivor, consistent with previous descriptions in PAN patients (16, 17).

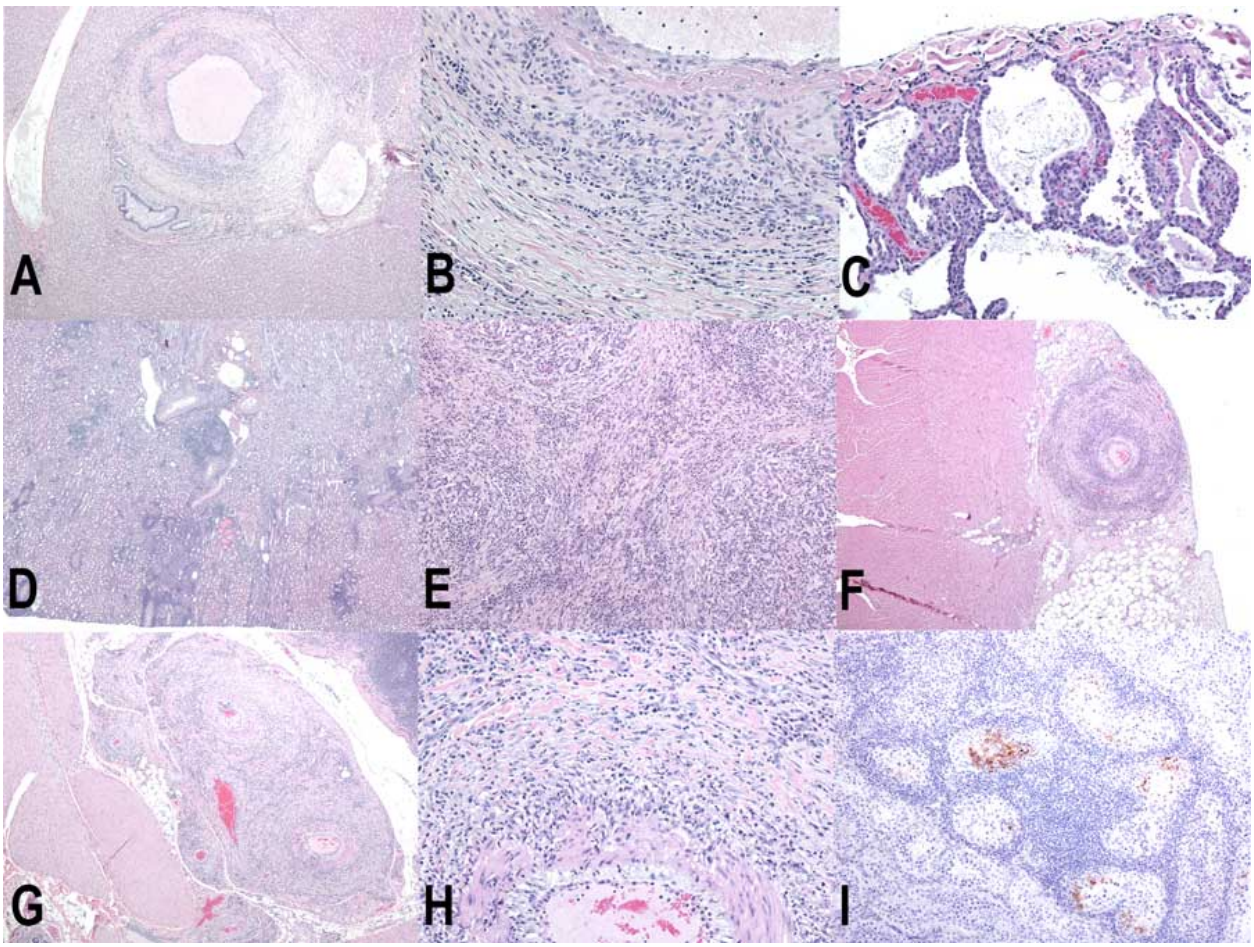


Fig. 3. Histopathologic findings in NHPs that survived to the end of study, 45 days post-exposure. *(A)* Necrotizing and proliferative vasculitis, liver, 45-day surviving NHP, 4x; *(B)* 20X view of the liver section in panel A; *(C)* Interstitial fibrosis with type II pneumocyte hyperplasia in the lung tissue of a surviving NHP 45 days post-exposure; *(D)* Membranous glomerulonephritis and vasculitis, kidney, 45-day survivor, 20x. *(E)* Profound chronic-active pancreatitis with fibrosis and duct hyperplasia, 45-day survivor, 10x. *(F)* Necrotizing and proliferative arteritis with narrowed lumen in the coronary artery, 45-day survivor, 10x. *(G)* Necrotizing and proliferative arteritis with narrowing of the lumen in the mesenteric artery, 45-day survivor, 4x. *(H)* Higher magnification of vascular changes noted in panel G, 20x. *(I)* Chronic-active orchitis, testicles, with cytoplasmic immunoreactivity in cells lining the seminiferous tubules in the male survivor, 45 days post-exposure, 10x.

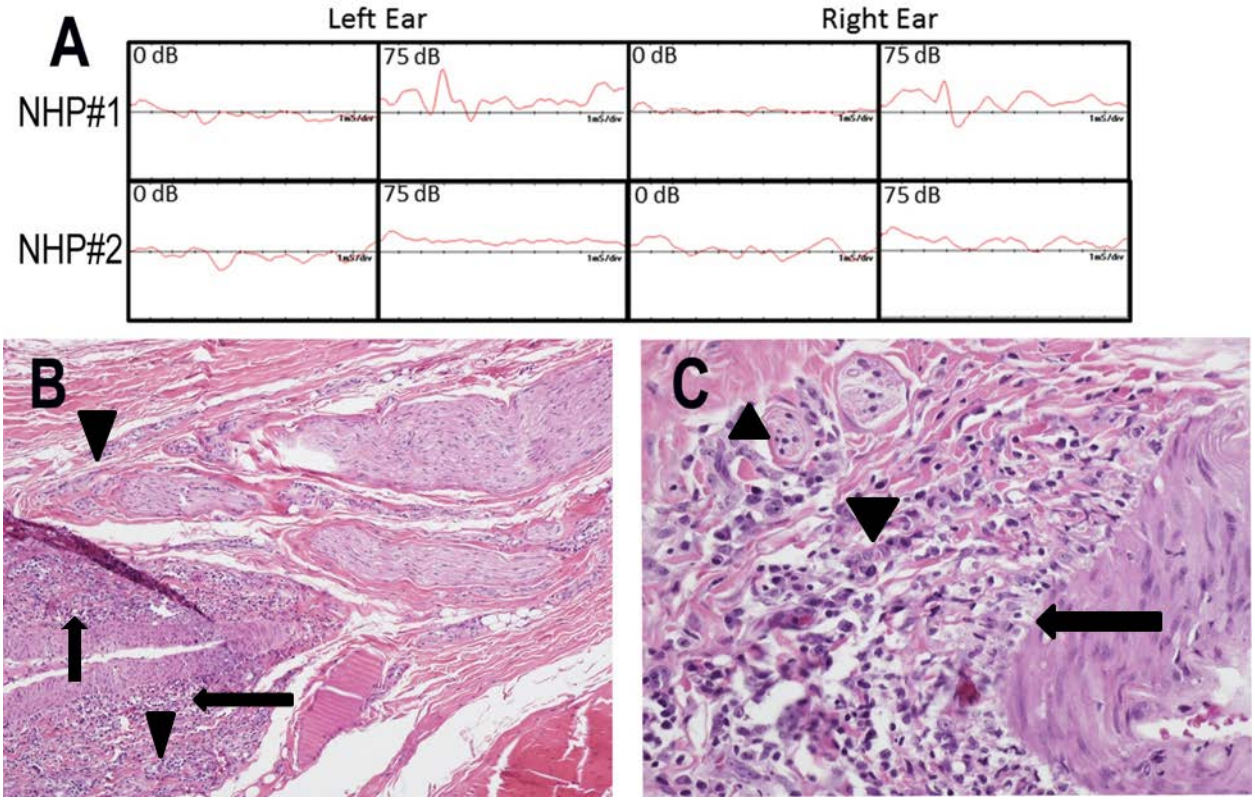


Fig. 4. Evidence for sensorineural hearing loss in a surviving NHP following LASV exposure. *(A)* A brainstem auditory evoked response device (BAERCOM) was used to assess the surviving NHPs for a hearing response on day 45 post-exposure. Clear waveforms indicating a hearing response was observed in both ears of the male survivor (NHP#1) at 75 dB; whereas, the female survivor (NHP#2) has an absence of waveforms for both the left and right ears at 75 dB. Histologic examination of inner ear *(B-C)* reveals inflammation of vessels and perivascular tissue in the female NHP at day 45 post-exposure. Chronic-active inflammation surrounding a vessel (arrow) extending around adjacent nerves (arrowhead) at 40x *(B)*. Histologic findings at high power 400x *(C)*.

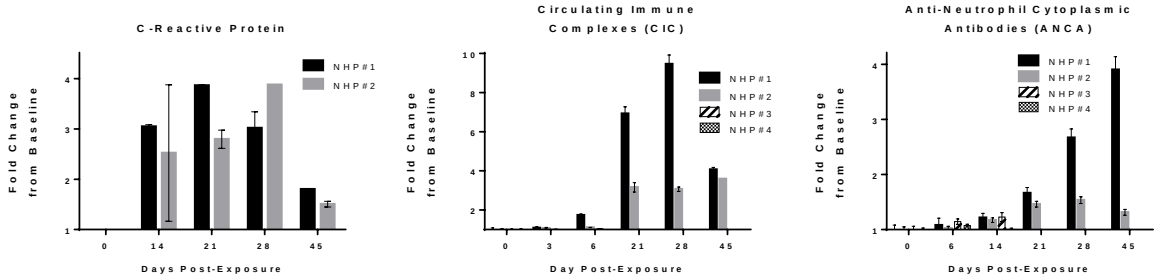


Fig. 5. Serological evidence for an autoimmune response in NHPs that survive LASV exposure**.** C-reactive protein *(A)* remains high throughout the course of disease for surviving NHPs despite being free of circulating virus in sera by day 28 post-exposure. *(B)* Surviving NHPs experienced a spike in CICs beginning on day 21 post-exposure and remained very high for the NHP that developed measureable deafness by BAERCOM testing, but decreased for the other surviving NHP. Both surviving NHPs had CIC levels significantly above baseline levels at the end of the study. *(C)* ANCA was present above background levels starting at day 21 post-exposure. One NHP experienced a large increase over baseline levels on days 28 and 45 post-exposure; whereas, the other had levels closer to, but still significantly above, baseline.

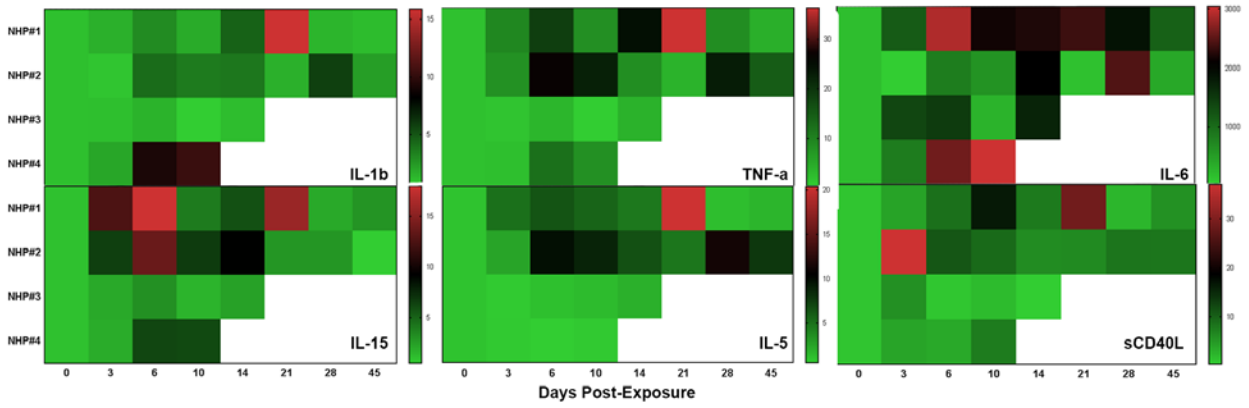
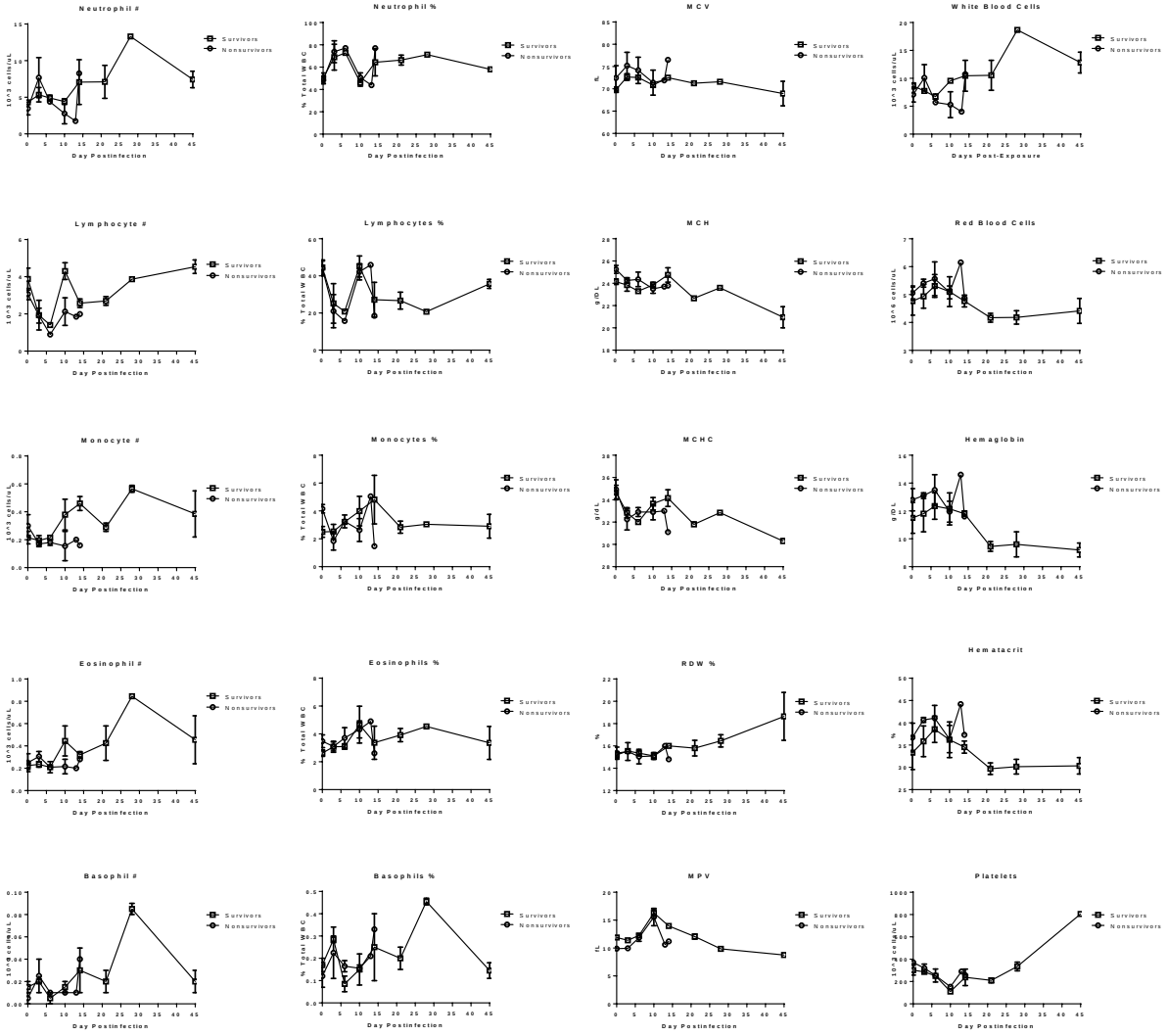


Fig 6. Heat map representation of cytokine and chemokine changes that support a systemic autoimmune response hypothesis. Proinflammatory cytokines IL-1b, TNF-a, IL-6 became elevated in acute phase of disease, but remained above baseline after viral clearance. Autoimmune mediators IL-15 and IL-5 became strongly elevated during the acute phase of disease in the NHPs that survived, but not the NHPs that succumbed. IL-15 returned to baseline and IL-5 differentially returned to baseline by the end of study, with the deaf NHP retaining highly elevated levels. sCD40L became highly elevated early post-exposure, and remained elevated from baseline throughout the study. All data is presented as fold change from baseline levels. The fold change scale for each cytokine and chemokine is included to the right of each panel.

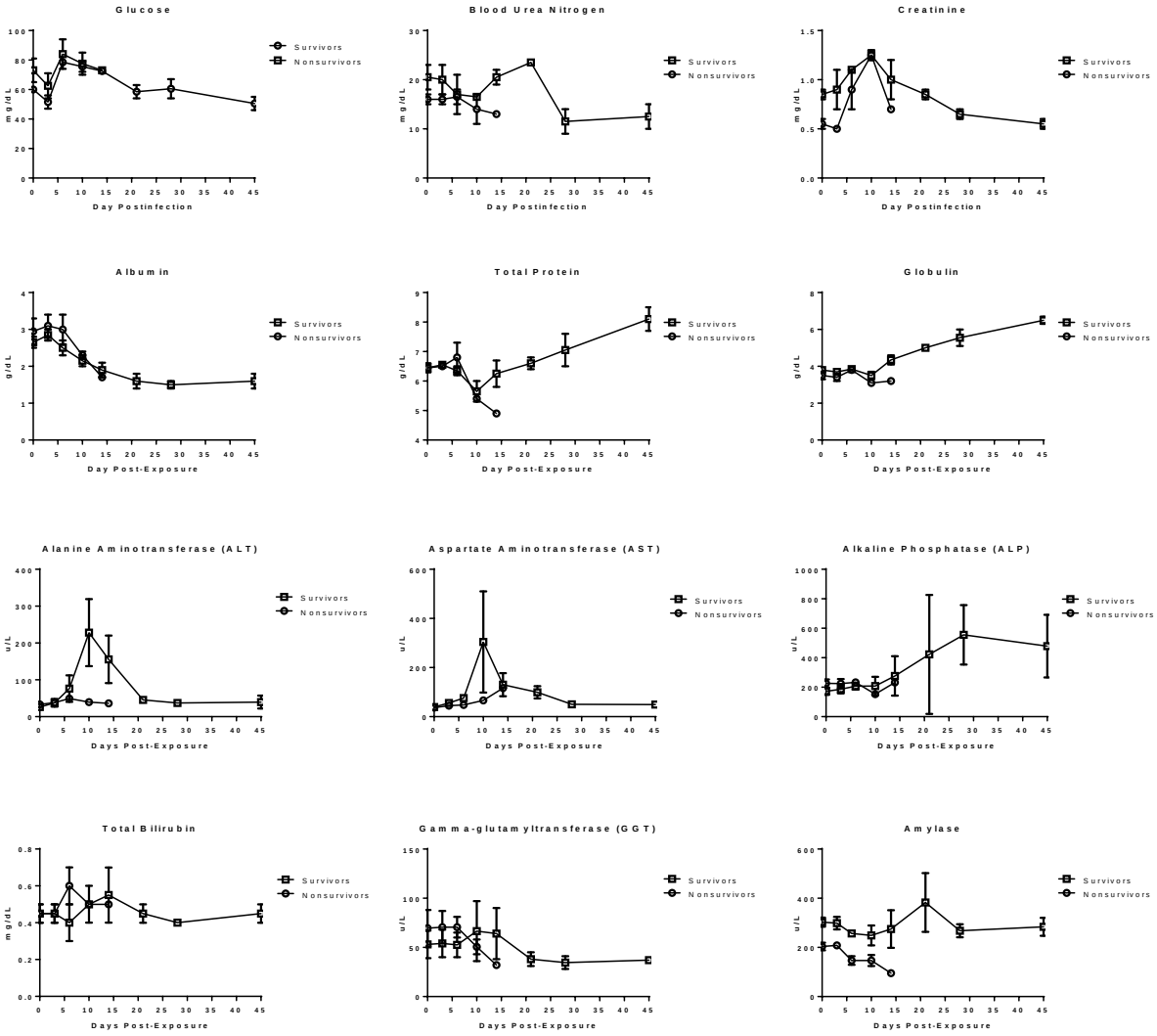
Table 1 – Summary of autoimmune-related Findings in LASV-exposed survivors.

		NHP#1 Survivor (male)	**NHP#2 Survivor (female)**
Tissue distribution of vasculitis lesions		All tissues except lungs	All tissues except lungs
Orchitis/epididymitis		Yes	N/A
Size of vessels affected		Small and medium sized vessels	Small and medium sized vessels
Glomerulonephritis		Yes	Yes
C-Reactive Protein elevated from baseline beyond day 14		Strongly elevated	Strongly elevated
TH-1 cytokines elevated from baseline beyond day 14	IL-2	Strongly elevated on day 21, mildly elevated after	Mildly elevated
	IFN-γ	Yes	No
	TNF-α	Strongly elevated on day 21, mildly elevated after	Strongly elevated on days 28 and 45
	IL-10	Strongly elevated on day 21, not elevated after	Mildly elevated on day 21, not elevated after
TH-2 cytokines elevated from baseline beyond day 14	IL-4	Strongly elevated on day 21, not elevated after	Not elevated
	IL-5	Strongly elevated on day 21, not elevated after	Strongly elevated on days 21, 28 and 45
	IL-10	Strongly elevated on day 21, not elevated after	Mildly elevated on day 21, not elevated after
	IL-13	Strongly elevated on day 21, lower but still strongly elevated on days 28 and 45	Not elevated
TH-17 cytokines elevated from baseline beyond day 14	IL-17A	Strongly elevated on day 21, mildly elevated after	Strongly elevated
	IL-10	Strongly elevated on day 21, not elevated after	Mildly elevated on day 21, not elevated after
B-cell activation cytokines elevated from baseline beyond day 14	IL-6	Strongly elevated on day 21, lower but still strongly elevated	Strongly elevated on day 28, returning to baseline by day 45
	IL-10	Strongly elevated on day 21, lower but still strongly elevated on days 28 and 45	Strongly elevated on day 28, lower but still strongly elevated on day 45
Chemotactic cytokines elevated	IL-8	Strongly elevated	Not elevated
	MCP-1	Strongly elevated	Strongly elevated at day 21,

from baseline beyond day 14			lower but still strongly elevated on days 28 and 45
	MIP-1α	Strongly elevated on day 21, mildly on day 29, not elevated on day 45	Not elevated on day 21, strongly elevated on days 28 and 45
	MIP-1β	Strongly elevated on day 21, not elevated on days 28 or 45	Not elevated
Immune Complexes in Serum		Yes	Yes
ANCA-Positive		Strongly positive from baseline	Weakly positive from baseline
Peripheral blood eosinophilia		Yes	Yes
Sensorineural hearing loss		No (at 75 dB)	Yes (at 75 dB)



Supplemental Figure 1 – Changes in complete blood count parameters in NHPs that survived LASV exposure compared against those from NHPs that succumbed to LASV disease.



Supplemental Figure 2 - Changes in blood chemistry parameters in NHPs that survived LASV exposure compared against those from NHPs that succumbed to LASV disease.