DYNAMICS OF ADAPTIVE IMMUNITY DURING LASSA VIRUS INFECTION
AND A PROPOSED MECHANISM FOR IMMUNE IMPAIRMENT

AN ABSTRACT
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OF
PHILOSOPHY BY

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ABSTRACT

Lassa virus (LASV) is a member of the Old World Complex of arenaviruses infecting an estimated 300,000-500,000 people each year primarily in the endemic region of West Africa. Lassa fever (LF), a neglected tropical disease, puts a heavy burden on the communities in which it is endemic. Reportedly, 15%-20% or up to 50% during epidemics succumb to LASV infection. Without U.S. Food and Drug Administration approved diagnostics, therapeutics, or vaccines, knowledge of this disease must advance in order to create affordable and reliable care. Unlike other viral infections, LF does not stimulate a robust humoral immune response. Many in vitro studies have demonstrated the ability of LASV nucleoprotein (NP) and Z protein to interrupt innate immune activation of important cytokines and costimulatory molecules, which can impair the adaptive immune response. To this end, we investigated the antibody and cytokine responses in LF confirmed patients. We then investigated the role NP plays in subverting the innate immune response, suggesting a possible mechanism for humoral immune impairment. Our findings demonstrate a prolonged IgM response and a dysregulated cytokine response in vivo. Additionally, we show LASV NP is capable of modulating the immune system by masking pathogen pattern signals, namely dsRNA, that initiate immune cascades via the Toll Like Receptor 3 pathway.
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INTRODUCTION

A. Overview

Lassa fever (LF) is an often fatal disease that affects an estimated 300,000-500,000 people each year in endemic areas of Sierra Leone, Guinea, Liberia, Nigeria, and other West African countries – some of the most impoverished nations [1-3]. According to the United Nations Human Development Reports, Sierra Leone ranks in the top ten countries for poverty and illiteracy rates [4]. Past studies have identified fatality rates of 5-20% in suspected Lassa fever patients [5]. Recent studies in Sierra Leone showed fatalities rates of greater than 60% in acutely ill patients presenting to a health care facility while viremic [6, 7]. Pleural and pericardial effusions, hemorrhaging, and spontaneous abortion are common acute complications, while bilateral deafness is reportedly a common, irreversible sequela of LF in convalescent patients [8-12]. In resource-poor settings, prevention is key, though very difficult due to the reservoir being a ubiquitous rat that is found in the fields of crops and in homes. The antiviral therapeutic ribavirin is available off-label for the treatment of LF and is considered somewhat effective if administered early in infection, though it is extremely toxic and not approved by the FDA or any other regulatory agency for treatment of LASV infections [13]. Fear of bioterrorism and weaponization resulting in nefarious release of the virus has resulted in significant U.S. government funding for research on LASV diagnostics, prevention, and therapeutics [14].
Research points to the T cell response as the dominant defense mechanism in overcoming LASV infection in non-human primates and in vitro models [15-17], whereas the humoral immune response does not appear to be effective in clearing the virus [5, 18-21]. Many investigations attribute this immune disruption to LASV protein influence on important immune modulators expressed within primary target immune cells such as dendritic cells and macrophages, which thereby interfere with the conduit between innate and adaptive immune mechanisms [22-28]. The study herein investigates the innate and adaptive immune responses in vivo and proposes a specific target in LASV immune disruption in vitro.

B. Lassa Fever

Lassa fever was first reported in Nigeria in 1969 after three missionary-nurses contracted the illness [29]. After the deaths of the first two nurses, a third nurse was evacuated from Nigeria and recovered in the United States where the etiological agent Lassa virus (LASV) was identified [30-32]. LASV is a member of the family Arenaviridae. Though it was discovered in man in 1969, LASV likely diverged from other members of the family Arenaviridae over 1000 years ago [33, 34]. Currently, LASV is comprised of four major lineages. Lineages I, II, and III circulate in Nigeria, while a newer lineage IV is responsible for disease in Sierra Leone and surrounding countries [35].

LASV belongs to the Old World arenavirus subfamily, grouped with the well-studied lymphocytic choriomeningitis virus (LCMV). The counterpart subfamily is comprised of the New World arenaviruses. Because the viruses of the New and Old World complexes share common features – they have highly conserved genomic
structures and gene sequence similarity and function – studies with one Arenavirus allow general predictions to be made about characteristics of related counterparts [36].

1. Transmission

a) Rodent-to-Human

LASV transmission can occur via many different modes. Culturally in endemic areas, LASV transmission is believed to occur by catching and consuming the persistently infected zoonotic host, multimammate mice [37, 38]. Multimammate mice (*Mastomys natalensis*) shed high titers of virus in their excreta and often urinate when frightened – often in the hands of hunters [37]. Due to poor hygiene practices in endemic areas, this occurrence accounts for one mode of rodent-to-human transmission. Secondarily, after the rodent is caught, the meat is roasted and usually to a temperature that does not kill all virus particles. Though a campaign against the practice of catching and eating rodents was initiated, they are a primary source of protein and in many areas are considered a delicacy [37]. Fortunately, this practice is in decline and is not the primary mode of transmission.

*M. natalensis* is known to be peridomestic and are often found scavenging inside homes [5]. Due to inadequate storage of food and water, rodents can contaminate these supplies by shedding virus in urine and feces [37]. Contaminated surfaces, including utensils, mattresses, eating surfaces, and floors, can infect individuals with cuts and scrapes [39]. Alternatively, studies have shown the potential for aerosolization of the virus from rodent excreta [40]. Regardless of the route, rodent-to-human transmission is a serious concern in LASV endemic areas.
b) Human-to-Human

Human-to-human transmission of LASV have caused several outbreaks in endemic areas, and namely in hospital settings. The virus is found in bodily secretions including blood and urine. Close contact with these fluids allows the virus to transmit easily from person to person, especially via mucosal tissues [39]. Anecdotally, a nurse became ill with LF after bodily fluid landed in her eye while delivering a baby from a woman infected with LASV. Therefore undertrained practitioners facilitate in the transmission either by becoming infected themselves due to a lack of barrier nursing practices or by transmitting the virus from one patient to another due to poor hygienic practices. Moreover, needles and other medical instruments are common sources of transmission. Accidental needle sticks or reusing needles or instruments contaminated with LASV inevitably introduce the virus into a new host.

Other instances of human-to-human transmissions occur outside the hospital – while caring for LASV infected individuals, having intercourse with infected individuals, or interacting with the body of individuals who have succumbed to LF, which is a common practice during funerals in endemic areas [39]. Even interaction with convalescent patients may be a risk as the virus may persist in the urine at low levels for weeks after it is no longer detected in the blood [41]. While management of human-to-human transmission seems more transparent compared to rodent-to-human transmission, high numbers of nosocomial infections persist and communities are still undereducated on the need for hygiene and isolation while caring for or interacting with LASV infected individuals.
2. **Prevention, diagnosis, and treatment of Lassa fever**

Currently, there are no approved vaccines, or therapeutics, and only one commercially available diagnostic for LASV infection [42]. The antiviral therapeutic ribavirin is available outside of the US as an off-label treatment for LF and increases survival if administered early in infection [5, 7, 43-45]. Ribavirin has not been approved by the United States Food and Drug Administration (FDA) for the treatment of LF as it has recognized side effects if improperly administered and can be toxic, particularly to fetuses. Therefore, preventing LASV transmission is the primary goal in endemic regions. The National Institutes of Allergy and Infectious Diseases (NIAID) classifies LASV as a Category A pathogen and a BSL-4 agent. Fear of nefarious release of LASV as a bioterrorism agent has resulted in significant interest in development of new and effective LASV diagnostics, vaccines, and therapeutics.

**a) Prevention**

LASV infection has a high prevalence in communities in West Africa according to serosurveys based on LASV-specific IgG antibodies. Reports from the latter half of the twentieth century identified that 7%-20% of people in endemic areas have been exposed to the virus [1, 46, 47]. However, recent studies of apparently health blood donors from districts of Sierra Leone previously thought to be non-endemic for LF showed even higher incidences of exposure (unpublished observations). These observations may be indicative of the ease with which the virus is transmitted from reservoir-to-human and human-to-human.

The natural reservoir for LASV is *Mastomys natalensis*, which is referred to as the multimammate mouse or rat [48]. This ubiquitous Sub-Saharan rodent is found
throughout LASV endemic regions and lives commonly near human dwellings and crop fields [1, 48, 49]. A study that examined the seroconversion of members of two mining communities and two agricultural communities showed that those of the mining communities had more exposure to LASV [1]. The report suggested that dwellings of the mining communities were constructed poorly, food was not stored safely, and homes in the communities were generally more “disheveled” than those of the agricultural communities [1]. The investigators surmised that rodent access to dwellings was an important part of the transmission chain. Though, the investigators were not able to show a correlation between the percentage of human seroconversion and the level of LASV-infected Mastomys in the communities. It is possible that human-to-human transmission contributed and continues to contribute to LASV seroprevalence [1].

Due to the lack of rodent-proof construction of most dwellings, and inadequate food and water storage, Mastomys are often found scavenging inside of West African homes. Mastomys, which shed LASV in their excreta, can contaminate surfaces in homes as well as food and water supplies (Figure 1) [1, 48, 50]. The excreta potentially infect humans either via oral or respiratory routes or through micro abrasions in the skin [40, 49]. Consumption of Mastomys as a food source has also been implicated in LASV infection [37]. Outreach groups in endemic regions attempt to combat these forms of transmission by educating communities on how to prevent rodents from entering homes, how to safely store food and water, and the importance of cooking meat to the proper temperature [51]. Moreover, efforts undertaken to create and implement rodent traps made from strictly local supplies may begin to curb Lassa fever transmission chains.
Figure 1. Lassa fever rodent vector

*Mastomys natalensis* (natal mastomys, or multimammate “rat”) is the main reservoir of Lassa virus, and are peridomestic and highly prolific breeders. As the name indicates female *Mastomys* have multiple mammary ducts (A) permitting the sustenance of large litters of offspring in each breeding cycle. Grain harvested from fields is processed, dried, and often stored in open spaces inside dwellings (B). Food stored in case homes inside containers often shows evidence of rodent entry (C). Dwellings are usually built with readily available local lumber or handmade bricks and are not rodent proof. Rats can burrow through weaknesses in dwellings, often through outside walls (D) or under doors that offer substandard barrier against entry by small animals (E). Lassa fever is acquired through contact with excreta or preparation of “rat” for food.

Nosocomial infections have caused outbreaks in Sierra Leone and Nigeria, and are another mode of preventable transmission [52-56]. The reuse of syringes has been the source of outbreaks in past settings [53, 54, 57]. An effort to educate healthcare providers as to the dangers of reusing needles – dangers to both themselves and to patients – has presumably slowed this route of transmission in hospital settings. Direct contact with blood or bodily secretions may also account for a large portion of nosocomial infections [53-55]. Proper barrier practices while treating LF patients is key to preventing transmission to healthcare workers. Recommendations of disposable coveralls, filter masks, and face shields provide adequate protection from both “aerosolization” of LASV droplets and fluid splatters [53, 57, 58]. Moreover, patient isolation and the use of an anteroom are important for preventing viral transmission to health staff and patients or visitors [57, 58]. Another factor that can limit transmission in healthcare settings is corpse decontamination. As observed for other VHFs, such as Ebola, ritual burials that involve the touching and “cleansing” of the corpse can contribute greatly to transmission. The handling of a corpse without proper protection can lead to transmission from infected body fluids. While practically more difficult in resource-limited settings, nosocomial transmission of LASV is preventable.

b) Diagnosis

Early detection of a disease is key to treating it and preventing its spread. The most sensitive diagnostic procedure for a virus infection is viral isolation in cell culture. In LF endemic regions in West Africa, high containment (BSL-4) laboratories with cell culture capabilities are exceedingly rare [39, 47, 59]. Therefore, the “gold standard” diagnostic procedure for LASV infection is viral RNA isolation from blood followed by
RT-PCR (reverse transcription PCR) using oligonucleotide primers to highly conserved regions of the GPC gene [2, 60, 61]. Endemic areas are often not equipped to perform PCR. As a result, doctors without this technology are left to diagnose patients based on clinical symptoms alone [8]. Often patients present with symptoms that are similar to other common infections in LASV endemic areas such as malaria, typhoid, or influenza. Only during advanced stages of LASV infection do patients experience the telltale symptoms of LF – mucosal bleeding, conjunctivitis, encephalitis, and seizures. These symptoms, though, are indicative of a poor prognosis when treatment with ribavirin is usually no longer beneficial.

In the early 1980s, immunofluorescent antibody assays (IFA) for screening patient samples for the presence of viral antigens were implemented [59, 62, 63]. IFA made it possible for laboratory diagnosis of LF, which provided important advantages over clinical diagnosis alone. However, similar to viral isolation, this method was not readily implementable in field settings and fell out of favor when PCR and ELISA were developed for the detection of LASV [2, 60]. Utilizing RT-PCR for the diagnosis of LF in endemic areas proved to be challenging because of a lack of a consistent power supply, easy contamination of samples, and personnel technical expertise shortcomings. More than these environment- and resource-dependent limitations, a failure to amplify divergent strains of LASV with a single primer set has been recorded, even from a geographically close sample group [64, 65]. Nevertheless, several laboratories in endemic areas successfully make use of RT-PCR as a diagnostic, including the Irrua Specialist Teaching Hospital in Nigeria and Kenema Government Hospital (KGH) Lassa Fever Laboratory (LFL) in Sierra Leone [66, 67]. Because LASV remains a threat to first world
countries by exportation and weaponization, microarray assays targeting LASV in addition to numerous other pathogens and toxins are under development [68].

The ELISA platform, while still requiring laboratory equipment, is much less prone to contamination, can better accommodate inconsistent power supply, and results in higher sensitivity and less cross-reactivity and subjectivity than IFA [39, 59]. Moreover, ELISA may be able to detect a broader range of viral strains because the assay depends on antibody-antigen interaction rather than the highly specific interactions of PCR primers with the viral genome [65]. The broader detection range is sustainable when employing polyclonal antibodies for both capture and detection of antigens, even if strain variants emerge as a result of point mutations or more significant antigenic shifts. The first ELISA for the detection of LASV antigen and LASV-specific antibody was developed in the mid-1980s [8, 69], and for the following few decades ELISA were used in some endemic areas. The definition of a positive LASV diagnosis was broad, though – a fourfold rise in IgG antibody titer, or LASV-specific IgM and/or IgG accompanying LF clinical symptoms [39] – given that the dynamics of the host humoral immune response remained relatively obscure. Since then, ELISA diagnostics have been optimized for sensitivity and strain-specificity, and have provided more insight into the host immune response. Recent studies employing the use of modern, recombinant protein based diagnostics have helped redefine the emergence and diagnostic role of immunoglobulins following infection with LASV [7].

In Sierra Leone, the KGH LFL diagnoses LF cases for the county. The laboratory employs three different diagnostic ELISA for the detection and monitoring of LASV [6, 7, 39, 59, 67, 70]. To determine if an individual is experiencing an acute infection, LASV
antigen-capture ELISA, which identifies the NP antigen, is performed for the detection of circulating antigen in biologic samples. LASV IgM and IgG antibody capture ELISA are performed to determine if an individual’s immune system is responding to the infection and if they have had previous LASV infections. Recombinant proteins coated on the ELISA plate are bound by LASV NP-, GPC-, and Z-specific IgM or IgG antibodies from patient samples [6, 70]. Though these diagnostics require significantly less time and specialized equipment than PCR, IFA and virus isolation, they are still prohibitive in LASV endemic areas that lack laboratory infrastructure.

A rapid, lateral flow immunoassay (LFI) that provides point-of-care (POC) diagnosis of LASV infection from a single finger prick has been developed to detect LASV infections in resource-limited settings. Based on principles similar to ELISA, the LFI captures LASV antigen from 30-40 μL of blood, serum, or plasma samples, obtained by pricking a fingertip with a safely lancet. This method reduces risk of exposure to the healthcare provider and reduces the amount of blood collected from the potentially hemodynamically unstable patient. The LFI can be interpreted visually for a dichotomous scoring of positive or negative, can be scored on a scale from 0 - 5 indicating the relative viral load, or can be semi-quantitated by an optical reader which provides a numeric value. The time from addition of the biological sample to reading of the result is between 10 and 15 minutes. The minimally invasive nature of sample collection for analysis, the visual assessment, the short time to result, and the portability and stability make the LFI an ideal diagnostic tool for the endemic, resource-limited settings. Though proving to be less sensitive than RT-PCR, this diagnostic is an adequate supplement to a clinical diagnosis in field settings where no laboratories are present. This test, ReLASV®
Antigen Rapid Test (Corgenix Medical Corporation), is a first-generation, CE marked rapid diagnostic platform. The ReLASV® LFI was developed with reagents that detect Sierra Leonean strains of LASV (Clade IV). While they are still capable of providing a broader range of detection due to strain variation than PCR, a second-generation product that is inclusive of all circulating strains in West Africa is under development (Pan LASV). Not only will this be advantageous to endemic countries whose citizens may cross neighboring borders and transmit a strain that is new to that region, but this can also aid in anti-terrorism measures. Being a Category A agent, weaponization of LASV is a concern of homeland defense. These diagnostics would be able to quickly detect the nefarious release of nearly any strain of LASV, allowing a swift response to a potential disaster.

c) Treatment

Ribavirin, a purine nucleoside-analogue drug, has activity against a number of RNA viruses including hepatitis C virus and respiratory syncytial virus. Its mechanism(s) of action have not been clearly determined, but is suggested to include inhibition of viral RNA replication, inhibition of inosine monophosphate dehydrogenase (IMPDH), endogenous guanosine 5’ cap binding of mRNA, immunomodulation, and mutagenesis [43]. Ribavirin is the only therapeutic currently used for the treatment of LASV infection. In a 1986 study of the efficacy of various therapies including oral and intravenous ribavirin and convalescent plasma transfusion from a LF survivor, intravenous ribavirin was shown to be the most effective. In the study, convalescent plasma to treat LF was used alone in pregnant women or concurrently with ribavirin in non-pregnant patients [43]. Despite showing advantageous results in cynomolgus monkeys [71, 72], in these
human clinical trials convalescent plasma was shown to be ineffective [43]. Additionally, the plasma was prohibitively expensive to isolate and difficult to store in endemic areas. Therefore, convalescent plasma has since been disregarded as a viable treatment option for LF, although it is the current treatment of choice for Argentine hemorrhagic fever caused by Junin virus, a New World arenavirus [73]. Similarly, oral ribavirin did not reduce mortality enough to warrant its use as the preferred therapy in acute patients, though it has long been considered a post-exposure prophylactic (PEP) treatment option (current opinions suggest oral ribavirin is ineffective as a PEP) [5, 13, 74-78]. Intravenous ribavirin treatment, on the other hand, significantly reduced mortality compared to patients who received no therapy for LASV infection, and reduced mortality nearly 5-fold when treated within the first six days of fever compared to those who received treatment after seven days since fever onset. Moreover, significant toxicity associated with administration of intravenous and oral ribavirin was not reported in the 1986 study [43]. These results were supported by studies using NHP models, though were never again formally investigated in humans because of the difficulty of conducting a clinical trial in endemic regions [72, 74-76, 78, 79]. Therefore, intravenous ribavirin is recommended for use in the early stages of acute LASV infection. It is also believed that fluid replacement and blood transfusions are beneficial, though they must be monitored carefully [80].

The pharmaceutical company SIGA Technologies, Inc. has discovered and evaluated several compounds believed to possess antiviral properties against arenaviruses [81]. Their studies have demonstrated protection and tolerance in animals that have received ST-193 following a lethal challenge with LASV. One additional compound has
been identified, ST-161, with subnanomolar activity against LASV and submicromolar activity against other arenaviruses in vitro. To date, SIGA Technologies has not reported the progression of these drugs to advanced pre-clinical studies in relevant animal models of LF.

Another potential alternative to the toxic ribavirin for treatment of LF is the drug favipiravir (T-705). This drug is a broadly active nucleotide analog, which is licensed in Japan and is currently undergoing Phase III clinical studies in the U.S. for the treatment of influenza infections. Favipiravir has been tested in a hamster model of Pichinde (PICV) virus infection (a New World arenavirus distantly related to LASV), with higher reported activity than ribavirin, reflected by a greater therapeutic index [82]. Studies on the efficacy of favipiravir in animal models of LF have not been reported.

A promising approach to antiviral drug development is the use of monoclonal antibody therapy. The reported ineffectiveness of convalescent human LF serum as a source of protective antibodies might have precluded efforts to develop immunotherapeutics for the post-exposure treatment of LASV infections. However, recent efforts to define and characterize protective and pathogenic human B cell epitopes in LF by the Viral Hemorrhagic Fever Consortium (VHFC) have led to the isolation of the largest known panel of human monoclonal antibodies (huMAbs) specific to LASV glycoprotein and nucleoprotein antigens. To date, greater than 125 huMAbs reactive with full length LASV GP1, GP2, and GPC have been independently derived from Sierra Leonean LF survivors. Selected LASV huMAbs with significant in vitro neutralizing activity have been tested in vivo. Antibodies that protect a minimum of 75% of guinea pigs are candidates for testing in NHP. Due to significantly larger amounts of each
antibody needed for NHP studies, constructs of interest have been stably transfected into serum-free medium-adapted mammalian NS0 (murine myeloma background) cells for generation of stable cell lines. The CHOLCelect system [83] generates stable NS0 cell lines for large-scale production of recombinant molecules in approximately two and a half months, thus reducing timelines to in vivo testing. To date, eight huMAbs protected 100% of guinea pigs against lethal LASV challenge, and six of the MAbs tested so far in NHP protected 75% - 100% of animals in lethal challenge studies, with each antibody administered independently and on the same day as infection with LASV (unpublished data). Moreover, most huMAbs have conferred complete protection in guinea pigs and NHP without visible signs of LF throughout the study, and with transient or undetected viremia (unpublished data). These encouraging results are driving the development of potent immunotherapeutic huMAb cocktails for the treatment and prevention of LF.

C. Lassa Virus

1. Natural History

LASV infection is characterized by a broad range of symptoms including fever, malaise, headache, sore throat, cough, myalgia, gastroenteritis, abdominal or retrosternal pain, myocarditis, pleuritis, and can progress to a late stage involving encephalopathy and hemorrhaging [5, 8, 29, 84, 85]. Symptoms typically present one to three weeks after exposure to LASV, and the differential presentation poses significant challenges to accurately diagnose LF. Major symptoms of LF, such as conjunctival injection, facial edema, and frank bleeding can occur and generally correlate with a poor prognosis [39, 80, 86]. Among positively diagnosed LF patients, those under the age of 29 have a higher case fatality rate without distinction between males and females [7]. However, pregnant
women with LF, particularly in their third trimester, appear to have a higher case fatality rate and a high rate of fetal abortion [6, 7, 67, 87, 88].

2. Viral Agent Overview

Arenaviruses have a bisegmented, single-stranded RNA genome encoding four genes in an ambisense fashion. The large (L) RNA encodes the viral RNA-Dependent RNA Polymerase (RDRP, also known as L polymerase) and the Zinc or RING finger matrix protein (ZP) [89-91]. The small (S) RNA encodes the nucleoprotein, possessing known 3’ – 5’ dsRNA exonuclease function and immune evasion activity [23, 24, 26, 27, 92-96] and the envelope glycoprotein complex (GPC) precursor. The GPC undergoes post-translational cleavage into two protein subunits (GP1 and GP2) and a virion-associated stable signal peptide (SSP) that interact [36, 89, 97, 98]. The SSP allows trafficking of the GP1-GP2 precursor out of the endoplasmic reticulum to the Golgi apparatus where SKI-1/S1P proteases mature the proteins by cleaving the polypeptide into distinct glycoprotein subunits [97, 99-102]. This process is essential for generating a glycoprotein complex that renders virions infectious [102-104]. The genome is packaged into a spherical, pleomorphic lipid-enveloped virion ranging in size from 50 to 300 nm in diameter [105]. Electron microscopy of LASV particles reveals a “sandy” morphology due to the presence of electron-dense structures within the virion that resemble grains of sand. These structures are believed to be host ribosomes based on the findings of abundant 28S and 18S ribosomal RNA [106], but there has not been a formal demonstration that arenaviruses incorporate intact and functional ribosomes.
3. **Viral Replication**

LASV as well as other Old World arenaviruses have a broad cell tropism due to the fairly ubiquitous alpha-dystroglycan (α-DG) [99, 107]. α-DG is a component of the extracellular matrix on many cells in humans, including cells that participate in the primary immune response. LASV GP1 recognizes and binds α-DG on the cell surface, initiating endocytosis of the virion (Figure 2)[108]. LASV and the other Old World arenaviruses are internalized into unique endosomes free of clathrin, caveolin, dynamin and actin [108-111]. Acidification of the endosome may trigger conformational changes in the GP spike allowing GP1 to dissociate from GP2 [112, 113]. GP2 and the stable signal peptide associated with GP2 are then able to mediate fusion of the endosome and viral membrane allowing the release viral genomic RNA and viral proteins including ribonucleoprotein and L polymerase into the cell cytoplasm [103, 104].

Once in the cytoplasm, L polymerase begins to transcribe and replicate the S and L genomes. The L polymerase has three distinct domains that incorporate a RNA-dependent RNA polymerase as well as a cap-snatching endonuclease that is involved only in transcription [91, 114-116]. Because both genomes are ambisense, one protein from each genome cannot be directly transcribed into mRNA. The L polymerase gene on the L genome and the NP gene on the S genome are in the antisense coding direction and are therefore translated directly from viral RNA. Z and GPC, on the other hand, must first be replicated into template strands and then transcribed into mRNA. Each genome possesses an intergenic region (IGR), which regulates transcription and regulation by acting as a transcription terminator [36, 117, 118].
Though transcription of each gene and replication of each genome occur concurrently during acute infection, NP mRNA and S genomic sense RNA accumulates more rapidly than the other genes and genome (Figure 2) [118]. The accumulation of resulting NP is important in regulating both transcription and replication over the course of acute infection [119]. NP encapsidates nascent RNA and, when there are sufficient levels of NP, shifts L polymerase function from transcription to replication by affecting the secondary structure of the IGR [120].

After transcription, viral mRNA moves into the endoplasmic reticulum where it is translated and post-translationally processed into functional protein. This processing is most important for GPC, which undergoes cotranslational cleavage by signal peptidase to form SSP and proteolytic cleavage by protease subtilase SKI-1/S1P to form GP1 and GP2 (Figure 2) [97, 105, 121]. While the posttranslational processing is not necessary for trafficking to and expression at the cell surface (that function is controlled by SSP [102]), it is required for its incorporation into virions, production infections viral particles, and cell-to-cell spread [99, 100].

Though L polymerase, NP, and GPC each provide a unique and important function in the LASV replication cycle, virions cannot form without Z. Many studies have shown that particles are capable of forming and budding from cells in the presence of only Z, and also that they cannot form or bud without Z [122-126]. Z accumulates at the cellular surface and strongly associates with the host membrane. The late domains of LASV Z, PTAP and PPXY, have been shown to be essential in budding. These two classes of late domains and one more class that LASV Z does not possess are common among budding viruses. Though, LASV Z is unique from other members of the
Arenaviridae in that it contains two late domains rather than one. Both domains are important for efficient budding, and a mutation in either of the two decreases the release of particles [122]. Moreover, myristoylation of a glycine residue at position two on the N-terminus of Z is also required for budding [124]. Once bud from the host cell and containing properly processed proteins, the LASV virion can go on to infect more cells or a new host entirely (Figure 2).
**Figure 2. LASV replication cycle**

LASV GP1 binds cellular α-DG and is endocytosed. A low pH environment in the endosome causes fusion of the endosome membrane and viral membrane allowing the viral genome to enter the cytoplasm. The viral proteins are transcribed and the viral genome is replicated (A), and then proteins undergo translation and post-translational processing in the cellular endoplasmic reticulum and Golgi apparatus (B). The proteins and viral genome are trafficked to the cellular membrane where they associate and bud into the extracellular space, forming a virion (C). Reprinted from “Envelope glycoprotein of arenaviruses,” by D.J. Burri, *et al.*, 2012, *Virus*, 4: p. 2162.
D. Pathogenesis

Disease severity of LASV can most likely be attributed to cell tropism and the virus’s impact on the cellular processes. LASV targets immune cells – monocytes, macrophages, and dendritic cells – and endothelial cells [22, 127-131]. Dendritic cells become heavily infected with LASV as over 99% of the cellular receptor, α-DG, in secondary lymphoid organs is associated with dendritic cells [132]. Upon identification of a foreign pathogen, these cells express pro-inflammatory cytokines and chemokines, thereby eliciting a strong innate immune response. However, LASV infected monocytes, macrophages, and dendritic cells have been shown to impair expression of TNF-α, IL-1β, IL-6, IFN-α, and IL-8 [128, 130, 131]. Even upon induction of macrophages and monocytes with lipopolysaccharide (LPS), a strong activator of the innate immune response, TNF-α and IL-8 levels did not increase in LASV infected monocyte-derived macrophages [128]. Interestingly, though, LASV appeared to activate expression of IL-8, MIP-1α, MIP-1β and MCP-1 in another cell type, monocyte-derived dendritic cells [130]. These molecules seem to selectively recruit macrophages, neutrophils and T cells to the site of infection. By selectively inhibiting inflammatory cytokines and activating expression of recruitment chemokines for specific immune cell types, LASV intricately avoids destructive immune detection, thereby advancing the infection.

Early investigations into the pathogenesis of LASV in non-human primates (NHP) revealed that LASV elicits a weak lymphocyte response. NHP were either previously infected with Mozambique virus (MOZV) or not, then challenged with LASV (75 days p.i. for those infected with MOZV). Those not previously infected became viremic and produced both IgM and IgG antibodies around day 12 post infection with
LASV, but eventually succumbed or were sacrificed due to severe disease state. NHP that were infected with MOZV then inoculated with LASV 75 days later never developed a fever and had no circulating virus by day 12 post infection. Both IgM and IgG cross-reactive antibodies against LASV and MOZV antigens were present early in infection and persisted for 68-70 days post infection, except for one NHP whose antibody levels against LASV decreased during convalescence [133]. This study suggested that protective antibodies where important in LASV clearance. However, given that NHP challenged with only LASV became febrile and succumbed to infection despite producing antibodies, it was evident that protective antibodies either developed over time or that the host was incapable of generating them upon primary LASV infection.

Another NHP study elucidated that, during severe disease state, there was a significant decrease in cellular immune response as evidenced by impaired lymphocytes even upon $ex$ $vivo$ stimulation mitogens phytohemagglutinin (PHA) and concanavalin A (conA), which have been shown to elicit a robust lymphocyte response. Moreover, this study revealed that lymphocyte and neutrophil levels were low upon initial infection, from days four to seven post infection, and then returned to pre-infection levels. Neutrophil levels rose steadily after day seven post infection, while lymphocyte levels remained consistent throughout the disease course until succumbing to LASV infection or being sacrificed [127]. These findings in NHP combined with the observation of impaired cytokine expression suggest that LASV is capable of controlling the host immune response from an early stage, ultimately leading to shock and multi-organ failure in severe cases (Figure 3).
Figure 3. Pathogenesis of viral hemorrhagic fevers

Viral hemorrhagic fevers disrupt the host immune response. The viruses interfere with innate immune activation leading to paralysis of subsequent immune systems and ultimately leading to shock in severe cases. Though this image is not a literal depiction of LASV pathogenesis, it is a compatible representation. Reprinted from “Clinical Aspects of Marburg Hemorrhagic Fever,” by M. Mehedi, et al., 2011, Future Virology, 6: p.1091.
1. Innate Immunity

The innate immune system comprises the primary line of defense in humans after a pathogen has crossed the epithelial barrier. Cells of innate immunity – macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and natural killer (NK) – can recognize and respond to a foreign pathogen [134]. Many pathogens possess ligands on their surface that target specific receptors on immune cells. Pathogen ligand binding to host cell receptors elicits an inflammatory response. During an inflammatory response, the immune cells secrete immune modulators called cytokines and chemokines to attract and activate more immune cells to the area of infection, as well as destroy infected cells, and effect vascular permeability and clotting function [135, 136]. For instance, chemokine groups such as MIP and MCP recruit macrophages and monocytes to the site of infection where they will become activated and consequently secrete more cytokines and chemokines themselves [137]. Alternatively, cytokines can have drastically different effects. The cytokine TNF attracts lymphocytes to the site of infection, induces cellular apoptosis, causes vasodilation, and increases vascular permeability, often leading edema due to an excess of immune cells extravasating into tissue and sepsis [130, 136, 138]. Cytokines and chemokines play an extremely important roll in controlling the initial infection. If not properly activated at the onset of infection, not only will the pathogen continue to replicate and infect more tissue, but the adaptive immune response could be negatively affected as well.

a) Activators of Innate Immunity

Receptors located on the surface or within immune cells recognize specific ligands of pathogens and become activated, initiating a signal cascade system, and
ultimately leading to the expression of cytokines and chemokines that propagate the immune response. Each immune cell possesses an assorted plethora of receptors such that the pathogen would be unable to escape recognition. These receptors, known as pattern-recognition receptors (PRRs), recognize a unique aspect or pattern of the pathogen. Though each pathogen is unique, PRRs are capable of recognizing fundamental components that are ubiquitous amongst microorganisms. Common pathogen-associated molecular patterns (PAMPs) include sugar groups, DNA, and RNA. These PAMPs trigger PRRs to induce expression of cytokines, chemokines and co-stimulatory molecules.

Carbohydrate recognition receptors include conglutinin, mannan-binding protein, CL-43, and pulmonary surfactant protein A [139]. Many pathogens possess carbohydrate structures. Viruses often collect them during the intracellular replication process. Post-translational modifications using the endoplasmic reticulum and Golgi apparatus can occur and add sugar groups to proteins. Budding viruses may bring carbohydrates from the host cell membrane with them. Thus, innate immune cells have receptors with affinities to galactosyl and lactosyl ceramides with certain fatty acids or high-mannose type N-glycans found on glycoproteins [139].

DNA and RNA compose the genomes of pathogens. Viruses are intracellular pathogens that rely on host cells for replication. During infection, the genomes of viruses are venerable to recognition by cellular PRRs. Common PRRs that detect genomic material include the retinoid acid-inducible gene I (RIG-I) -like receptors (RLRs) and some members of the Toll-like receptor family (TLR). RIG-I and melanoma differentiation-associated gene 5 (MDA5) are two important receptors that activate an
immune response in the presence of a pathogen like LASV. RIG-I and MDA5 localize in the cytosol and can detect the presence of dsRNA viruses via their genomes or genomic replication byproducts.

The TLR family comprises 13 known mammalian members [140]. Each member contains a type 1 transmembrane protein that anchors to either the cell membrane or endosome surface and is exposed to various environments where pathogens and their PAMPs are present. Some TLRs recognize a wide variety of PAMPs while others recognize only one. A majority of the TLRs recognize bacterial PAMPs. TLRs -3, -7, -9, and -13 are virus-specific receptors that recognize genome- and replication-specific ligands – dsRNA, ssRNA, CpG-DNA, and a ribosomal RNA sequence, respectively [140, 141]. TLRs -3, -7, and -9 localize on the cell surface of epithelial cells, fibroblasts, and astrocytes [142-144] and reside on endosomes in monocytes, macrophages and dendritic cells with the receptor domain oriented towards the interior milieu [140, 141, 145]. TLRs utilize Toll/IL-1 receptors (TIR) to transduce signals. Upon activation, TLRs recruit TIR-containing adaptor proteins such as MyD88, TRAM, TIRAP and TRIF [146]. These proteins initiate a signal cascade that leads to transcription and expression of cytokines, chemokines, costimulatory molecules, and major histocompatibility complex (MHC) [141, 147].

In viral diseases such as West Nile Virus (WNV) and Dengue Virus (DENV), TLR-3 has a protective role in limiting viral replication and activating the anti-viral immune response [148-152]. TLR-3 was shown to regulate WNV entry into brain cells such as astrocytes [148, 150]. These studies determined that pro-inflammatory cytokines produced as a result of TLR-3 stimulation lead to a break down of the blood brain barrier.
in mice causing encephalopathy, though they experienced low peripheral viremia. Contrastingly, TLR-3 knockout mice had high viral titers in their peripheral organs, with little brain pathology [148, 150]. Later, the WNV nonstructural protein (NS1) was implicated in abrogating the signal transduction of TLR-3 and subsequent transcriptional activation of pro-inflammatory cytokines [149]. This exemplifies the importance of PRRs, particularly TLR-3, in controlling viral replication.

The cytokine subfamily of type I interferons (IFN-I – IFN-α and -β) are products of activated PRRs and are closely associated with viral clearance and an effective adaptive immune response (Figure 4) [153, 154]. While transcription of IFN-I is activated via several PRR pathways throughout the course of infection, the RLRs and TLRs mediate expression during the beginning stages of infection to control viral replication and turn on other immune response pathways [154]. These two families of receptors ultimately control two different arms of the virus-specific immune response and provide the link between the innate and adaptive immune systems. A study examined the roles RLRs and TLRs played during infection with LCMV. Mavs−/− and Unc93b13d/3d mice were used to demonstrate the differential roles of RLRs and TLRs, respectively. MAVS is an adapter protein through which RIG-I and MDA5 activate IFN-I expression. UNC93B localizes TLRs 3, 7 and 9 to endosomes where they come in contact with viral genomes and replication products. The Mavs−/− mice failed to control LCMV replication and had a weakened CD8+ cytotoxic T cell response, while Unc93b13d/3d mice experienced diminished antibody responses against LCMV and exhausted supplies of CD8+ T cells [154]. This demonstrates that while both RLRs and TLRs mediate IFN-I activation, the ultimate functional roles of each are different.
Figure 4. IFN-I activation and downstream activity

b) LASV Innate Immune Impairment

Because activation of IFN-I is important for viral clearance early in infection, investigators wanted to study how this arm of the immune system responds to LASV. Investigators discovered that LASV NP was capable of impairing expression of IFN-I (IFN-α and IFN-β). Though, at the time, the specific mechanism by which NP disrupted IFN-I production was not known, residues were identified that, when mutated, rescued the expression of IFN-I and altered viral transcription [23-25, 93, 95, 155, 156]. After the crystal structure of LASV NP was elucidated, NP was found to have a similar structure to the DEDD family of exonucleases. Investigators discovered that some residues that rescued IFN-I expression overlapped the 3’-5’ exonuclease domain of NP [94, 95]. The NP exonuclease activity is specific for dsRNA, a byproduct of viral transcription and replication that activates an antiviral response in cells. The overlap in residues that are both a functional part of the exonuclease domain and play a roll in the IFN-I response suggests that exonuclease activity contributes to LASV impairment of the host immune system; however, none have been identified to date.

In 2008, Habjan et al. determined that 5’ processing of viral genomic dsRNA masked the PAMP from recognition by RIG-I [157]. This study highlighted the importance of RIG-I in viral infection, and also suggested a method by which RNA viruses may avoid immune detection. Combining this information with the knowledge that LCMV and LASV NPs down-regulated the expression of IFN-I, exonuclease activity on RIG-I activation was investigated [156, 158]. Dendritic cells, macrophages, and HEK-293T cells were used to determine the effects of wild type LASV (LASV-WT) and a mutated LASV on RIG-I activation by dsRNA. Point mutations were introduced through
reverse genetics at D389A and G392A of NP (LASV-ExoN). In previous studies, these mutations abrogated exonuclease activity of LASV NP and demonstrated increased levels of IFN-I in LASV infected immune cells [94, 95, 155]. When infected with LASV-ExoN, the levels of costimulatory molecules and cytokines dendritic cells and macrophages increased significantly over LASV-WT and mock infections. Additionally, RIG-I mRNA levels increase in dendritic cells when infected with LASV-ExoN when compared to LASV-WT. To define the role of RIG-I in LASV infection, 293T cells were transfected with either with or without RIG-I siRNA, then infected with LASV-WT or LASV-ExoN, and expression of cytokines and RIG-I were measured by qPCR. Only upon infection with LASV-ExoN did cytokine mRNA levels and RIG-I mRNA levels increase. Cells transfected with RIG-I siRNA attenuated mRNA levels of cytokines and RIG-I. This study provides evidence that link the exonuclease domain of LASV NP and immune evasion via RIG-I, but does not conclusively show that the exonuclease activity directly affects the activation of RIG-I.

Other mechanisms by which LASV NP is capable of disrupting IFN-I expression have been previously described. A study determined that NP colocalizes with interferon regulatory factor 3 (IRF3), which disrupts IFN-I expression. Investigators in this study infected cells with Sendai virus (SeV), and in the presence of NP found that IRF3 accumulates in the cytoplasm rather than translocating to the nucleus [23, 93]. This event correlated with a quiescence of IRF3 transcriptional activation and IFN-β mRNA production [23]. LASV NP has also been shown to bind directly to IKKe to inhibit hyper-phosphorylation, translocation, and promoter activation, which blocked activation of NF-κB [26, 27].
2. Linking LASV Innate Immune Impairment and Adaptive Immunity

An efficient and effective adaptive immune response requires the appropriate activation of the innate immune response. The cells of adaptive immunity, T cells and B cells, interact with and take direction from innate immune cells (Figure 5). Properly activated antigen presenting cells (APC) not only initiate expression of cytokines and chemokines, but also activate T cells and consequently B cells [159, 160]. Though several innate pathways have been implicated as LASV targets for immune disruption, the TLR3 pathway is of particular interest.
**Figure 5. Interaction of innate and adaptive immune systems**

LASV is known to infect and replicate in dendritic cells, which contain TLR3. TLR3 localizes to endosomes and recognizes and binds only dsRNA in acidic environments [161]. Therefore, the environmental conditions created by LASV endocytosis and low-pH mediated uncoating are ideal for TLR3 stimulation by dsRNA. Moreover, LASV NP, one of the first proteins transcribed during replication accumulates in great quantities in the cytoplasm while viral replication takes place and has exonuclease activity specific for dsRNA [95]. Once stimulated TLR-3, activates cytokine production via two distinct pathways. Pro-inflammatory cytokine production occurs through the TRIF/TRAF6 pathway, which causes phosphorylation of IKK complex and nuclear translocation of NF-κB to initiate transcription of target genes [162]. TLR-3 can also activate expression of IFN-I through an alternative signaling pathway that requires phosphorylation of IKKe and activation of IRF3 [162, 163]. Interestingly, a group demonstrated that both IRF3 and NF-κB are differentially activated by RLRs and TLRs in dendritic cells. RLRs generate a strong IRF3 response in the presence of pathogen ligands, whereas TLRs preferentially activate NF-κB [164]. This preferential activation may explain the results discussed in the “Activators of Innate Immunity” section whereby Mavs−/− (RIG-I deficient) mice failed to control LCMV replication and had a weakened CD8+ cytotoxic T cell response, while Unc93b13d/3d (TLR3 deficient) mice experienced diminished antibody responses against LCMV [154]. This could be a possible explanation for the deficient humoral immune response observed in LASV infection.

3. **Adaptive Immunity**

TLR3 is capable of maturing dendritic cells into APC and activating expression of IFN-I, CD86, IL-12, TNF-α, and IL-6 [165]. Once in secondary lymphoid organs,
activated dendritic cells can interact with T cells via MHC molecules, which present antigen fragments to naïve T cells, and co-stimulatory molecules to initiate cellular immunity [166] and eventually humoral immunity.

IFN-I plays a major roll in the activation of the adaptive immune response [167]. Studies have shown that IFN-I and granulocyte macrophage colony-stimulating factor (GM-CSF) is more efficient at differentiating peripheral blood monocytes into dendritic cells in vitro than IL-4 and GM-CSF [168, 169], though these studies could not be confirmed in vivo [170-172]. However, IFN-I is an important autocrine survival factor for dendritic cells in vivo [170-172], and is important for modulating co-stimulatory molecules necessary for activating T cells. Not only do IFN-I-producing dendritic cells efficiently activate T cells, but also they are capable of activating certain T cell subsets that lead to a more robust and longer-lived humoral response [169, 173-175]. Additionally, they protect B cells from antigen-induced apoptosis [175]. While these observations have not been directly studied in LASV infections, correlations suggest that IFN-I plays an important role in these pathways.

Dendritic cells acting as APC influence the pathway naïve T cells take. MHC molecules, co-costimulatory molecules, cytokines and chemokines determine T cell fate (Figure 6). MHC class I (MHC I) presents processed antigen to naïve CD8+ T cells, and with the help of CD40, CD86, IL-2, and IL-12 activate and lead to differentiation of CD8+ T cells into effector cytotoxic T lymphocytes (CTL) [176, 177]. Interestingly, interaction of the co-stimulatory molecules of CD86 and CD28 on mature dendritic cells and naïve CD8+ T cells, respectively, are not necessary for functional activation of cytotoxic T cells [177-181]. Moreover, naïve CD8+ T cells need relatively short
interactions with the antigen to become activated, proliferate and differentiate into
effector cells [182]. Thus, even with weak immune stimulation, antigen-specific CD8+ T
cells are readily generated. Upon infection with LCMV, mice deficient in both CD40L
and CD28 still mounted an effective cytotoxic T cell response [166], demonstrating that
CD8+ T cells can become activated despite viral impairment of the immune response.

CD4+ T cells, as opposed to CD8+ T cells, necessitate MHC class II (MHC II)
molecules presenting antigen, CD40 and CD86 co-stimulatory molecules along with IL-2
and other cytokines that direct differentiation [176, 183]. In mice deficient in CD40L and
CD28 and infected with LCMV, CD4+ T cell activation was severely compromised
compared to CD8+ T cells [166]. Activated CD4+ T cells perform a number of roles. They
prime immature APC so that they, in turn, can activate CD8+ T cells [184-187], they can
stimulate B cell proliferation and class switching activities [188-190], and they express
cytokines such as IL-2, IL-4, IFN-γ, and TGF-β that further guide immune function [183,
191-194]. The effects of presenting antigen to CD4+ T cells without proper co-
stimulation from CD40, CD86, and cytokines could be detrimental in terms of a viral
infection. Studies have shown that immature dendritic cells pulsed with antigen and co-
cultured with T cells were capable of activating proliferation; however, antigen-specific T
cell activation was short-lived, T helper cells (T_H cells) did not become polarized, or T
effector cells (T_E cells) became tolerant of antigen stimulation and protective function
ceased [195, 196]. Though not necessary for initial activation and proliferation of CD8+ T
cells, polarized T_H cells prime APC, creating a more robust CTL response, especially
upon a secondary challenge [182, 197-202]. Therefore, while viral impairment of the
immune response may not affect CD8+ T cell stimulation during an initial infection, upon
a secondary infection, CD8$^+$ T memory cells cannot properly resume their effector function.

For an effective, long-lasting humoral response, B cells rely mainly on T\textsubscript{H} cells for activation (Figure 6). Mature naïve B cells recognize and bind antigen via immunoglobulin receptors on the cell surface. Once internalized, B cells degrade the antigen, and MHC II molecules present the peptide to T cells at the T-B cell border in secondary lymphoid organs. T\textsubscript{H} cells recognize the peptide displayed on the B cell surface, bind, and become activated. After activation of the T\textsubscript{H} cell and along with the interaction of co-stimulatory molecules CD40/CD40L (CD154) and cytokines, the B cell becomes activated. Activated B cells proliferate and can follow various pathways including differentiation into terminal plasma cells or memory cells. Plasma B cells can express IgM molecules, which act as immediate, but short-lived antibody producing cells. However, as a plasma B cell progresses through the germinal center of the secondary lymphoid organ, it can encounter signals that will cause class-switch recombination (CSR). CSR will rearrange the genes of an antibody such that the resulting molecule can signal different functions. IgG molecules are important for long-term immunity and IgG-producing plasma cells can last for months following an initial infection [203-207].
Figure 6. Cytokine dependent activation of T and B cells

a) LASV and Adaptive Immunity

Investigators have discovered LASV infection is capable of diminishing immune cell maturation and activation. LASV impairs dendritic cell function and maturation resulting in a marked depression of the T cell response [16, 17, 25, 43, 127, 130, 131, 208-211]. Many studies examining in vivo responses to LASV either in humans or NHP have shown immunosuppression and transient lymphopenia [6, 16, 67, 70, 79, 127, 209, 210, 212, 213]. Dendritic cells infected with LASV failed to express pro-inflammatory cytokines and co-stimulatory molecules, particularly CD86 and CD83 [130, 131]. Moreover, both immature and mature dendritic cells challenged with LASV were unable to propagate an effective immune response [211]. In a mixed lymphocyte assay, dendritic cells infected with LASV failed to cause proliferation of T cells [130].

These in vitro results corresponded with fatal infection in NHP models. Cynomolgus monkeys that were challenged with LASV and survived showed a higher lymphocyte count than those who succumbed [16]. While both cohorts initially showed diminished levels, lymphocyte populations in survivors became elevated 9 to 16 days post infection [16]. Upon examining CD25⁺/CD8⁺ T cell populations and CD69⁺/CD4⁺ T cell populations, investigators determined that high counts of T cells within the first 3 to 6 days post infection correlated with survival [16]. These increases in T cell populations during early stages of infection in monkeys that survived LASV infection also corresponded with elevated levels of IFN-α. Interestingly, levels of IFN-α in fatalities increased three days after what was seen in survivors, but fatalities maintained high levels of IFN-α, whereas the levels in survivors diminished a few days later [16].
While a causal link between elevated IFN-I levels and T cell activation was not provided in this NHP study, an *in vitro* by Pannetier et al. study demonstrated the importance of IFN-I in T cell activation [214]. Synthesis of IFN-I correlates with the presence of chemokines CXCL10 and CXCL11 in dendritic cells [215]. These chemokines are recognized by CXCR3 found on activated T cells [216, 217], thereby attracting T cells in secondary lymphoid organs and subsequently to peripheral sites [218], and have been shown to stimulate T cell proliferation [219]. In their study, Pannetier et al. blocked the IFN-I receptor with neutralizing antibodies. Neutralization of the IFN-I receptor abolished the production of CXCR10 and CXCR11 and prevented T cell priming as seen by inadequate T cell stimulation upon a second exposure to infected dendritic cells. This demonstrated the importance of IFN-I in the induction of the adaptive immune response. Furthermore, Pannetier and others found that CXCL10 and CXCL11 were elevated in both peripheral blood mononuclear cells (PBMC) and in lymph nodes in LASV infected NHP [16, 214], and elevated levels of CXCL10 have been shown to correlate with survival [214, 220]. However, compared to the similar but non-pathogenic Mopeia virus (MOPV), LASV induced lower levels of CXCL10 and CXCL11 *in vitro* in macrophages, emphasizing the ability of LASV to impair the host immune system [214]. Interestingly, recombinant LASV (rLASV) with a mutated NP at the exonuclease site (NP D389A/G392A) rescued production of CXCL10 and CXCL11 in dendritic cells. Though a correlation was not made, this implicates the LASV NP exonuclease activity as a mediator in host immune impairment [214]. Lastly, the authors of this study noted that, while elevated levels of CXCL10 correlated with survival in
NHP, one monkey experiencing increasingly high levels of these chemokines in lymph nodes on day nine post infection succumbed to LASV.

While T cell activation is believed to be important in viral clearance, it has shown to be detrimental when over-activated, as perhaps was the case in the previous report [17, 127, 214]. In HLA-humanized C57BL/6 mice (HHD), infection with LASV moderately controlled the virus, but resulted in a severe disease state. Contrastingly CD8⁺/CD4⁺-depleted HHD mice did not clear the virus – it persisted – but resulted in less severe to no symptoms. Moreover, HHD mice primed with MOPV, a closely related virus, was protective against subsequent LASV infection [17]. Therefore, to be protective, the adaptive immune response must maintain a fine balance dictated by the innate immune cells, and particularly dendritic cells, as acquired immunity appears to be productive at viral clearance but deleterious if the inflammatory response is over-activated. If viral replication continues uncontrolled, infected cells can enter the circulatory system where they travel to organs and inflict further damage resulting in LF disease manifestations.

Upon proper activation, CD4⁺ T cells assume distinct roles that are dictated by their interaction with APC and cytokines [221]. One such role that remains relatively ambiguous in LASV infection is the activation of B cells. Many investigators have reported that the humoral response is not protective in LF [16, 18, 45, 84, 120, 127, 208, 222, 223]. Early studies of LF positive individuals demonstrated a delayed humoral response with little to no neutralizing antibodies (NAb) [19]. Moreover, convalescent immune plasma used to treat LF positive individuals proved to be ineffective despite showing positive results in NHP [20, 43]. Recent studies in NHP revealed a complete absence of NAb after immunization and challenge with LASV [18, 21].
Interruptions seen in the humoral immune response in LF, including exceedingly small amounts of NAb, is likely the result of immune impairment by LASV beginning with the innate immune response and ultimately affecting T cells and B cells. It has been shown that compared to MOPV, LASV infection downregulates CD40L+ T cells [6, 211]. This would certainly weaken humoral immunity in LF. However, recent reports demonstrate that antibodies may be more important in LASV infection than previously thought. Using antigen- or antibody-capture ELISA (Ag-ELISA, Ab-ELISA), Shaffer et al. found that LF patients that were positive for LASV Ag (indicating and active, acute infection) and were α-LASV IgM negative, but α-LASV IgG positive had a lower case fatality rate than those without α-LASV IgG [7]. Additionally, the mortality rate of LF patients who received the drug ribavirin versus those who did not was not significantly different if they were both α-LASV Ag and α-LASV IgM positive, indicating that antibodies may be protective. Interestingly, it was noted in several reports that LASV may impair CSR as a persistence of α-LASV IgM was detected well into convalescence [6, 7, 69, 70]. These new interpretations of the humoral response have encouraged development of huMAbs to LASV for therapeutic purposes, as mentioned earlier in this chapter. These huMAbs were selected for their in vitro neutralization capabilities and have shown to be protective to lethal LASV challenge. In guinea pigs, 8 of the neutralizing huMAbs were 100% protective, and 6 were 75%-100% protective in NHP when administered on the same day as challenge [Branco et al., unpublished data]. These recent data highlighting the importance of antibodies in LF have prompted investigators to examine how LASV impairs the humoral immune system, making advancements toward novel methods for treating the disease.
E. LASV Recombinant Proteins for LF Diagnosis and Advancement in Understanding of Pathogenesis

In 2005, the VHFC began work towards a field-deployable ELISA for detection of LASV Ag and Ab. Branco et al. recombinant LASV NP, GP1, and GP2 [224]. The first-generation Ab-ELISA was composed of individual proteins coated on 96-well plates to capture antibodies from the serum acute and convalescent patients. After its initial deployment at KGH LFL, it became clear that GP1 and GP2 produced in bacteria were insufficient for capturing LASV-specific Abs, as the post-translational processing was quite different than native antigen. Therefore, the GP gene without the transmembrane domain was cloned into a mammalian expression vector. This allowed the mammalian cellular proteins to process the GPC as it would in the natural disease state. The ReLASV® IgG/IgM ELISA Test kits utilized these proteins in a microwell plate format [Boisen et al., manuscript in preparation]. These assays have been in use since early 2010 to diagnose patients at KGH Lassa Fever Ward (LFW) and have provided invaluable information regarding the impact of LF in communities across Sierra Leone [6, 7, 67, 70, 225, 226].

The VHFC further characterized the recombinant LASV NP protein through X-ray crystallography. Hastie et al. elucidated that the structure of LASV NP was similar to the DEDD family of exonucleases [95]. Point mutations discovered to abrogate IFN-I activity were later known to be located in the exonuclease domain of the C’-terminus of LASV NP [23, 24, 93]. Experiments to determine the specificity of exonuclease function revealed that this domain could exclusively bind dsRNA and degrade in a 3’ – 5’ direction [95]. Recombinant proteins isolating the exonuclease region, NPΔ340WT, and
mutants that had been shown to abrogate IFN-I expression, NPΔ340^{D389A}, NPΔ340^{G392A}, NPΔ340^{R393A}, and NPΔ340^{QuadA} were used to in these exonuclease experiments. NPΔ340^{WT}, NPΔ340^{R393A}, and NPΔ340^{QuadA} retained exonuclease function, while NPΔ340^{D389A} and NPΔ340^{D389A} lost function [95]. Moreover, it was determined that the N’-terminus of LASV NP possessed a structure capable of binding ssRNA [94]. In the virion NP binds the genomic ssRNA, but mutational analysis also reveals that this binding domain may be necessary for transcriptional activity [94]. Proteins used for the exonuclease analyses were cloned into the pET46 vector and expressed in Rosetta 2(DE3)pLysS E. coli cells as truncated proteins. Removal of the first 340 residues allowed for a higher expression concentration and allowed the exonuclease function to be teased out from the cap-binding function. As was predicted, mutations made to certain residues in the exonuclease domain abrogated dsRNA degradation in the 3’ – 5’ direction [95]. These point mutations in truncated LASV NP also rescued IFN-I production compared to truncated LASV-WT NP suggesting that the exonuclease function impairs the host immune response [94, 95].

F. Research Objective

The aim of this study is to demonstrate that LASV impairs the host immune response during infection. The innate immune response is believed to be the main mediator in managing the LASV infection. This may be due to the virus’s ability to interrupt important innate immune pathways that regulate adaptive immunity. Several of the viral proteins have been implicated in disruption of immune activation; however, LASV NP exonuclease activity on dsRNA and its stimulation of TLR3 in dendritic cells has yet to have been explored. The study presented herein examines the antibody and
cytokine responses in LASV acute and convalescent patient samples to elucidate trends throughout the disease course. Additionally, the impact of NP exonuclease activity on TLR3 is explored.
MATERIALS AND METHODS

A. Analysis of host immune response to LASV infection

1. Human Subjects

Suspected LF patients, close contacts, and healthy volunteers were eligible to participate in these studies as outlined in Tulane University’s Institutional Review Board (IRB) protocol for this project, National Institutes of Health/ National Institutes of Allergy and Infectious Diseases guidelines governing the use of human subject for research, and Department of Health and Human Services/ National Institutes of Health/ National Institute of Allergy and Infectious Diseases Challenge and Partnership Grant Numbers AI067188 and AI082119. This project was approved by the Tulane University IRB. Adult patients in this manuscript have given written informed consent for the publication of their case details. Written informed consent was obtained from all subjects.

2. Sera from suspected LF patients and healthy volunteers

Small blood volumes (typically five milliliters), for serum separation were collected from study subjects with consent from the attending physician. Blood from healthy Sierra Leonean volunteers were used as normal controls. Three groups of normal donors were assembled for this study: 1) non-febrile volunteers comprised of Lassa program staff from Kenema district and nursing staff from a hospital in Bo, Sierra Leone, who reported to be in generally good health at the time of blood collection; 2) volunteers from randomly-chosen, historically non-endemic villages in the District of Moyamba...
(N=101) and the District of Bombali (N=13) that also reported to be in generally good health, not having any recent illnesses or having travelled to Africa’s Eastern Province. Both patients and healthy volunteer samples received a coded designation and were collected in serum vacutainer tubes and allowed to coagulate for 20 minutes at room temperature. Serum was separated from coagulated blood by centrifugation (200 x g, 20 minutes at room temperature). The serum fraction was collected for analysis, and aliquots were stored in cryovials at -20°C. Sera from a panel of 50 randomized U.S. male donors were purchased from Bioreclamation, Inc., and a similar panel of 50 U.S. female donors was obtained from SeraCare Life Sciences, Inc. All U. S. donors were at least 18 years of age and reported their ethnic groups as Caucasian, Hispanic, or African American.

3. Detection of LASV antigen and LASV-specific antibodies

Serum levels of Lassa nucleoprotein (NP) -specific antigen were determined using a sensitive antigen-capture ELISA employing either a murine monoclonal or caprine polyclonal capture antibody, followed by a peroxidase-labelled caprine reagent and tetramethylbenzidine (TMB) substrate. A standard curve was generated with rLASV NP in order to quantify serum levels of virus-associated NP.

4. IgM and IgG levels to rLASV proteins ELISA

Individual LASV proteins and combinations optimized for detection of virus-specific IgM and IgG levels in serum were coated in stripwell plates, blocked, dried, and packaged with desiccating packs (Corgenix Medical Corp.). For analysis, sera were diluted 1:100 in a proprietary sample dilution buffer and incubated in wells for 30 minutes at room temperature, washed, and incubated with optimized HRP-labelled α-human IgG or IgM conjugates for an additional 30 minutes. After washing, detection was
performed with TMB substrate for 10 minutes at room temperature, stopped with sulphuric acid, and read at A450 in a BioTek ELISA plate reader.

5. Laboratory confirmation of LF

All serum samples were tested for LASV-specific Ag and IgM and IgG antibodies by ELISA, as outlined above. ReLASV NP was serially diluted and used to generate a standard curve in Ag-capture ELISA and LFI formats. Sera from previously diagnosed LF patients were used as IgM and IgG Ab-capture ELISA calibrators. As discussed in detail herein, patients who presented to the KGH LFW with a febrile illness were assessed by the following criteria: Patients were considered to have acute LF if a reaction above background levels developed on rLASV LFI modules and/ or LASV Ag-capture ELISA, and/ or LASV-specific RT-PCR generated a GPC gene-specific amplicon using oligonucleotides previously described by Olschläger et al. [61]. Subjects testing only as IgM+ using rLASV IgM Ab-capture ELISA were not considered acute LF cases and often were not administered a course of ribavirin. The ultimate decision to administer a course of ribavirin to Ag-IgM+ patients was at the discretion of the attending KGH LFW physician. Subjects testing only as IgG+ using rLASV IgG were also not considered acute cases, but rather were considered to have been previously exposed to LASV and were deemed to be suffering from a non-Lassa febrile illness. Similarly, patients who were dual IgM+IgG+ and LASV Ag- at the time of testing were considered to be suffering from an unrelated febrile illness. For these patients additional testing and disease diagnosis was performed, as per the capabilities available at the KGH.
6. **Patient database analysis**

A database was generated with coded patient designations and corresponding LASV antigen, IgM and IgG status at the time of testing and admission. In total 1,909 suspected LF patient data were collected and a subset of those data were used in this study. All patients in the database were suspected of having LF based on clinical presentation, or for failure to respond to anti-malarial and/or antibiotic drug regimens over the course of a prolonged febrile illness.

Limitations of this study include lack of randomization, limited number Lassa cases with multiple sample draws or sample analysis across all platforms, potential patient by treatment interaction, and the limited duration of the study. Random assignment of subjects to comparison groups was not possible due to the uncontrollable nature of the outcome. As a result, some of the results that we observed may be due to inherent characteristics of our study subjects.

7. **Flow Cytometry**

The kinetics of eleven serum cytokines were analyzed daily using an Accuri® C6 benchtop cytometer (Accuri Cytometers Inc.) with an eBioscience FlowCytomix Human Th1/Th2 11-plex Kit (Bender MedSystems GmbH). Serum aliquots collected and frozen throughout the monitoring timeline were analyzed concurrently at the end of the study.

8. **Peripheral Blood Mononuclear Cells and FlouroSpot Analysis**

Whole blood was drawn by venepuncture and collected into K2-EDTA vacutainer tubes (BD). PBMCs were separated by density centrifugation using Lymphocyte Separation Medium (MP Biomedicals). PBMCs were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium, HEPES supplemented with 1X Gluta-Max,
10% heat-inactivated fetal bovine serum (ΔFBS), 100μg/mL Gentamycin, and 1X Pen/Strep. Cells were counted and seeded onto FluoroSpot for Human IFN-γ/IL-2 plates (Mabtech Inc.) at about 100,000 cells/well. Stimulants α-CD28, rLASV NP, sGP1, and GPC were added to cells and incubated overnight at 37°C, 5% CO₂. The detection of the spots was performed according to manufacturer’s instructions and was read on an AID iSpot FluoroSpot Reader System and analyzed by AID EliSpot software (AID GmbH).

9. Detection and measurement

ELISA data were plotted as mean ±SD, where each mean was based on two replicates, and error bars were used to represent the standard deviations. Cutoffs were set at three times the mean of panel of sera from U.S. (USN) or Sierra Leonean donors without detectable LASV Ag, IgM, and IgG titers. Cytokine levels were calculated by applying curve fitting techniques to data generated with quantified standards for each analyte.

10. Statistical analysis

Box plots of IgM and IgG serum responses were generated for comparison groups. Box plots of serum responses for IgM and IgG profiles were generated using the Vertex42 Microsoft Excel template. The Kruskal-Wallis test was used to compare mean serum responses and subsequent pairwise comparisons were conducted using Dunn’s post-test. Wilcoxon’s signed rank test was used to assess changes in median IgM and IgG serum levels between baseline and convalescent end points. Simple linear regression was used to assess the relationship between IgM and IgG serum level responses and the number of days post discharge. The response data for these regression models was log-transformed to conform to linear regression assumptions. Logistic regression models
were used to make patient subgroup comparisons with respect to survival outcome. Grouped column graphs were plotted as the mean ±SD, and analyzed by two-way ANOVA with a Bonferroni post-test. All of the statistical analyses were conducted using GraphPad InStat 3 (GraphPad Software, Inc.), Prism (GraphPad Software, Inc.), the SAS System (SAS Institute, Inc.), and JMP (SAS Institute, Inc.). The α level was set at 0.10 statistical significance threshold for all of the analyses was set at \( p < 0.05 \).

**B. Evaluation of LASV NP exonuclease activity on dsRNA and its role in immune suppression via the TLR3 pathway**

1. **Cells, plasmids, recombinant gene constructs, and antibodies**

HEK-293T/17 cells were maintained in complete high glucose Dulbecco’s Modified Eagle Medium (cDMEM) supplemented with non-essential amino acids (NEAA) and 10% ΔFBS. HEK-Blue™ hTLR3 Cells and HEK-Blue™ Null1 Cells (IvivoGen) were maintained according to manufacturer’s instructions.

Plasmid constructs expressing NPΔ340 WT, LASV NP 1-340, and mutants NPΔ340 D389A, NPΔ340 G392A, NPΔ340 R393A, and NPΔ340 QuadA from the Josiah strain were described elsewhere [95].

Genes for clade II (LASV237-Nig-2010) [Andersen et al., unpublished data] clade III (Nig08-A18) [33] LASV NP were cloned into pcDNA3.1+intA vector as PCR products (LASV237-Nig-2010 Forward 5’ – ATCGATGCTAGCCACCATGAGTGCTTCTAAGGAAGTGAAG, LASV237-Nig-2010 Reverse 5’ – ATCGATGCGGCCGCTTAGCTGTGATGATGATGATGGCTGCTGCCCTTGTC; Nig08-A18 Forward 5’ – ATCGTCATCCAGGACGACCTTTGGGGCT; Nig08-A18 Reverse 5’ – ATCGTCATCCAGGACGACCTTTGGGGCT;...
ATCGATGCTAGCCACCATGAGTG CCTCCAAGGAAGTGAAA, Nig08-A18
Reverse 5’ –
ATCGATGCGGC GCTTTAGCTGTGATGATGATGATGATGGCTGCTGCCCTTGTC
ATCGTCATCCAGGACGACTTTAGGTGT). Restriction digest of the cDNA and vector
was performed with NheI and NotI for both genes. The recombinant plasmids were
propagated in Escherichia coli strain TOP10 cells (Invitrogen) according to the
manufacturer’s instructions and confirmed by double stranded DNA sequencing
(Genewiz).

For western blot analysis, polyclonal antibodies (PAb) against recombinant LASV
NP were generated in goats and affinity purified by Autoimmune Technologies LLC,
New Orleans. 6x-His Epitope Tag MAb was purchased from Thermo Scientific.
Horseradish peroxidase (HRP)-conjugated secondary antibodies specific for goat and
mouse IgG-gamma were purchased from Kirkegaard and Perry Laboratories.

2. Expression and Purification

LASV237-Nig-2010 and Nig08-A18 plasmids were transfected in HEK-293T/17
cells seeded in HYPERFlask® Cell Culture Vessels (Corning®) using jetPRIME®
(Polyplus Transfection™) according to manufacturer’s instructions. On day three post
transfection, cell supernatant was removed and saved for later analysis and cells were
treated with lysis buffer consisting of 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP40,
and 0.1% protease inhibitor. Cellular debris was centrifuged at 15,000 x g for 10 minutes,
and supernatant retained. The 6XHis-tagged recombinant LASV NPs were purified by
affinity chromatography using HisTrap HP columns (GE Healthcare Life Sciences). The
purified protein was subsequently dialyzed into 50mM HEPES buffer.
LASV Josiah plasmids were transformed into Rosetta™ 2(DE3) Competent Cells (Novagen) and expression induced with Overnight Express™ Autoinduction System 1 (EMD Millipore) overnight at 37°C, with shaking at 250rpm. Expression cultures were harvested, centrifuged to pellet cells, and lysed using BugBuster (Novagen) supplemented with DNase I, RNase free (Novagen) and Protease Inhibitor Cocktail Set III, EDTA-Free (Calbiochem) according to the manufacturer’s instructions. The cleared lysates were purified stepwise with Ni-NTA Superflow Columns (Qiagen), dialyzed into 1X PBS with Slide-A-Lyzer™ Dialysis Cassettes, 10K MWCO (Thermo Scientific), then further purified with Ni-NTA Magnetic Agarose Beads according to manufacturer’s instructions, and subsequently dialyzed onto 10mM Tris pH 8.5, 300mM NaCl using Slide-A-Lyzer™ Dialysis Cassettes.

3. SDS PAGE, western blot, and proteomic analysis

Purified LASV237-Nig-2010, Nig08-A18, and Josiah strain NP proteins were analyzed by SDS PAGE for protein purity. Five to ten µg of total NP protein were denatured, reduced, and resolved on 10% NuPAGE Novex Bis-Tris gels, according to the manufacturer’s specifications (Novex). The gel was stained overnight in Commassie Brilliant Blue G-250 (Sigma Aldrich).

A second 10% NuPAGE Novex Bis-Tris gel was electrophoresed according to the method described above, then proteins were transferred to 0.45-µm nitrocellulose membranes, blocked, and probed in 1X PBS, pH 7.4, 5% non-fat dry milk, 1% heat inactivated fetal bovine serum, 0.05% Tween-20, and 0.1% thymerosal. Membranes were then incubated in LumiGlo chemiluminescent substrate (KPL) and exposed to Kodak BioMax MS Film.
NPΔ340 WT, NP 1-340, and NPΔ340 D389A purified proteins were resolved and visualized by one dimensional gel electrophoresis outlined above and sent to Nevada Proteomics Center at the University of Nevada, Reno for LC/MS proteomic analysis. Scaffold 4 (Proteome Software) was used to analyze data.

4. Radiolabeled dsRNA synthesis

Synthesis of radiolabeled, short dsRNA was described elsewhere [95]. The same technique was used to radiolabel pUC19 digested-Sau3A I ladder (Invitrogen). Long dsRNA was synthesized from the filovirus L gene in the pIDTBlue vector (Integrated DNA Technologies). “Sense” and “antisense” PCR products were generated using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Inc.) with primers inclusive of the T7 and T3 promoter regions according to manufacturer’s instructions (Sense Forward 5’ – GTGAGCGCGCGTAATACGACTCACTATAGGG, Sense Reverse 5’ – ACCCTCACTAGGATCCAGGGAACAAAAGCTG; Antisense Forward 5’ – CACAGGAAACAGCTATGACCATGATTACGCCAAGC, Antisense Reverse 5’ – ACTCACTATAGGATCCGGGCGAATTGGGTAC). The PCR products were purified using PureLink® PCR Purification Kit (Invitrogen) before performing a BamHI restriction digest (New England BioLabs, Inc.). Following the restriction digest and DNA purification, Sense and Antisense cDNAs were transcribed into ssRNA using T7 and T3 polymerases, respectively. T7 RNA Polymerase-Plus™ Enzyme Mix (Invitrogen) using UTP Alpha $^{32}$P according to manufacturer’s instructions generated radiolabeled sense ssRNA. T3 RNA Polymerase (Thermo Scientific) was used without radiolabeled nucleotides to produce antisense ssRNA according to manufacturer’s instructions. These ssRNA products were purified using 2% E-Gel® SizeSelect™ Agarose Gels (Invitrogen).
The products collected from electrophoresis were desalted and dialyzed with illustra MicroSpin G-25 Columns (GE Healthcare Life Sciences). The radiolabeled sense ssRNA and antisense ssRNA were heated to 95°C for five minutes to denature the RNA, immediately combined using an excess of antisense ssRNA, annealed at 65°C for 10 minutes, and cooled to room temperature and placed on ice until ready to use.

5. Exonuclease activity assay

Standard reactions contained approximately a molar ratio of 200X protein to dsRNA in reaction buffer of 20mM Tris, pH 7.5, 150mM NaCl, and 5mM MgCl₂. Approximately 1000cpm of short dsRNA was added to 10µM of NPΔ340 WT, NP 1-340, and the four NPΔ340 mutants in reaction buffer. Approximately 1200cpm of long dsRNA was added to 75µM of protein in reaction buffer. At two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours, an aliquot of standard reaction was added to RNA Loading Dye (New England BioLabs, Inc.), flash frozen on dry ice and transferred to -80°C. All reactions were thawed on ice and resolved on a 4%-20% continuous gradient urea/TBE gel (Jule, Inc.) and exposed overnight to Kodak BioMax MS Film.

6. HEK-Blue™ hTLR3 Cells assay

10µM NPΔ340 WT, NP 1-340, and NPΔ340 mutants D389A, G392A, R393A, and QuadA were serially diluted from 10µM in 1X PBS. The EC₉₀ of high molecular weight (HMW) and low molecular weight (LMW) polyinosinic:polycytidylic acid (PiC) (Invivogen) was added to the protein dilutions and incubated at room temperature for 30 minutes. These reactions were resuspended in HEK-Blue™ hTLR3 Cells and HEK-Blue™ Null1 Cells in HEK-Blue™ Detection medium according to manufacturer’s
instructions. After 16 hours in 37°C, 5% CO₂ incubator, the optical density (OD) was measured by a spectrophotometer at 630nm.

7. **Statistical analysis**

For all HEK-Blue™ experiments, the mean A₆₃₀ of HEK-Blue™ hTLR3 Cells was subtracted from the matched mean A₆₃₀ of HEK-Blue™ Null1 Cells. The EC₅₀ of LMW and HMW PiC minus basal response were calculated with the four parameter logistic nonlinear regression model. The mean A₆₃₀ were used to generate logistic regression models with the error bar indicating one standard error from the mean. The IC₅₀ of each protein was calculated using four parameter logistic nonlinear regression subtracted for basal response with a bottom constraint of zero. All statistical analyses were performed in JMP (SAS Institute, Inc.) and Prism 5 (Graphpad Software, Inc.).
RESULTS

A. Antibody response to LASV infection reveals dysregulation of IgM and IgG as determined by α-LASV NP and GPC ELISA

1. Antibody response in LASV positive patient G-1180

Blood samples were collected daily, except for day of illness 12. Day 14 post onset of illness was the last day that diagnostic tests were conducted on patient G-1180. The NP antigen capture ELISA detected LASV NP through day 11 (Figure 7A). LASV NP antigen dropped rapidly over 3 days following the onset of ribavirin treatment. Additionally, IgM levels to recombinant LASV proteins (NP alone, GPC alone, and NP, GP1, GPC combination) were detected by ELISA, with low levels of immunoglobulin to NP and GPC (Figure 7B). A blood sample for follow-up testing was obtained on day 74 post onset of illness. At that time, IgM titers against GPC and NP had risen relative to day 14. IgG titers against NP rapidly increased by day 10 and was significantly elevated between the last draw during acute illness and day 74 (Figure 7C).

Two contacts of G-1180 (G-1180-A and G-1180-B) tested positive for LASV NP antigen and IgM, and were subsequently transported to the KGH LFW for ribavirin treatment (Figure 7A and B). Despite detecting antigen and IgM in both patient sera by ELISA, neither presented with classical symptoms of the disease. Additional contact samples (G-1180-C through –L) revealed no antigen, except for one contact, and varying
levels of IgM and IgG levels despite reporting no recent febrile illness Figure 7A, B, and C).

Normal samples from four Sierra Leonean individuals and three American individuals were used as negative controls and displayed no LASV NP antigen (Figure 7A). Two Sierra Leone samples contained elevated levels of IgM and IgG without any reported cases of recent illness (Figure 7B and C). Additionally, a previous positive LASV case and contact, G-1177 and G-1177-A, respectively, were used as positive controls (Figure 7A, B, and C).
Figure 7. ELISA detection of LASV NP antigen and virus-specific immunoglobulins M and G in normal donors, G-1180, G-1180 contacts, and in two additional patient and contact sera (G-1177 and G-1177-A)

(A). LASV NP antigen was not detected in normal sera from Sierra Leone and U.S. origin, or in most G-1180 contacts. The level of LASV NP antigen (◆) in G-1180 dropped significantly during the first 3 days of ribavirin administration, to nearly undetectable levels by day 11. Conversely, antigen levels in G-1180-A and –B did not drop over the course of three days of ribavirin treatment. G-1180-F registered a significant level of antigen but did not seek treatment. G-1177 succumbed to acute Lassa fever, with very high levels of viral antigen detected before expiry. LASV-specific IgM (B) and IgG (C) were detected in a recombinant ELISA plate format, coated with NP, sGP1, and GPCdTM (●), or with individually coated proteins (▲, ■, and ×, respectively). IgM and IgG assays were performed similarly to antigen-capture ELISA. Most Sierra Leonian sera showed significant levels of IgM, IgG, or both, whereas U.S. normals did not. IgM and IgG levels in G-1180 rose throughout the course of the illness, and remained high on day 74-post onset of infection (black arrow). For G-1180 data are also plotted with IgM (▲ NP, ■ sGP1, and × GPCdTM) and IgG (▲ NP, ■ sGP1, and × GPCdTM) responses to individual LASV proteins. The IgM and IgG responses were raised primarily against NP, with a low IgG titer to GP1 detected on day 74. Data are plotted as mean $A_{450} \pm SD$, N=2. Adapted from “Capacity building permitting comprehensive monitoring of a severe case of Lassa hemorrhagic fever in Sierra Leone with a positive outcome: case report,” by J.N. Grove, et. al., 2011, Virology Journal, 8: p. 314.
Figure 7. ELISA detection of LASV NP antigen and virus-specific immunoglobulins M and G in normal donors, G-1180, G-1180 contacts, and in two additional patient and contact sera (G-1177 and G-1177-A)
2. **Antibody response in LASV positive patient G-1442**

   A blood specimen collected on patient G-1442’s day of admission was positive for LASV NP Ag by quantitative NP capture ELISA, with a level of 2.265 µg/mL NP (Figure 8A). IgM levels to individual recombinant LASV proteins (NP, sGP1, GPCdTM, Z) were determined by ELISA, with low but detectable levels of immunoglobulin to NP and Z (Figure 8B). This data suggests the patient was naïve to LASV exposure prior to this incident. Levels of low IgM response to GP1 and GPC were detected on days 11-20 when compared to naïve negative controls and G-1442 sera from days 7 - 10. Low levels of NP-specific IgG were not detected until at least day 12 post onset of illness (Figure 8C). During the monitoring period G-1442 did not develop significant IgG titers against GP1, GPC, and Z. Control serum from two healthy and one suspected Sierra Leoneans were used as negative controls and displayed no LASV NP antigen and varying levels of immunoglobulins M and G to LASV proteins (Figure 8 A, B, and C).
Figure 8. NP antigen, virus-specific IgM and IgG detection by ELISA in G-1442, normal, and contact G-1446 sera

An antigen capture ELISA was used to detect LASV NP antigen (NP Ag, ♦) in patient sera (A). LASV NP antigen was not detected in normal sera from Sierra Leonean origin, or in contact G-1446. The level of LASV NP antigen in G-1442 dropped significantly during the first 3 days of ribavirin administration, and was undetectable by day 10. LASV-specific IgM (B) and IgG (C) were assayed in a recombinant ELISA plate format, with individually coated NP (▲), sGP1 (■), GPCdTM (●), or Z (♦) proteins. One Sierra Leonean serum registered a high IgG titer to NP (NHS015), whereas the other had moderate IgM titers to NP (NHS023), but both were negative for IgG and IgM to Z and glycoproteins. NP-specific IgM and IgG levels in G-1442 rose throughout the course of the illness, through day 20. Contact G-1446 did not have measurable IgG titers, and only registered a low IgM titer to Z. Data are plotted as mean A450±SD, N=2. Adapted from “Lassa hemorrhagic fever in a late term pregnancy from northern Sierra Leone with a positive maternal outcome: case report,” by L.M. Branco, M.L. Boisen, K.G. Andersen, J.N. Grove, et al., 2011, Virology Journal, 8: p. 404.
Figure 8. NP antigen, virus-specific IgM and IgG detection by ELISA in G-1442, normal, and contact G-1446 sera

A.

B.

C.
3. Immunoglobulins M and G in LASV positive patients measured over time

The levels of LASV-specific serum IgM and IgG were compared among U.S. and West African normal controls (USN, MOY NHS and BOM NHS, respectively), surviving LF patients (LF FU), acute fatal (LF F), and nonfatal LF (LF NF) patients (Figure 9). IgM levels to LASV antigen were significantly higher in the surviving population than both LF NF and LF F cohorts (Figure 9A). Additionally, LF patient IgM and IgG titers were measured for months to years into convalescence (Figure 10). These comparisons were aimed at establishing (1) how long into convalescence LASV-specific IgM levels persist; (2) if humoral responses between fatal and nonfatal LF differed significantly at time of admission and testing, thus serving as a prognostic marker; (3) if surviving LF patients mounted a significant humoral response during treatment that subsides upon recovery from acute infection. Studies on LF carried out between 2006 and 2011 revealed LASV-specific IgM levels persist in convalescence lasting from months to years after initial infection (Figure 10A). The immunological profile was analyzed in 34 LF convalescent patients who donated whole blood for analysis between 8 weeks and 2.2 years post-discharge. The mean time between discharge and follow-up analysis for these donors was 263±221 days (ranging from 51 to 785 days). Corrected levels of LASV-specific IgM did not correlate linearly with time post discharge ($R^2 < 0.0001$, $p = 0.986$), suggesting that IgM does not subside rapidly following the rise of IgG titers in convalescence (Figure 9B). Similarly, LASV-specific IgG levels did not correlate linearly with time post discharge ($R^2 = 0.0195$, $p = 0.431$). A moderate to high level of IgG was recorded in all follow-up sera (Figure 9B and 10B).
Figure 9. IgM and IgG responses for normal donors, LF and NL febrile subjects

Box plots of LASV-specific IgM (A) and IgG (B) levels determined by ELISA, are displayed as mean $A_{450}$ with corrected cutoff values based on the 95$^{th}$ percentile of established negative control sera. Each display shows the minimum non-outlying value, three quartiles, maximum non-outlying value, and outlying values. The comparison groups include U.S. normals (US N), Moyamba district normals (MOY NHS), Bombali district normals (BOM NHS), convalescent LF follow-up patients (between 8 and 108 weeks post discharge, LF FU), nonfatal acute LF cases (LF NF), and fatal LF cases (LF F). IgM and IgG levels for patients in the LF FU sera group were significantly higher than those in for all of the other comparison groups, save for those in the BOM NHS cohort. There were no significant differences between LF NF and LF F cases for both IgM and IgG responses. BOM NHS showed relatively high levels of LASV-specific IgM and IgG, which did not significantly differ from those for LF FU patients. Outliers are indicated with red asterisks (※). Significant $p$ values for pairwise comparisons are displayed as * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. Adapted from “Emerging trends in Lassa fever: redefining the role of immunoglobulin M and inflammation in diagnosing acute infection,” by L.M. Branco, J.N. Grove, et al., 2011, Virology Journal, 8: p. 478.
Figure 9. IgM and IgG responses for normal donors, LF and NL febrile subjects
Figure 10. Regression analysis for IgM and IgG responses against number of days post-discharge for convalescent LF patients

Corrected mean $A_{450}$ values for LASV-specific IgM (A) and IgG (B) levels in LF convalescent patients did not reveal any dependence with time post-discharge for immunoglobulins responses. Hypothesis tests for the slope of each regression line revealed zero slopes for both profiles, suggesting that IgM and IgG responses for convalescent patients remained relatively constant after discharge. The fitted intercept for the regression line shown in (A) was 0.46 (SE = 0.09), which showed prolonged elevation in IgM responses for convalescent LF patients. The fitted intercept of 1.14 (SE = 0.06) for (B) was indicative of a prolonged mature humoral response (IgG) in convalescent LF patients. Adapted from “Emerging trends in Lassa fever: redefining the role of immunoglobulin M and inflammation in diagnosing acute infection,” by L.M. Branco, J.N. Grove, et al., 2011, Virology Journal, 8: p. 478.
4. Characterization of antibody response in LASV positive individuals over time with respect to phase of illness, immunoglobulin class, and epitope specificity

When permitted by patient consent and health stability, whole blood was drawn and serum was separated on multiple days for use in LASV antigen detection assays. Additionally, immunoglobulin M and G α-LASV NP and GPC ELISAs were performed. If patients survived, additional samples during the convalescent phase were taken and antibody levels were determined. For this investigation, individuals who provided samples on multiple days during acute illness (N=161), and also those who provided multiple samples from acute illness and provided at least one sample during convalescence (N=68) were analyzed by antibody capture ELISA. Samples from both the acute and convalescent stages were categorized based on changes in antibody response. During the acute phase, individuals with a negative antibody response on the first draw and a positive response (OD_{450} > negative control*3) on a subsequent draw were assigned to the “Increase” category; those who had all positive or all negative antibody responses during the acute phase were “Positive” or “Negative,” respectively; patients with an initial sample of positive and switched to negative during the acute phase were categorized as “Decrease.” During the convalescent phase, individuals were categorized based on either the change from their last acute phase sample to their convalescent sample, or based on the change of only convalescent phase samples. From these data, contingency tables and odds ratios (OR) with a 95% confidence interval (CI) were generated. We determined that regardless of immunoglobulin class (IgM or IgG) and phase (acute or convalescent), NP is the predominant immunogenic antigen. Both
immunoglobulins M and G against LASV NP are at least 3.4 times more likely to be positive during acute and convalescent phases (Table 1A). Additionally, IgM against LASV NP is not more likely to be positive in the acute phase than the convalescent phase of illness (Table 1B). However, IgM antibodies against GPC tend to decrease in the convalescent phase, which is consistent with a typical humoral response (Table 1B). During the acute phase of illness, α-LASV NP IgM is more likely present than NP-IgG, which again is consistent with the typical humoral response; however, in convalescence, neither immunoglobulin class against NP is predominant (Table 1C). These results indicate a disruption in the humoral immune response, particularly with regards to IgM antibodies against NP, during LF.
Table 1. Analysis of antibody dynamics in LASV positive individuals

The characteristics of LASV positive individuals over time were compared for antibody profiles, namely \( \alpha \)-LASV NP IgM, \( \alpha \)-LASV NP IgG, \( \alpha \)-LASV GPC IgM, and \( \alpha \)-LASV GPC IgG. Subjects with multiple sample draws over the course of acute illness or in the convalescent stage were categorized exclusively based on whether their antibody status increased, was positive over multiple sample draws, decreased, or was negative. Antibodies to LASV GPC, regardless of phase of illness or antibody class – acute or convalescent, and IgM or IgG – are more likely to be negative than positive compared to antibodies to NP (A). Interestingly, during acute illness \( \alpha \)-LASV GPC IgM is more likely to be negative than to decrease compared to NP-IgM antibodies during acute illness (A). Analyzing antibody status in acute and convalescent stages reveals that NP-IgM is more likely to be negative than to decrease during acute illness as opposed to convalescence (B). During the acute phase of illness, GPC-IgM antibodies are more likely to be positive than decrease, and more likely to be negative than decrease compared to convalescence (B). Unsurprisingly, IgM antibodies to LASV NP and GPC during acute illness were more likely than IgG antibodies to be positive than negative (C). Significant \( p \) values for OR with CI are denoted as * \( p < 0.05 \); ** \( p < 0.01 \).
Table 1. Analysis of antibody dynamics in LASV positive individuals

### A.

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
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<th>GPC (%)</th>
<th>NP (%)</th>
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### B.

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<th>Convalescent (%)</th>
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### C.

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<th>IgG (%)</th>
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<td>Negative</td>
<td></td>
<td>5 (11)</td>
<td>3 (6)</td>
<td>NS</td>
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</tbody>
</table>

### Notes:
- The OR (odds ratio) values are given along with their 95% confidence intervals (CI).
- Asterisks indicate statistical significance: *p < 0.05, **p < 0.01.
5. **Summary**

We determined that the host humoral response to LASV infection does not follow the trends of a typical immune response to pathogens. Whereas in a prototypical infection the IgM response appears shortly after infection and begins to wane weeks to a month post onset of symptoms while the IgG response raises, LASV infection alters this response such that IgM is maintained into convalescence as the IgG response gradually increases. Moreover, the IgG response does not appear to diminish as time post acute illness goes on. While immunoglobulins are produced to both LASV NP and GPC antigens, NP is the immunodominant antigen. These data suggest that there is an impairment of the host humoral immune system. We hypothesized that LASV was interfering with normal immune regulation upstream of B cell activation. To determine how the innate and cellular systems respond to LASV infection, we examined the cytokine profiles of LF patients.

B. **Circulating inflammatory mediators in Lassa fever**

1. **Cytokine profiles of patient G-1180**

Cytokine profiles were analyzed by flow cytometry on three healthy individuals and G-1180. IL-12p70, IL-1β, TNF-α, IL-6, IL-8, IL-10, and IFN-γ levels were measured during the course of acute illness (Figure 11). At the time of admission to KGH LFW at day 6-post infection, IL-12p70, IL-6, and IL-10 were elevated in G-1180 compared to the healthy controls. G-1180’s IL-12p70, IL-6 and IL-10 levels returned to normal on the following day after the commencement of intravenous ribavirin and supportive care. On day 10, a sudden increase in IL-6, IL-8, IL-10 and TNF-α levels was noted. These levels
returned to near-baseline as determined by the healthy donor controls on day 11. IL-6 was again elevated on day 13.
Figure 11. Serum cytokine levels analyzed by multiplex Flow Cytometry for G-1180

Serum cytokine levels were analyzed with a BenderMed Systems Human 11-Plex Inflammatory Cytokine kit, and an Accuri C6 Flow Cytometer equipped with dual laser detection. Data was processed and quantitated with Flow Cytomix Pro software, and plotted on linear scales. G-1180 presented with elevated levels of IL-12p70 (A), IL-6 (B), and IL-10 (B), all of which decreased by the following day. On day 10-post onset of disease a significant but transient spike in IL-1β (A), TNF-α (A), IL-6 (B), IL-8 (B), and IL-10 (B) occurred, which rapidly decreased overnight to near background levels.

Figure 11. Serum cytokine levels analyzed by multiplex Flow Cytometry for G-1180

A.

B.
2. **Cytokine profiles of patient G-1442**

Cytokine profiles were performed on serum samples collected daily (Figure 12). G-1442’s IFN-γ levels were highly elevated on the day of admission, but decreased to baseline levels by the following day and did not rise above normal levels over the ensuing 12 days of monitoring. A significant quiescence in IL-6 and IL-10 levels was noted on day 8, but levels fluctuated throughout the course of hospitalization. IL-8 levels dropped significantly on days 9 and 10, followed by a spike on day 11, and a steady decrease thereafter. IL-12p70 and -1β, and TNF-α were not detected or were present in the serum of G-1442 at very low levels at all time points analyzed. On day 20, the last day of monitoring, all cytokines with the exception of TNF-β were at or near normal levels. Interleukin-8 and TNF-α were elevated in one healthy control serum (LS004) but were within normal levels in the other healthy control serum (LS022). All other cytokines were at baseline levels in both control sera (Figure 12A and B).
Figure 12. Serum cytokine levels analyzed by multiplex Flow Cytometry for G-1442

Data generated with a Human 11-Plex Inflammatory Cytokine kit was quantified with Flow Cytomix Pro software, and plotted on linear scales. G-1442 presented with elevated levels of IL-6, IL-8, IL-10, and notably IFN-γ (B), all of which decreased by the next day, following initial treatment with ribavirin on day 7. Cytokine levels were also measured in normal Sierra Leonean controls, for comparison (LS004, LS022). Significant changes in the cytokine profile were not noted following delivery of a stillbirth fetus on day 13. Adapted from “Lassa hemorrhagic fever in a late term pregnancy from northern Sierra Leone with a positive maternal outcome: case report,” by L.M. Branco, M.L. Boisen, K.G. Andersen, J.N. Grove, et al., 2011, Virology Journal, 8: p. 404.
Figure 12. Serum cytokine levels analyzed by multiplex Flow Cytometry for G-1442

A.

B.
3. Evaluation of pro- and anti-inflammatory cytokines as related to survival

A cytokine panel revealed statistically significant differences between LF patients and non-LF controls in the levels of IL-6, IL-8, and IL-10 (Figure 13). IFN-γ showed a wider range of increased expression in fatal cases than nonfatal cases, but the differences in median expression levels were not statistically different. Similarly, statistical differences were not observed between fatal and nonfatal LF cases for levels of IL-1β, which tended to be lower than normal for both fatal and nonfatal LF groups compared to convalescent individuals. IL-6 and IL-10 were significantly higher for fatal LF cases than LF NF and normal donor levels, but differences were not observed between nonfatal LF and normal controls. Additionally, IL-12p70 appeared to be elevated in LF F individuals compared to normal controls. TNF-α did not appear to be influential in the pathogenesis of LF (data not shown). The LF convalescent group displayed cytokines and metabolic markers similar to those recorded in normal individuals, with the exception of IL-1β and IL-6, which showed a higher range than normal donors.
Figure 13. Levels of relevant cytokines in LF pathogenesis

Serum levels of IL-6 and IL-10 showed significant differences between LF NF and LF F patients on admission. For LF NF patients, IL-8 levels were reduced when compared to LF F subjects, and significantly reduced when compared to normal, and LF FU subjects. Elevated levels of IL-6 and IL-10 were recorded in LF F patients, but were significantly lower in LF NF subjects. IFN-γ was significantly higher for LF F patients than normal donors and convalescent patient controls, but IFN-γ levels did not differ between LF F and NF patients. Similarly, IL-12p70 levels were significantly elevated in LF F when compared to normal donors, but did not differ from the other comparison groups. IL-1β was significantly increased in convalescent subjects when compared with normal controls, LF F, and LF NF individuals. Outliers are denoted with black dots (●). Significant p values for pairwise comparisons are denoted as * p < 0.05; ** p < 0.01; and *** p < 0.001. Adapted from “Emerging trends in Lassa fever: redefining the role of immunoglobulin M and inflammation in diagnosing acute infection,” by L.M. Branco, J.N. Grove, et al., 2011, Virology Journal, 8: p. 478.
Figure 13. Levels of relevant cytokines in LF pathogenesis
4. Evaluation of pro- and anti-inflammatory cytokines as related to antibody status

The cytokines of LASV positive individuals, whom were categorized based on the change in their antibody status, were measured to establish trends in the dynamics of the humoral response. Patients were grouped into the same categories as described in Table 1. Briefly, individuals who received multiple blood draws were analyzed for IgM and IgG responses against LASV. Samples from the acute phase (N=58) of illness were categorized based on changes in antibody response and compared to normal (N=8) and convalescent samples (N=8). From the acute phase, samples were grouped according to their change in either IgM alone, IgG alone, or change in both IgM and IgG (IgM+IgG) (Figure 14). IL-8 levels were significantly lower in individuals who were either positive or negative for antibodies, with the exception of IgM, compared to normal controls. There was not a significant difference between any individuals who underwent either an increase or decrease during the course of testing. IL-1β and TNF-α levels were significantly higher in the convalescent cohort than normal controls and in IgM+IgG increase, positive, and negative individuals. IL-1β was also significantly higher in convalescent individuals compared to the IgM positive group, and IgG increase, positive, and negative groups. Individuals who were negative for IgM throughout acute illness experienced elevated IFN-γ levels compared to the convalescent cohort. IL-6 was significantly higher in patients who had decreasing IgG levels compared to those who had an increasing, positive, or negative IgG response, and normal individuals. IL-10 did not appear to correlate with antibody responses.
Figure 14. Levels of relevant cytokines with respect to the adaptive immune response

The cytokine levels of LASV positive individuals over time were measured in individuals during acute illness. The overall antibody response, the IgM response, and the IgG response were compared within their respective categories or to normal and convalescent samples. Subjects with multiple sample draws over the course of acute illness or in the convalescent stage were categorized based on whether their antibody status increased, was positive over multiple sample draws, decreased, or was negative. IL-1β and TNF-α levels were significantly higher in convalescent patients. IL-1β was lower in normal individuals, LASV positive patients with an increasing, positive, and negative overall antibody response and IgG response, and a positive IgM response compared to convalescent samples. TNF-α levels in convalescent samples were significantly higher than levels in normal samples and individuals with an overall antibody response that was increasing, positive, and negative. There was little difference observed in the levels of IFN-γ and IL-10. IL-6 levels were significantly higher in individuals who experienced decreasing IgG levels during acute illness compared to increasing, positive or negative, and normal samples. IL-8 levels were generally significantly lower than normal samples. Outliers are denoted with black dots (●). Significant p values for pairwise comparisons are denoted as * p < 0.05; ** p < 0.01; and *** p < 0.001.
Figure 14. Levels of relevant cytokines with respect to the adaptive immune response.
Figure 14. Levels of relevant cytokines with respect to the adaptive immune response (con’t)
5. T cell activity upon restimulation with rLASV proteins in convalescent PBMCs

PBMCs were collected from three convalescent patients and two control individuals (one U.S. – USN, one Sierra Leone – SLN) and seeded on plates coated with antibodies against IL-2 and IFN-γ. The cells were then stimulated with α-CD28, commonly used to co-stimulate T cells, rLASV antigens NP, sGP1, or GPC, or no stimulant for approximately 16 hours. Though α-CD28 is not a true positive control because there needs to be antigenic stimulation to generate cytokine production, it does provide a good baseline for T cell activation in the absence of antigen. Cells secreting IL-2, IFN-γ, or both were counted via fluorospot analysis. IL-2 secreting CD4+ T cells represent proliferative Th0 cells, while IFN-γ cells represent CD4+ Th and CD8+ T cell populations and, and T cells that secrete both IL-2 and IFN-γ are indicative of memory T cell subsets [227-229]. Convalescent patients restimulated with rLASV protein do not produce more IL-2 than normal control samples (Figure 15A). The only elevation in IL-2 levels observed was in convalescent patient one stimulated with sGP1 (significant compared to α-CD28 and no stimulant, p < 0.05), indicating that T cells are undergoing proliferation in response to this antigen (Figure 15A). Alternatively, stimulation with rLASV proteins caused increased IFN-γ in two of the three convalescent patients (Figure 15B). Convalescent patient 1 experienced significantly elevated levels in response to rLASV NP compared to USN (p < 0.01). Additionally, the levels produced in response to NP antigen were also significantly higher than all other stimulants. In the second convalescent patient, IFN-γ levels were higher in cells stimulated with all three rLASV proteins compared to both USN and SLN (p < 0.01). rLASV stimulants NP, sGP1, and
GPC each produced significantly elevated IFN-γ levels in convalescent patient 2 compared to cells stimulated with α-CD28 or with nothing (p < 0.01, p < 0.001, and p < 0.001, respectively). The rLASV proteins induced a strong T_H1 or CTL response in these convalescent patients. However, there appeared to be very little memory T cell stimulation by rLASV proteins (Figure 15C). Only convalescent patient 1 experienced significantly elevated levels of dual-secreting T cells in response to sGP1 compared to those same cells stimulated with NP, GPC, or nothing (p < 0.05 each). Memory T cells are not only important in controlling a secondary infection, but also signify a robust immune response. The absence of these cells in LASV convalescent individuals indicates an impaired immune response. One caveat that may explain the low memory T cell population and proliferation of CD4^+ T cells observed in these individuals is co-infection with HIV, as sub-Saharan Africa is the most afflicted region [230]. Though we were unable to test for HIV co-infection, establishing a proper positive control such as α-CD3 or PMA and ionomycin will be important for future investigations.
Figure 15. Convalescent T cell response to rLASV antigen stimulation

PBMCs from convalescent patients (Convalescent 1-3) and normal individuals (USN, SLN) stimulated with α-CD28, rLASV NP, sGP1, or GPC, or nothing were analyzed for IL-2 and IFN-γ secretion by fluorospot. We observed very little IL-2 (Figure 15A) secretion or co-secretion with IL-2 or IFN-γ (Figure 15C). However, stimulation with the rLASV proteins induced significantly high levels of IFN-γ in two convalescent patients compared to the sample controls (USN and SLN) and to the stimulant controls (α-CD28 and none) (Figure 15B). Results are displayed as the mean +SD of duplicates, and significant p values are denoted as * p < 0.05; ** p < 0.01; and *** p < 0.001.
Figure 15. Convalescent T cell response to rLASV antigen stimulation

A.

B.

C.
6. Summary

Cytokines relevant to the production of antibodies were studied in LF patients to elucidate trends and determine how LASV may be impacting the humoral immune response. IL-12p70 appeared to be suppressed in LASV infection showing no statistically significant difference between individuals with LF and those without, except for the LF F cohort. The other interleukins, IL-1β, IL-6, IL-8, and IL-10, reveal varying levels of activation during acute illness. Interestingly, IL-1β is not expressed at high levels during acute illness, but appears to increase in convalescence. IL-8 levels are increased in the early stages of infection when the infection is still not under control; however, once the humoral immune response is established, IL-8 tends to decrease. Similarly, IL-6 and IL-10 appear to fluctuate early in infection, though they diminish with time and the presence of activated B cells. There is a significant difference between LF F and LF NF cohorts for both of these cytokines whereby individuals who succumb tend to have significantly elevated levels of IL-6 and IL-10. IL-10, an anti-inflammatory molecule, is capable of suppressing T cell activation, while IL-6 stimulates CD4⁺ T cell proliferation independent of IL-2 [231]. This pro- and anti-inflammatory dysregulation may be detrimental to LF patients. In surviving patients months out from acute illness, we observed low levels of IL-2 induction by stimulation with rLASV proteins. Additionally we saw very little memory cell activation with NP, sGP1 or GPC with the exception of one convalescent patient. Therefore, depressed levels of cytokines, which likely result in diminished CD4⁺ T cell activation and B cell class switching events, may in fact be advantageous. To determine what mechanism may be causing the impaired, albeit potentially beneficial, immune response, we investigated early events that initiate important immune cascades.
LASV readily infects dendritic cells, and NP is known to downregulate IFN-I in this cell type. With these considerations, we hypothesized that the exonuclease activity of NP, which is specific for dsRNA, is downregulating IFN-I and consequently effecting the adaptive immune response via the TLR3 pathway.

C. LASV WT and mutants possess exonuclease activity specific for long dsRNA resulting in abrogation of TLR3 signaling

1. LASV NP production in mammalian and bacterial cells

   a) Clade II and III NP production in mammalian cells

   In order to diversify the repertoire of LASV proteins available to ensure consistent exonuclease activity, we cloned, expressed, and purified clade II and III LASV NP, which are currently circulating in large areas of West Africa (Supplemental Figure 1). Using sequences derived from patients in Nigeria at two different time points, NP genes from LASV237-Nig-2010 and Nig08-A18 were cloned into the mammalian expression vector pcdna3.1+intA such that a 6xHis tag was expressed either on the N terminus, C terminus, or both termini of the protein for purification purposes. The first successful PCR products were genes tagged with the 6xHis on either the N terminus or the C terminus; therefore, these four products were ligated into the vector and transformed. Once transformed, bacterial colonies were chosen for sequencing analysis. The expression constructs that were selected contained the 6xHis tag on the C terminus. The DNA plasmids were transfected into HEK-293T/17 cells and harvested on day three. Cells were lysed and pelleted, and the resulting supernatant was purified by affinity chromatography using an α6xHis column. The protein was eluted from the column with a
range of acidic elution buffers or buffers containing imidazole (Supplemental Figure 2). Upon dialysis into buffers suitable for downstream assays, the protein precipitated. At this point, we chose to utilize plasmids generously provided to us by the Saphire Lab at The Scripps Research Institute.

b) LASV NP clade IV Josiah strain production in bacterial cells

Plasmids containing wild type (WT) genes coding either the first 340 residues of NP Josiah (NP1-340) or residues 314-569 (NPΔ340) were utilized. NP1-340 possesses only the viral RNA binding domain while NPΔ340 possesses only the exonuclease domain. Additionally, point mutations in the exonuclease domain have resulted in the following mutants: NPΔ340D389A, NPΔ340G392A, NPΔ340R393A, and NPΔ340QuadA (four residue mutations KΔ516 KΔ517 KΔ518 KΔ519). Rosetta 2(DE3)pLysS competent cells were cultured in 250 mL Terrific Broth supplemented with Overnight Express™ Autoinduction System (Novagen) in 1L baffled flasks, which eliminated the need for induction of plasmid expression by IPTG and yielded grams of cell paste. Cultures were harvested by centrifugation, cells were lysed, and the supernatant was clarified for α-His tag affinity purification. The protein was first passed through an α-His gravity column and then further purified with nickel magnetic beads to yield a highly pure protein solution (Supplemental Figure 3) at several milligrams per culture. All expression and purification steps were performed under RNase-free conditions to ensure downstream experiments were not contaminated with agents that also possessed exonuclease function. NPΔ340WT, NP1-340, and NPΔ340D389A purified protein samples underwent one dimensional gel electrophoresis followed by liquid chromatography/mass spectrometry (LC/MS) analysis to ensure there was no contamination in the purification process (Supplemental Figure 4).
The band at 37kDa from the first lane and bands at 28kDa and 25kDa from the second and third lanes were excised. Excised bands 28kDa and 25kDa were combined from each of the two lanes for proteomic analysis. LASV NP Josiah was the predominant protein found in these samples (data not shown). Subsequently, the remaining contents of lanes were combined and analyzed for protein content. LASV NP again the predominant protein in the sample followed by some cellular contaminants (Supplemental Figure 4). However, none of these extraneous proteins possessed exonuclease activity. Therefore, activities recorded from these samples are a result of the LASV NP function.

2. The exonuclease activity of LASV NPΔ340\(^{\text{WT}}\), NP\(_{1-340}\), and NPΔ340 mutants with short dsRNA, long dsRNA, and LMW PiC substrates and the effect on TLR3 activation

In previous studies using these proteins, mutants NPΔ340\(^{D389A}\) and NPΔ340\(^{G392A}\) have been shown to lose exonuclease activity compared to NPΔ340\(^{\text{WT}}\) [94, 95]. Similarly, NP\(_{1-340}\) has no reported exonuclease activity; this portion of the protein does not possess the exonuclease domain. However, mutants NPΔ340\(^{R393A}\) and, to a lesser extent, NPΔ340\(^{\text{QuadA}}\) maintain exonuclease activity compared to NPΔ340\(^{\text{WT}}\) [95]. These previous studies, however, visually measure activity on dsRNA only shorter than 22bp. The studies performed herein use the same short dsRNA (18bp) as well as a long dsRNA oligonucleotide (591bp) generated to mimic a more realistic size of dsRNA in vivo (Supplemental Figure 5). These short and long oligonucleotides were radiolabeled and resolved on a polyacrylamide gel to establish the amount of degradation by NP and mutants over time.
Additionally, the effect of exonuclease activity on TLR3 activation was measured using LMW PiC and HEK-Blue™ hTLR3 Cells. Briefly, this cell line is co-transfected with human TLR3 (hTLR3) and a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of IFN-β minimal reporter. Upon stimulation of hTLR3 by LMW PiC, SEAP is secreted and quantified to measure the level of hTLR3 activation. The EC\textsubscript{90} of LMW PiC (average molecular size 600bp) was determined in the absence of protein (data not shown). 1.8 \mu g/mL LMW PiC (9nM calculated using the average molecular weight) was incubated in the presence of serially diluted proteins.

NP\Delta340\textsuperscript{WT} was efficient at degrading both short and long radiolabeled dsRNA (Figure 16A). Nearly half of both dsRNA substrates were fully degraded by 30 minutes. The lack of signal at two hours and suggest that NP\Delta340\textsuperscript{WT} is capable of degrading dsRNA into fragments less than 8nt. As controls, substrate was incubated with LASV GPC (Non-NP Protein), having no known exonuclease activity, and in the absence of protein (dsRNA only) (Figure 16A). Both short and long dsRNA did not degrade over time. Of note, the Non-NP Protein sample volumes at time points six and 18 hours was nearly half (6\mu L versus 10\mu L) of the former time point samples. As a result, it the bands appear lighter. Moreover, there is smearing of all bands from the two control samples indicating there is likely some exogenous RNase contamination and/ or that RNA synthesis and annealing was not completely efficient, resulting in shorter sense strand fragments or the folding of sense ssRNA onto itself.

NP\Delta340\textsuperscript{WT} also successfully diminished TLR3 signaling in co-transfected HEK cells compared to cells activated with 9nM LMW PiC (PiC) alone and the negative
control (no PiC, NC). 10μM protein inhibited the EC$_{90}$ of LMW PiC from activating TLR3.

NP$_{1-340}$ has no reported exonuclease activity; however, it has been suggested that this domain of the protein is capable of binding cap structure m7GTP and that mutations to the N terminus severely diminished RNA transcription [94]. In previous studies, NP$_{1,340}$ showed no exonuclease activity on short dsRNA [95]. This supports our results using short radiolabeled dsRNA (Figure 17A). However, NP$_{1-340}$ was capable of degrading long radiolabeled dsRNA to about 20bp at the same pace as NPΔ340$^{WT}$. This result was supported by HEK-Blue™ hTLR3 cell assay (Figure 17B). NP$_{1-340}$ degraded LMW PiC more efficiently than NPΔ340$^{WT}$ causing diminished activation of TLR3.

The mutant, NPΔ340$^{D389A}$, was unable to degrade short dsRNA, however, it regained exonuclease activity in the presence of long dsRNA (Figure 18A). Similar to NP$_{1-340}$, NPΔ340$^{D389A}$ degraded the substrate to about 20bp fragments, yet is not as efficiently. The cellular assay revealed that NPΔ340$^{D389A}$ mutant downregulated stimulation of TLR3 by LMW PiC, though to a lesser degree than NPΔ340$^{WT}$ (Figure 18B). Mutant NPΔ340$^{G392A}$ possessed analogous functionality to NPΔ340$^{D389A}$. While NPΔ340$^{G392A}$ degraded long dsRNA to 20bp fragments, it was unable to degrade short dsRNA (Figure 19A). It also demonstrated the ability to degrade LMW PiC such that TLR3 stimulation was inhibited (Figure 19B).

The mutants that retained all or some exonuclease activity in previous studies [95] – NPΔ340$^{R393A}$ and NPΔ340$^{QuadA}$, respectively – maintained efficient function in the presence of long dsRNA. NPΔ340$^{R393A}$ degraded both long and short substrates to fragments smaller than 8bp, though less efficiently than NPΔ340$^{WT}$ (Figure 20A).
Moreover, NPΔ340^{R393A} began to diminish LMW PiC stimulation of TLR3 at about 1µM (Figure 20B). While NPΔ340^{QuadA} appeared to degrade long and short dsRNA at a rate similar to NPΔ340^{WT} in the radioassays (Figure 21A), it demonstrated diminished, albeit bona fide, function compared to NPΔ340^{WT} (Figure 21B).
Figure 16. NPAΔ340WT exonuclease activity effect on short and long radiolabeled dsRNA and on stimulation of hTLR3 by LMW PiC

Short and long radiolabeled dsRNA was incubated with NPAΔ340WT for two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours. NPAΔ340WT quickly degraded both long and short dsRNA compared to dsRNA incubated with the control protein, GPC, and no protein (A). While 100nM and below of NPAΔ340WT failed to prevent stimulation of hTLR3 by LMW PiC EC90, background levels of hTLR3 stimulation were observed at 10µM and 1µM of protein (B). Values are mean ±SE.
Figure 16. NPΔ340<sup>WT</sup> exonuclease activity on short and long radiolabeled dsRNA
and stimulation of hTLR3 by LMW PiC
Figure 17. NP<sub>1-340</sub> exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC

Short and long radiolabeled dsRNA was incubated with NP<sub>1-340</sub> for two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours. NP<sub>1-340</sub> quickly degraded long dsRNA at a rate comparable to NPΔ340<sup>WT</sup> (A). However, NP<sub>1-340</sub> was not able to degrade fragments shorter than about 20bp as seen by the accumulation of long radiolabeled dsRNA at the bottom of the gel and by lack of any short dsRNA degradation (A). NP<sub>1-340</sub> appeared to degrade LMW PiC more efficiently than NPΔ340<sup>WT</sup> as demonstrated by levels of hTLR3 stimulation (B). Values are mean ±SE.
Figure 17. NP_{1-340} exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC
Figure 18. NPΔ340^{D389A} exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC

Short and long radiolabeled dsRNA was incubated with NPΔ340^{D389A} for two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours. NPΔ340^{D389A} was capable of degrading long dsRNA; however, the protein was not able to degrade fragments smaller than about 20bp as seen by the accumulation of long radiolabeled dsRNA at the bottom of the gel (A). NPΔ340^{D389A} was unable to degrade short dsRNA (A). NPΔ340^{D389A} appeared to degrade LMW PiC at a rate similar to NPΔ340^{WT}, though ultimately not to the same level as demonstrated by hTLR3 stimulation (B). Values are mean ±SE.
Figure 18. NPΔ340D389A exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC
Figure 19. NPAΔ340G392A exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC

Short and long radiolabeled dsRNA was incubated with NPAΔ340G392A for two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours. NPAΔ340G392A was capable of degrading long dsRNA; however, the protein was not able to degrade fragments smaller than about 20bp as seen by the accumulation of long radiolabeled dsRNA at the bottom of the gel (A). NPAΔ340 G389A was unable to degrade short dsRNA (A). NPAΔ340G392A appeared to degrade LMW PiC at a similar rate to NPAΔ340WT as demonstrated by hTLR3 stimulation (B). Values are mean ±SE.
Figure 19. NPΔ340<sup>G392A</sup> exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC.

A.

B.
Figure 20. NPΔ340R393A exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC

Short and long radiolabeled dsRNA was incubated with NPΔ340R393A for two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours. NPΔ340R393A quickly degraded long dsRNA, albeit at a slower rate than NPΔ340WT (A). NPΔ340R393A also efficiently degraded short dsRNA (A). NP1-340 degraded LMW PiC at a rate similar to NPΔ340WT as demonstrated by levels of hTLR3 stimulation (B). Values are mean ±SE.
Figure 20. NPΔ340R393A exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC
Figure 21. NPΔ340\textsuperscript{QuadA} exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC

Short and long radiolabeled dsRNA was incubated with NPΔ340\textsuperscript{QuadA} for two minutes, five minutes, 30 minutes, one hour, two hours, six hours, and 18 hours. NPΔ340 Quad quickly degraded long dsRNA (A). NPΔ340\textsuperscript{QuadA} also efficiently degraded short dsRNA (A). NPΔ340\textsuperscript{QuadA} degraded LMW PiC at a seemingly slower rate than NPΔ340\textsuperscript{WT} as demonstrated by levels of hTLR3 stimulation (B). Values are mean ±SE.
Figure 21. NPΔ340^{QuadA} exonuclease activity on short and long radiolabeled dsRNA and stimulation of hTLR3 by LMW PiC.
3. **Exonuclease activity of proteins compared using HMW PiC**

HMW PiC elicits a stronger hTLR3 response and therefore was used to compare the cohort of LASV proteins. A dilution series of LASVΔ340 WT, NP1-340, and NPΔ340 D389A, G392A, R393A, QuadA was incubated with HMW PiC EC_{90} for 30 minutes and applied to HEK-Blue™ hTLR3 Cells and Null1 Cells overnight. Similar to the previous results, NP1-340 appeared to degrade dsRNA more efficiently than NPΔ340 WT or any of the mutants, as demonstrated by a lower A_{630} at 100nM and 1µM (Figure 22A). The IC_{50} of NP1-340 was 0.09µM (Figure 22B). NPΔ340 WT and mutant proteins except for NPΔ340 R393A, however, degrade HMW PiC at similar rates according to the CI of IC_{50} for the respective mutants (Figure 22A and B).
Figure 22. Exonuclease activity of proteins compared using HMW PiC

NPΔ340\textsuperscript{WT} and mutants degraded HMW PiC at similar rates such that hTLR3 stimulation was at near background levels (A). NP\textsubscript{1-340}, however, degraded HMW PiC at a faster rate indicating that this portion of the protein may function as more than an RNA binding domain (A). The IC\textsubscript{50} were determined by nonlinear 4 parameter logistic regression with a bottom constraint of zero and 95% CI (B).

\begin{itemize}
  \item \textbf{A.}
  \begin{figure}
  \centering
  \includegraphics[width=\textwidth]{figure22a.png}
  \caption{Graph showing the exonuclease activity of various proteins compared using HMW PiC.}
  \end{figure}

  \item \textbf{B.}
  \begin{table}
  \centering
  \begin{tabular}{|c|c|c|c|c|c|}
    \hline
    \textbf{IC\textsubscript{50} (µM)} & \textbf{WT} & \textbf{NP 1-340} & \textbf{D389A} & \textbf{G392A} & \textbf{R393A} & \textbf{QuadA} \\
    \textbf{(CI, 95%)} & (0.642, 1.09) & (0.066, 0.121) & (0.481, 7.05) & (0.585, 2.39) & (1.17, 2.77) & (0.931, 1.24) \\
    \hline
  \end{tabular}
  \caption{IC\textsubscript{50} values for various proteins degraded by HMW PiC.}
  \end{table}
\end{itemize}
4. Summary

The exonuclease activity of LASV was characterized using long dsRNA in parallel with the already defined effects of short dsRNA. It has been shown that NP\textsubscript{1-340}, and NP\textDelta{340} mutants D389A and G392A, do not degrade short dsRNA [95], whereas NP\textDelta{340}\textsuperscript{WT}, NP\textDelta{340}\textsuperscript{R393A}, and NP\textDelta{340}\textsuperscript{QuadA} exhibit exonuclease activity in the presence of short dsRNA, or maintain exonuclease activity despite mutations to the exonuclease domain [95]. To depict more realistic conditions, we synthesized long dsRNA and found discrepancies between the results using short dsRNA or long dsRNA. NP\textsubscript{1-340}, NP\textDelta{340}\textsuperscript{WT}, and each of the mutant proteins were able to degrade long dsRNA. Interestingly, the proteins that could not degrade short dsRNA were unable to degrade dsRNA fragments smaller than about 20bp, while NP\textDelta{340}\textsuperscript{WT}, NP\textDelta{340}\textsuperscript{R393A}, and NP\textDelta{340}\textsuperscript{QuadA} degraded dsRNA to a size that could no longer be detected.

Examination of the exonuclease effects LMW PiC and HMW PiC revealed that LASV is capable of immune suppression via the TLR3 pathway. Cells incubated with either LMW PiC or HMW PiC produced elevated levels of SEAP, which is under the control of IFN-β minimal promoter and activated by hTLR3 stimulation. However, when LMW PiC or HMW PiC is incubated with NP\textsubscript{1-340}, NP\textDelta{340}\textsuperscript{WT}, or any of the mutants and then added to the HEK-Blue\textsuperscript{™} hTLR3 Cells, SEAP levels diminish with increasing molar concentrations of protein.
DISCUSSION

The immunological arms necessary to generate an efficient and productive response to LASV infection have largely remained a mystery until recently. Historically, LASV viremia has been the primary marker of acute infection as assessed by Ag-capture ELISA, LASV RNA detected by RT-PCR or virus culture. Alternatively, elevated LASV-specific IgM has served as a surrogate marker of a recent infection when LASV Ag could no longer be detected [220]. With little known about LASV-specific humoral immune responses and immunopathology, the rationale for considering IgM positivity as acute LASV infection was based on observations in other pathogenic infections in which a drop in antigen levels coincided with increasing IgM titers that class-switched to a predominantly IgG status within weeks of initial infection. However, data presented herein and recent discoveries suggest IgM may not represent a recent infection and they may be indicative of viral impairment of the host immune system.

When patient G-1180 presented to the KGH LFW on day six post onset of symptoms, levels of NP-specific IgM were low indicating naiveté to LASV. The levels progressively increased throughout the monitoring period. Conversely, the GPC-specific IgM titers were not statistically above background levels throughout the same period (Figure 7B). On day 10, low IgG titers were detectable in G-1180. Immunoglobulins were again measured on day 74 post onset of illness in G-1180, and surprisingly, IgM titers against GPC and NP had risen relative to day 14, rather than subsiding. An
expected increase in IgG titers to LASV proteins was also observed (Figure 7C); however, IgM and IgG levels against GPC were not significant on day 74.

The two contacts of G-1180 that were admitted to the KGH LFW presented with lower, albeit significant, levels of LASV NP antigen compared to G-1180 (Figure 7A). Surprisingly, and despite presence of NP antigen in the serum of both G-1180-A and -B contacts by ELISA, levels did not decrease over a two day period following treatment with ribavirin. Neither contact presented with or developed classical symptoms of LF during their 10 day hospitalization period. Contact G-1180-A presented with very low but detectable IgM titer, while G-1180-B presented with significant IgM (Figure 7B). In both contacts, IgG titers were detectable from the outset, but remained unchanged throughout the three days of testing (Figure 7C). Thus, potential previous exposure to LASV and development of protective humoral or cellular immunity may have contributed to the observed Ag positive but asymptomatic status of these two patients.

ELISA data suggested that patient G-1442, similar to G-1180, was also naïve to infection as very low LASV-specific IgM were recorded to all viral proteins analyzed at seven days post onset of symptoms (Figure 8B). G-1442 then began showing a consistent increase in NP-specific IgM, and a low level IgM response against GPC starting on day 11, which continued throughout the monitoring period. Only IgG to NP developed over the analysis timeline (Figure 8C).

A larger cohort of patients revealed trends similar to G-1180 and G-1442. While IgM levels were elevated throughout the cohort, including normal African controls (MOY NHS and BOM NHS), there was a statistically significant elevation in the convalescent group compared to those with acute infection (Figure 9A). Moreover,
acutely ill patients, regardless of their survival status (LF NF and LF F), had LASV IgG titers comparable to U.S. normal subjects (US N). Only in NHS and convalescent samples were IgG levels significantly elevated (Figure 9B). There was no correlation with the level of immunoglobulins and time from acute illness. IgM levels, expected to decrease over time, remained elevated in convalescence, and were invariably paralleled by rising, though temporally delayed, IgG titers (Figure 10A and B).

To identify a source of humoral immune disruption, we analyzed cytokine profiles of LF patients. The most remarkable aspect of the cytokine profile obtained from patient G-1180 at the time of admission was the significant level of IL-12p70 measured in serum (Figure 11A). This pleiotropic pro-inflammatory cytokine is produced by dendritic cells, B cells, and other antigen-presenting cells. In dendritic cells, it becomes activated by PRRs in the presence of PAMPs. IL-12 can modulate adaptive immune responses, promote Th1 cell development, and induce of IFN-γ and other cytokines in peripheral blood T and NK cells. Acting as a positive feedback loop, IFN-γ further enhances production of additional IL-12 and other pro-inflammatory cytokines by phagocytic cells [232]. Remarkably, on day 7, IL-12p70 was not detected, but its decrease coincided with a rise in IFN-γ levels, which remained elevated through day 10 (Figure 11A and B). Another increase in IL-12p70 levels was measured on days 11 and 12, the latter coinciding with a second spike in IFN-γ. Though IL-12p70 may have some impact on pathogenesis of Lassa fever, its role in arenaviral pathogenesis has not been described.

Despite this profile early in the monitoring period of G-1180, other pro-inflammatory cytokines such as IL-1β and TNF-α were undetectable (Figure 11A), and IL-6 and IL-8 fluctuated on the lower end of the assay range (Figure 11B). Elevated
levels of IL-8 have been previously reported by Mahanty et al. to correlate with a positive outcome in acute Lassa fever infections [220], in addition to IFN-induced IP-10, which was not measured in these studies. A very notable albeit short increase in pro-inflammatory cytokines was measured on day 10, when IL-6 and spiked to very high levels, in addition to a small spike in IL-1β and TNF-α. These levels dropped to baseline or were undetectable 24 hours later.

Concurrent with this observed increase in pro-inflammatory cytokines, a significant spike was measured in the IL-10 levels (Figure 11B), an anti-inflammatory cytokine, and potent inhibitor of Th1 cytokines and cellular responses. This spike coincided with the onset of a significant increase in the levels of LASV-specific IgM and IgG. Notably, IL-10 levels were elevated throughout the seven days of monitoring. Thus, it appears that G-1180 experienced a dynamic interplay of pro- and anti-inflammatory cytokines, and their physiological effects were ongoing throughout the extended analysis. Though this patient survived, there was considerable supportive care given in addition to treatment with ribavirin. Therefore, an important caveat to all of these results is the effect of the course of therapy provided during acute illness.

The marked absence of TNF-α, a potent inducer of endothelial activation [233] and thrombocytopenia [234] throughout the monitored course of illness in G-1180 (Figure 11A), with the exception of a spike on day 10, suggests a somewhat regulated and effective immune response at play. This applies to the absence of IL-1β during the same period. It has been suggested that an early pro-inflammatory cytokine response followed by downregulation to baseline levels, which has been characterized in Ebola
patients, may also be important in a regulated and balanced immune response and outcome in LF [235].

At the time of admission, patient G-1442 presented with elevated serum levels of IFN-γ, IL-6, and IL-8 (Figure 12B). Elevated IFN-γ and IL-6 levels are common in non-lethal LF and other febrile illnesses alike, but are highly variable in fatal cases of LF [220, 236]. Elevated IL-8 levels have been associated with positive outcomes in acute LF, but are also common in native Sierra Leonean healthy controls [220]. The anti-inflammatory cytokine IL-10 was elevated in the serum of G-1442 throughout the treatment period (Figure 12B). IL-1β and IL-12p70 was not detected in G-1442 throughout the course of recovery from LF, while TNF-α was present at very low levels at admission and quickly subsided (Figure 12A). The levels of serum cytokines in patient G-1442 in conjunction with those obtained for G-1180 strengthen the hypothesis that an imbalance between pro- and pre-inflammatory cytokines plays an important role in the development of LF and could contribute to a deficient adaptive immune response.

In examining a larger sample size grouped by survivors (LF NF) and fatalities (LF F), we discovered that low levels of IFN-γ, IL-6, IL-8, and IL-10 might be predictive of survival (Figure 13). Although there was not a significant reduction in the overall levels of IL-8 between normal donors and LF F patients, a significant drop in the levels of this cytokine was observed in most patients who survived LASV infection. The frequent exposure of populations to parasitic infections, and probable co-infections, is a likely factor in the elevated levels of recorded IL-8 in the sampled normal and convalescent groups. Thus, our data from this report and others [236-238] suggest that a regulated quiescence of the inflammatory response may be central to a successful outcome in
symptomatic acute LF. In three surviving LF patients who were months into convalescents, their PBMCs stimulated with rLASV proteins showed little activation of memory T cells (Figure 15C) or proliferation of T\textsubscript{H} cells (Figure 15A). However, IFN-\(\gamma\) became elevated with the addition of rLASV antigen. This can be attributed to either the activation of T\textsubscript{H}1 cells or CTL; however, given previous investigations [16, 17, 43, 127, 130, 131, 208-211] and our findings that the humoral response is impaired and there is marked cytokine dysregulation, particularly with respect functional T\textsubscript{H} cells, we believe the elevation of IFN-\(\gamma\) is due to CTL stimulation. Further investigations will need to be performed in order to support this.

To understand the dynamics of the immune response, we collected samples from LASV infected individuals at several time points during acute illness and in convalescence. We discovered individuals in both the acute and convalescent stages are more likely to be positive for NP-specific IgM antibodies as opposed to negative. Similarly, IgG antibodies present in individuals during the acute and convalescent stages are more likely to be positive against the NP antigen (Table 1A). We did not observe any significant changes (increase or decrease) in the IgM status against NP except during the acute phase. During this phase NP-IgM was more likely to decrease than be negative, meaning the patient developed a response that subsided before reaching convalescence (Table 1A, 1B). Otherwise, patients tended to develop an early and strong NP-IgM response that likely persisted into convalescence (Table 1A). Interestingly, individuals that mounted a GPC-IgM response during acute illness were more likely to experience a decrease in those levels in convalescence as opposed to being strictly positive or negative for GPC-IgM (Table 1B). Additionally, immunoglobulins to GPC during acute illness are
far more likely to be of the M class than G (Table 1C). This suggests that LASV positive
individuals capable of developing an IgM response against GPC likely won’t maintain
those levels into convalescence. Ultimately, these data suggest a sustained IgM response
with specificity primarily to LASV NP. The IgG response, though delayed, tends to be
against NP as well, and while GPC elicits an IgM response during acute infection, albeit
at levels lower than NP, the occurrence of class switching is rare.

To understand the effects the inflammatory response has on the dynamics of the
humoral response, the cytokine profiles of these same sample groups were also analyzed
(Figure 14). IFN-γ was elevated in individuals who were IgM negative compared to
convalescent samples. IL-1β and TNF-α levels were elevated in convalescent samples
compared to subjects who had either and increasing, positive, or in some cases negative
antibody response, whereas IL-8 levels were higher in normal individuals than
convalescent subjects or those with a positive or negative antibody response. Additionally, those with a decreasing IgG response had elevated levels of IL-6. This
indicates that subjects with a decreasing antibody response tended to have elevated
cytokine levels, which we previously demonstrated to be associated with a poor outcome.
Notably, cytokine levels in many convalescent patients are elevated, which may
contribute to prolonged IgM responses and delayed class switching events seen in many
survivors.

There are several interpretations for the sustained LASV-specific IgM titers seen
in LF patients [239-243]: 1. IgM may be indicative of a recent infection for which class
switching has not fully occurred; 2. a prolonged and as of yet largely uncharacterized
inhibition of class switching could be prevalent in LASV infections; 3. sustained IgM
titers could be generated by IgM+ memory B cells; 4. impaired CD4+ T_H lymphocyte function during LASV infection and a failure to produced memory T cells, with long-term impact on class switching may be central to humoral immunity aspects in LF.

Evidence for these mechanisms can be found in the literature. Bergthaler et al. noted that non-neutralizing virus-specific IgM were crucial for impedance of viral persistence in LCMV infection, suggesting that IgM-producing cells have remained largely obscure and underappreciated in the characterization of arenaviral infections [244]. Additionally, the late appearance of neutralizing antibodies in arenaviral infections has been tied to high viral antigen-to-B cell ratios and low T cell help, which resulted in a normal IgM response but reduced the efficiency of class switching [245]. Lowering the antigen-to-B cell ratio and increasing T cell help resulted in rescuing of class switching and emergence of neutralizing IgG specificities. The roles of IgM and T_H lymphocytes have been studied in controlled cynomolgus macaque models of simian immunodeficiency virus (SIV) infection [246]. These studies noted a strong inverse correlation of the immunoglobulin and CD4+ T_H cell counts after the primary peak of IgM response, reflecting the prevalence of mature plasma cells that have not undergone class switching. Moreover, a strong correlation was observed in the same studies between pre-infection immune status and disease progression. It is therefore possible in arenaviral infections, as in the SIV model, that a normal, pre-infection CD4+ T_H cell threshold may regulate normal B cell responses, with concomitant class switching, emergence of strong neutralizing IgG titers, and quiescence or elimination of short lived IgM-producing plasma cells. Therefore, what we have observed in LASV infected patients may be the
result of a normal, early IgM response in LASV infections, followed by an impaired T\textsubscript{H} cell response leading to an IgM\textsuperscript{+} B memory cell population.

To elucidate the cause of the dysregulated humoral and inflammatory response, we targeted cells that are understood to initiate these events. Dendritic cells are main sites of LASV replication. They possess many receptors that recognize and respond to viral infection. One receptor of interest, TLR3, recognizes dsRNA and upon stimulation, activates expression of IL-12, TNF-\(\alpha\), and IL-6, and IFN-I matures dendritic cells into APC [162, 163, 165]. LASV infection has been shown to impair expression of TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-8, and IFN-I in dendritic cells [23, 93, 128, 130, 131]. We proposed that LASV NP exonuclease activity contributes to the dysregulation of the immune response by degrading dsRNA such that the ligand is unable to stimulate TLR3 (Figure 23).
Figure 23. Depiction of NP exonuclease activity disruption of TLR3 signaling in dendritic cells and in HEK-Blue™ hTLR3 Cells

In dendritic cells, LASV binds to its receptor, α-DG, and is internalized by endocytosis. Acidification of the endosome allows the membranes of LASV and the endosome to fuse and also creates an environment favorable to dsRNA interaction with TLR3 [161, 247]. Transcription and translation of the NP gene occurs rapidly such that there is an excess of NP [118, 119]. The excess of NP potentially subverts detection by TLR3 as it degrades dsRNA byproduct from the viral genomic replication (A). In HEK-Blue™ hTLR3 Cells, PiC stimulates hTLR3 which activates transcription and expression of SEAP under the control of IFN-β minimal promoter (B).
In a previous study, recombinant proteins NPΔ340WT, and mutants NPΔ340R393A and NPΔ340QuadA possessed or retained exonuclease activity as seen by degradation of radiolabeled 18bp dsRNA, while NP1-340, and mutants NPΔ340D389A and NPΔ340G392A did not have or lost exonuclease function [95]. While our experiments duplicated these results using radiolabeled short dsRNA, we observed different results with radiolabeled long dsRNA (Figures 16A-21A). The N-terminus of NP (NP1-340), which has to date only been known to bind ssRNA, in addition to NPΔ340WT and its mutants all were capable of degrading long dsRNA. To confirm that the recombinant protein samples did not contain any contaminants that may be contributing to these results, proteomic analysis was performed and revealed no other proteins with exonuclease activities. Interestingly, the recombinant proteins that displayed no exonuclease activity on short dsRNA were incapable of degrading long dsRNA smaller than about 20bp (Figures 17A-19A). This is most likely why they previously demonstrated no exonuclease activity. Contrastingly, NPΔ340WT, NPΔ340R393A, and NPΔ340QuadA degraded dsRNA past 8pb (Figures 16A, 20A, and 21A).

Using these proteins, we assessed the effects they had on TLR3. All six proteins degraded both LMW PiC and HMW PiC to a point where they were no longer capable of stimulating TLR3 and activating expression of SEAP (Figures 16B-21B and 22A). Though SEAP production stimulated by LMW PiC was lower compared to HMW PiC, the proteins followed approximately the same slope. Jelinek et al. demonstrated that TLR3 stimulation and resulting protein expression was dependent on the size of the dsRNA [165]. Therefore, the EC90 of LMW PiC produced lower levels of SEAP, as determined by the A630, because its average size is 0.6kb, whereas the average size of
HMW PiC is 4.75kb. Using HMW PiC, this experiment demonstrated that \( \text{NP}_{1-340} \) was the most efficient at dsRNA with an IC\(_{50} \) of 0.09\( \mu \)M, while \( \text{NP}_{\Delta 340}^{\text{WT}} \) was approximately a log higher at 0.83\( \mu \)M, followed by the \( \text{NP}_{\Delta 340} \) mutants (Figure 22B).

Though results demonstrating the ability of \( \text{NP}_{1-340} \), \( \text{NP}_{\Delta 340}^{D389A} \), and \( \text{NP}_{\Delta 340}^{G392A} \) were unexpected, there are possible explanations. Firstly, and most evidently as exhibited by the assays herein, these proteins may be structurally incapable of binding and/or degrading dsRNA smaller than about 20bp. Secondly, separating the protein into its two functioning parts may eliminate either competitive inhibition or structural changes made to one domain when binding of the other occurs. Studies examining the ability of full length LASV NP (FL NP) [23, 24], \( \text{NP}_{1-340} \), and \( \text{NP}_{\Delta 340}^{\text{WT}} \) [95] to regulate IRF3 nuclear translocation demonstrated that FL NP and \( \text{NP}_{\Delta 340}^{\text{WT}} \) reduced the level of innate immune activation, while \( \text{NP}_{1-340} \) did not. Additionally, FL NP mutants D389A and G392A did not reduce the level of IRF3 translocation, while FL NP mutants R393A and QuadA moderately did [95]. This indicates either a change in exonuclease functionality when the N-terminus is removed, or that the ability of NP to regulate the innate immunity is not attributed to exonuclease activity in these assays. Another study that examined the effect of FL NP and FL mutants on PiC induction of a transfected IFN-\( \beta \) construct with a luciferase reporter exhibited similar results [94]. Moreover, this study identified the N-terminus (equivalent to \( \text{NP}_{1-340} \)) as a cap binding structure where mutations to the binding site arrest genome transcription. The cap-binding structure may be capable of endonuclease activity, resulting in the appearance of dsRNA degradation to a size of about 20bp.
The exonuclease and TLR3 assays presented herein should be repeated with full length protein to elucidate functional differences in NP<sub>1-340</sub> and NPΔ340 partial proteins. However, these studies have demonstrated important capabilities of LASV NP. Through analysis of the host immune response during LASV infection, we have identified a dysregulation of humoral immunity, namely a prolonged IgM response with preferential specificity to NP antigen. Additionally, elevated cytokine levels that we identified to be associated with fatality also tend to be associated with decreasing levels of antibodies to LASV antigen. This phenomenon prompted us to investigate the cause, wherein we discovered LASV NP is capable of disrupting important initial events in the immune activation cascade. The exonuclease activity of NP inhibits PiC stimulation of transfected hTLR3 in HEK-293T cells, and resultantly, the IFN-β promoter does not become activated. Ultimately, this represents a possible failure to activate any expression products of the TLR3 pathway in dendritic cells – cytokines and costimulatory molecules important in maturing the immune response. Inefficient dendritic cell maturation leads to impairment of the CD4<sup>+</sup> T<sub>H</sub> cell response and an inability to facilitate class switching events. However, this immunosuppression may be beneficial and necessary in surviving LF; however, further investigation is needed to support this hypothesis.
Supplemental Figure 1. Josiah, LASV237-Nig-2010, and Nig08-A18 LASV NP

Pearson Alignment

Three strains from three different clades – II, III, and IV – of LASV NP amino acid sequences were aligned with CLUSTALW using a global alignment algorithm.

| LASV_NP_Josiah   | NSEAYKFSYFLWQTQSLLRSLGCSNKLQLQVYDAQQALLGDLFSERVNQLKLKKKSD |
| LASV_NP_Nig08-A18 | NSEAYKFSYFLWQTQSLLRSLGCSNKLQLQVYDAQQALLGDLFSERVNQLKLKKKSD |
| LASV237-NIG-2010_NP | NSEAYKFSYFLWQTQSLLRSLGCSNKLQLQVYDAQQALLGDLFSERVNQLKLKKKSD |
| LASV_NP_Josiah   | DNLELRSLDNLQAVALNVELLSTQGSLILVQGTLSDLILAADELHLKSLVYVTRPPL |
| LASV_NP_Nig08-A18 | DNLELRSLDNLQAVALNVELLSTQGSLILVQGTLSDLILAADELHLKSLVYVTRPPL |
| LASV237-NIG-2010_NP | DNLELRSLDNLQAVALNVELLSTQGSLILVQGTLSDLILAADELHLKSLVYVTRPPL |
| LASV_NP_Josiah   | SAGTYGMLGSLAQDLQRALLLNGSGLGQGAAGDGVYVSUVVNAEELNQNGTHF |
| LASV_NP_Nig08-A18 | SAGTYGMLGSLAQDLQRALLLNGSGLGQGAAGDGVYVSUVVNAEELNQNGTHF |
| LASV237-NIG-2010_NP | SAGTYGMLGSLAQDLQRALLLNGSGLGQGAAGDGVYVSUVVNAEELNQNGTHF |
| LASV_NP_Josiah   | SLTACLTLQCGVLDNDVQALTDLGLIGYATKYPENTSDDLWLQGHPILNHIKPSSLN |
| LASV_NP_Nig08-A18 | SLTACLTLQCGVLDNDVQALTDLGLIGYATKYPENTSDDLWLQGHPILNHIKPSSLN |
| LASV237-NIG-2010_NP | SLTACLTLQCGVLDNDVQALTDLGLIGYATKYPENTSDDLWLQGHPILNHIKPSSLN |
| LASV_NP_Josiah   | IGSYFSLGAAVAGACMLDGGNLETTKVSFTQHDGILSLVYKALGFMISSTQGER |
| LASV_NP_Nig08-A18 | IGSYFSLGAAVAGACMLDGGNLETTKVSFTQHDGILSLVYKALGFMISSTQGER |
| LASV237-NIG-2010_NP | IGSYFSLGAAVAGACMLDGGNLETTKVSFTQHDGILSLVYKALGFMISSTQGER |
| LASV_NP_Josiah   | NFFTENLYICLSGDQWYIATSTSIWRAWVNTVDLEDGQPPQADSNSHSSKLQSAG |
| LASV_NP_Nig08-A18 | NFFTENLYICLSGDQWYIATSTSIWRAWVNTVDLEDGQPPQADSNSHSSKLQSAG |
| LASV237-NIG-2010_NP | NFFTENLYICLSGDQWYIATSTSIWRAWVNTVDLEDGQPPQADSNSHSSKLQSAG |
| LASV_NP_Josiah   | FTAGLYQSMJTLRDMLQLDPAWKTWMDIEGREPDVEALIQGSSQGCY1HFPEEPDL |
| LASV_NP_Nig08-A18 | FTAGLYQSMJTLRDMLQLDPAWKTWMDIEGREPDVEALIQGSSQGCY1HFPEEPDL |
| LASV237-NIG-2010_NP | FTAGLYQSMJTLRDMLQLDPAWKTWMDIEGREPDVEALIQGSSQGCY1HFPEEPDL |
| LASV_NP_Josiah   | SQFQQFAQFYSROGQTVTFLFAQFQGLTsAVIALPNNHVTDOQGQLSSEDSILEESESQGKDK |
| LASV_NP_Nig08-A18 | SQFQQFAQFYSROGQTVTFLFAQFQGLTsAVIALPNNHVTDOQGQLSSEDSILEESESQGKDK |
| LASV237-NIG-2010_NP | SQFQQFAQFYSROGQTVTFLFAQFQGLTsAVIALPNNHVTDOQGQLSSEDSILEESESQGKDK |
| LASV_NP_Josiah   | LDIALSFTEYRHYNAWVQDVLCHMHTGVVVEKRGKGEETTHPPACLDMCNDVFDAA |
| LASV_NP_Nig08-A18 | LDIALSFTEYRHYNAWVQDVLCHMHTGVVVEKRGKGEETTHPPACLDMCNDVFDAA |
| LASV237-NIG-2010_NP | LDIALSFTEYRHYNAWVQDVLCHMHTGVVVEKRGKGEETTHPPACLDMCNDVFDAA |
| LASV_NP_Josiah   | VSGGLTTSVLALAPVPRMDMVFRTSSTPVRV |
| LASV_NP_Nig08-A18 | VSGGLTTSVLALAPVPRMDMVFRTSSTPVRV |
| LASV237-NIG-2010_NP | VSGGLTTSVLALAPVPRMDMVFRTSSTPVRV |
Supplemental Figure 2. Analysis of LASV237-Nig-2010 and Nig08-A18 NP expression and purification

Proteins expressed in HEK-293T/17 cells and purified by affinity chromatography were resolved on SDS-PAGE (top panel) and Western Blot analysis (bottom panel). The proteins were probed with an α-6xHis MAb.
Supplemental Figure 3. Analysis of LASV NP Josiah NPΔ340WT, NP1-340s

NPΔ340D389A, G392A, R393A, QuadA

After expression in bacterial cells, the proteins were purified by α-6XHis column purification then by nickel magnetic beads. Panel (A) SDS-PAGE analysis; panel (B) Western Blot analysis of column-purified proteins probed with αLASV NP PAb; and panel (C) Western Blot analysis of bead-purified proteins probed with αLASV NP. Lanes: 1. NP1-340 2. NPΔ340WT 3. NPΔ340D389A 4. NPΔ340G392A 5. NPΔ340R393A 6. NPΔ340QuadA 7. Full length NP WT (B) 8. Protein ladder
Supplemental Figure 3. Analysis of LASV NP Josiah NPΔ340WT, NP1-340, NPΔ340D389A, G392A, R393A, QuadA

A.  

B.  

C.
Supplemental Figure 4. One dimensional gel electrophoresis and LC/MS analysis

NP<sub>1-340</sub>, NPΔ340<sup>WT</sup>, and NPΔ340<sup>D389A</sup> were resolved by polyacrylamide electrophoresis and bands were excised for analysis by LC/MS to ensure there was no contamination by RNases. The bands that are not labeled below were excised and combined for analysis.

<table>
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<tr>
<th>Protein</th>
<th>Percentage of total spectra</th>
<th>Molecular Function</th>
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<tbody>
<tr>
<td>Nucleoprotein/ Lassa virus</td>
<td>3.50%</td>
<td>RNA binding</td>
</tr>
<tr>
<td>Nucleoprotein/ Lassa virus</td>
<td>1.78%</td>
<td>RNA binding</td>
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<tr>
<td>Elongation factor Tu I</td>
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<td>translation regulator, nucleic acid binding</td>
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<tr>
<td>Glutamine-fructose-6-phosphate aminotransferase</td>
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<td>carbohydrate binding</td>
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<tr>
<td>Nucleoprotein/ Mopeia virus</td>
<td>0.42%</td>
<td>RNA binding</td>
</tr>
<tr>
<td>2-oxoglutarate dehydrogenase E1 component</td>
<td>0.42%</td>
<td>catalytic activity in citric acid cycle</td>
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</table>
Supplemental Figure 5. Long dsRNA sequence

Representation of long dsRNA sequence synthesized for exonuclease experiments. Each “U” on the sense strand represents $^{32}$P-rUTP.

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REFERENCES


81. SIGA, Research & Discovery, Arenavirus.


143.


BIOGRAPHY

Jessica N. Hartnett graduated from Tulane University in 2008 with a Bachelor of Science degree in Cell and Molecular Biology and French. After rotating in molecular biology and biochemistry labs during her undergraduate career, she decided to apply for doctoral programs, and was accepted to the Biomedical Science program at Tulane University, School of Medicine in the fall of 2008. She joined Dr. Robert Garry’s lab where the Lassa fever diagnostic and B-cell programs were in the early stages. Jessica coordinated trips and traveled to Sierra Leone six times throughout her graduate career on behalf of these programs and for work towards her dissertation. In late 2014 during the Ebola outbreak, she, along with Dr. Luis Branco, Dr. Robert Cross, Augustine Goba, and Mambu Momoh, validated Ebola virus rapid diagnostics (EBOV RDT) for Corgenix Medical Corporation in Kenema, Sierra Leone. This validation resulted in the first Ebola rapid diagnostic approval from the World Health Organization and Emergency Use Authorization from the Food and Drug Administration. Following completion of the Biomedical Science program, Jessica will continue working with Dr. Garry to advance the Lassa and Ebola programs.