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Novel Strategies for Development of Hemorrhagic Fever Arenavirus Live-Attenuated Vaccines

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Summary

Several arenaviruses, chiefly Lassa virus (LASV), cause hemorrhagic fever (HF) disease in humans and pose significant public health problems in their endemic regions. Moreover, HF arenaviruses represent credible biodefense threats. There are not FDA-approved arenavirus vaccines and current anti-arenaviral therapy is limited to an off-label use of ribavirin that is only partially effective. Live-attenuated vaccines (LAV) represent the most feasible approach to control HF arenaviruses within their endemic regions. Different platforms, including recombinant viral vectors expressing LASV antigens, and the use of attenuated reassortant arenaviruses, have been used to develop LAV candidates against LASV with promising results in animal models of LASV infection, but none of them has entered a clinical trial. These vaccine efforts have been the subject of recent reviews and will not be examined in this review, which is focused on new avenues for the development of safe and effective LAV to combat HF arenaviruses.

Keywords

viral hemorrhagic fever; arenavirus; Lassa virus (LASV); Junin virus (JUNV); live-attenuated vaccine; Candid1; reverse genetics; codon deoptimization; tri-segmented arenavirus

1. Arenaviruses and their impact in human health and biodefense readiness

Arenaviruses cause chronic infections of rodents across the world, and human infections occur through mucosal exposure to aerosols or by direct contact of abraded skin with infectious materials (1). Both viral and host factors contribute to a variable outcome of arenavirus infection, ranging from virus control and clearance by the host defenses to subclinical chronic infection, to severe disease (1). Several arenaviruses cause hemorrhagic fever (HF) disease in humans and pose a serious public health problem in their endemic

Declaration of Interests

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regions (1, 2). Thus, the Old World (OW) arenavirus Lassa virus (LASV) infects several hundred thousand individuals yearly in West Africa resulting in a large number of Lassa fever (LF) cases associated with high morbidity and mortality (1, 2). Recent evidence indicates that LASV endemic regions continue to expand with a current population at risk of ~200 million people. These findings have raised concerns about the emergence of novel HF-causing arenaviruses outside their current endemic regions, and increased traveling has led to the importation of LF cases into non-endemic metropolitan areas around the globe (3). Notably, new studies (4, 5) have indicated that LASV case fatality rate (CFR) might be significantly higher, above 60%, than previously documented (6). Differences in the criteria used to diagnose LF may have contributed to significant differences in the estimated CFR. In addition, while rodent-to-human transmission is the major mechanism of human LASV infection (4, 6), modeling studies examining the relative contribution of zoonotic and anthroponotic transmission of LF have indicated that a significant proportion of LF cases (~20%) likely arise from human-to-human transmission associated with super-spreading events (7). Hence, with Dengue fever exception, the estimated global burden of LF is the highest among viral HF (8). Likewise, Junin virus (JUNV) causes Argentine HF (AHF), a disease endemic to the Argentinean Pampas with hemorrhagic and neurological manifestations and a case fatality of 15–30%, whereas Machupo (MACV) and Guaranito (GTOV) arenaviruses emerged as causative agents of HF in Bolivia and Venezuela, respectively (9). In addition, evidence indicates that the worldwide-distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical significance (10, 11). Moreover, because their stability, high morbidity, potential for aerosol transmission and unrestricted source from their natural rodent hosts, several arenaviruses including LASV and JUNV represent credible biodefense threats and are considered Category A agents (12).

2. The need for vaccines to combat HF-causing arenaviruses

There are not FDA-approved vaccines against HF arenaviral diseases and current anti-arenaviral therapy is limited to an off-label use of ribavirin that is only partially effective and has several limitations, including the need of intravenous and early administration for optimal efficacy, and significant side effects (6, 13–15). The JUNV live-attenuated Candid1 strain has been shown to be an effective vaccine against AHF (16, 17). However, in the US Candid1 has only investigational new drug (IND) status and studies addressing long-term immunity and safety have not been conducted. Moreover, Candid1 does not protect against LASV. Despite significant efforts dedicated to the development of LASV vaccines, not a single LASV vaccine candidate has entered a clinical trial.

Several LASV vaccine candidates based on the expression of LASV-specific antigens using replication competent viral-based vectors including vaccinia virus, vesicular stomatitis virus (VSV) and yellow fever 17D have shown promising results in animal models, including non-human primates (NHP), of LASV infection (8). In addition, the reassortant ML29/Jos carrying the L segment from the non-pathogenic Mopeia virus (MOPV) and the S segment from the pathogenic Josiah (Jos) strain of LASV was successfully tested in immune compromised NHP (18) and shown to induce cross-reactive CMI after a single vaccination (19). ML29/Jos was also shown to be stable, elicited sterilizing immunity and protected

animals against genetically distantly related LASV lineages, and was effective in post-exposure applications (20). Despite its excellent profile as a candidate live-attenuated vaccine (LAV) for LASV, ML29/Jos was isolated and produced in cells that are not approved by the US Food and Drug Administration (FDA) and has no traceable records of passages in vitro, which has prevented the clinical development of ML29/Jos. Moreover, the mechanisms of ML29 attenuation remain poorly understood and additional mutations, including reversions, in ML29 enhanced virulence. Nevertheless, because their ability to frequently induce robust and long-term protective both humoral and cell mediated immunity (CMI) following a single immunization, LAV remain the most feasible and attractive approach to combat HF arenaviral diseases, including LF (8). In addition, considering the evidence that a significant number of LF cases may involve human-to-human transmission, improved herd immunity via implementation of a LAV would further contribute to the control of LF.

3. Mechanisms of immune-mediated protection against LASV

Control of LASV infection seems to be mediated mainly by CMI, and significant titers of LASV neutralizing antibodies (NAbs) are usually observed only in patients who have clinically recovered. However, passive antibody transfer has been shown to induce protection in animal models of LF (21–23) and in limited human studies (24) suggesting that a vaccine capable of inducing the right combination of cellular and humoral responses might be the preferred candidate.

4. Arenaviruses reverse genetics: implications for novel strategies to develop live-attenuated vaccines to combat HF arenaviruses

Arenaviruses are enveloped viruses with a bi-segmented negative-stranded (NS) RNA genome and a life cycle restricted to the cell cytoplasm (1) (Figure 1). Each genomic RNA segment, L (ca 7.3 kb) and S (ca 3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by a non-coding intergenic region referred to as IGR (1). The S RNA encodes the viral glycoprotein precursor (GPC) and the viral nucleoprotein (NP). GPC precursor is co-translationally cleaved by signal peptidase to produce a 58 aa stable signal peptide (SSP) and GPC that is post-translationally processed by the cellular site 1 protease (S1P) to yield the two mature virion glycoproteins GP1 and GP2 that form the spikes that decorate the virus surface (1). GP1 is located at the top of the spike and mediates virus receptor recognition and subsequent cell RNA dependent RNA polymerase (L), and the small RING finger protein Z that is a bona fide matrix protein (25, 26).

The development of state-of-the-art reverse genetics systems for the prototypic arenavirus LCMV (27, 28) and subsequently for other arenaviruses including JUNV and LASV (27–31), has provided investigators with a novel and powerful tool for the investigation of the viral *cis*-acting sequences and *trans*-acting factors that control cell entry, RNA replication, gene expression, assembly and budding of arenaviruses. Likewise, it is now feasible to rescue recombinant infectious arenaviruses with predetermined mutations in their genomes and examine their phenotypes both in cultured cells and well-established animal models of

arenavirus infection (27, 31). In addition, the use of reverse genetics approaches has facilitated the development of recombinant tri-segmented arenaviruses expressing additional genes of interest (27, 31, 32), as well as single-cycle infectious recombinant LCMV in which GPC is replaced by GFP (rLCMV GPC/GFP) (33). Genetic trans-complementation via transfection with plasmids or the use of stable cell lines expressing arenavirus GPCs of interest facilitates the production of the corresponding GPC-pseudotyped rLCMV GPC/GFP that can be used to assess NAb responses to HF-causing arenaviruses using a Biosafety Level 2 (BSL2) platform (33). Progress in arenavirus molecular and cell biology and pathogenesis, together with the use of robust arenavirus reverse genetics systems would allow investigators to engineer and rescue recombinant HF arenaviruses with optimal features as LAV.

5. Novel strategies for the development of safe and effective HF arenavirus LAV

5.1. Codon deoptimization-based LAV

Redundancy in the genetic code results in many amino acids (aa) being encoded by more than one codon, and codon usage bias refers to differences in the frequency at which synonymous codons are used by an organism to incorporate the same aa into a protein (34–38). Codon optimization is a frequently used strategy to improve expression of genes in heterologous systems (31, 39–42), but all mammals exhibit essentially the same codon bias (43, 44). Conversely, replacement of commonly used codons with non-preferred codons (codon deoptimization, CD) can dramatically decrease gene expression (38, 45–48). Protein expression of mammalian viruses is subjected to the codon usage bias of the cells they infect and thereby introduction of unfavorable host codons into a viral genome is predicted to adversely affect viral protein translation thus resulting in viral attenuation. Accordingly, RNA viruses can be effectively attenuated by codon deoptimization of a single or a limited number of viral gene products (38, 45–48). Therefore, recoding of the arenavirus genome in a way that preserves the wild type (WT) aa sequence but creates a suboptimal arrangement of codons could facilitate the development of safe, immunogenic, stable and protective LAV to combat HF arenaviruses. The use of genome-scale changes in codon-pair bias (38, 45–48) has been successfully used to generate attenuated viruses exhibiting properties amenable for the development of LAV. This approach, however, requires the use of computer algorithms to design viral genomes with appropriate pair-deoptimized codons. In addition, whether the use of alternative codon pair deoptimization results in different degree of attenuation remains to be elucidated. Likewise, it is unclear if the same arrangement of pairs of codons would result in viral attenuation with different viral strains and therefore each virus would need to be evaluated individually before the vaccine could be used (38, 45–48). These difficulties can be overcome using the more direct approach of engineering recombinant arenavirus with open reading frames (ORF) where many of the aa are encoded by the less frequently used codon, which is predicted to result in reduced protein expression and, therefore, viral attenuation (49). A CD-based approach for development of LAV would have the following unique advantages: **1)** CD LAV and WT virus would have the same protein sequence and hence identical immunogenic properties. **2)** CD LAV would contain a large number (>100s) of silent mutations, which would make highly unlikely, if not impossible, a reversion to a

virulent viral sequence, thus overcoming a safety concern affecting current LAV approaches where LAV and WT genomes differ by a very limited number of mutations. **3)** Reassortants between a circulating virulent WT arenavirus and its attenuated CD LAV cannot generate variants with a combination of mutations or arrangement of genes that could result in increased virulence. **4)** Generation of CD arenavirus LAV can be rapidly achieved combining *de novo* synthesis of CD genes with currently available state-of-the-art arenavirus reverse genetics technologies.

The feasibility of a CD-based approach as a novel strategy to develop arenavirus LAV has received strong support from results obtained with the prototypic arenavirus LCMV (49). Expression levels of LCMV NP in transfected cells correlated well with the degree of codon deoptimization incorporated into the NP open reading frame (ORF) (Figure 2). As expected, CD mediated reduced expression levels of NP resulted in the corresponding reduced levels of replication and gene expression of an LCMV minigenome, biosynthetic processes that require NP (49). Similarly, reduced CD NP-mediated inhibition of activation of the interferon beta (IFN β) promoter upon Sendai virus (SeV) infection correlated with the corresponding reduced expression levels of NP (49). The use of reverse genetics approaches facilitated the rescue of rLCMV containing NP with different degrees of CD that exhibited variable levels of decreased fitness in cultured cells (49), which might have reflected differences in species codon usage, or in tRNA availability among cell lines, or a higher decreased in fitness in type I IFN (IFN-I) competent cells compared to IFN-I-deficient cells.

The lack of in vivo virulence while retaining the ability to induce a protective immune response represents a first and necessary step to evaluate LAV candidates. In this regard, it was possible to identify rLCMV/NPcd whose degree of NP CD conferred them with key features of an LAV candidate (49), including: 1) robust multiplication in cultured cells, including cell substrates with FDA approval for production of human vaccines, 2) complete attenuation in a mouse model of fatal LCM disease, and 3) ability to induce protective immunity against a subsequent lethal challenge with LCMV WT (Table 1). Genetic stability is another critical feature that should be exhibited by a LAV candidate. Genetic and phenotypic characterization of rLCMV/NPcd that were serially passed in Vero cells demonstrated that rLCMV/NPcd exhibited the stability required for LAV candidates (49).

5.2. Reorganization of the arenavirus L segment IGR as a general molecular approach for the generation of recombinant arenaviruses with optimal features of LAV

The IGR presence in each of the arenavirus genome segments plays critical roles in arenavirus transcriptional control of gene expression and production of infectious particles (50). Recently it was documented that it is possible to rescue an rLCM virus, called rLCMV(IGR/S-S), containing the same S-IGR in both the S and L genome segments. This rLCMV(IGR/S-S) grew to high titers in cultured cells (Figure 3), whereas in vivo it was highly attenuated but able to induce protection against a lethal challenge with WT LCMV (Figure 4) (51). These findings have uncovered a potential general molecular strategy for arenavirus attenuation. Subsequent studies investigated the sequence plasticity of the arenavirus IGR and revealed that rLCMV whose S-IGR were replaced by S-IGR of a different arenavirus including the closely related LASV, or the very distantly related

reptarenavirus Golden Gate virus (GGV), as well as an entirely non-viral S-IGR like sequence (Ssyn) were viable (52), indicating that the function of the S-IGR tolerates a high degree of sequence plasticity. In addition, rLCMV's whose L-IGR were replaced by Ssyn were viable and severely attenuated in vivo but able to elicit protective immunity against a lethal challenge with wild type LCMV. These findings indicated that replacement of L-IGR by a non-viral Ssyn could serve as a universal molecular determinant of arenavirus attenuation (52).

5.3. The use of tri-segmented recombinant Candid1 (r3Can) to develop a safe and effective polyvalent live-attenuated vaccine to combat both LF and AHF

Both, the live-attenuated Can (rCan) and pathogenic Romero (rRom) strains of JUNV have been rescued from cloned cDNAs using reverse genetics approaches and shown that guinea pigs infected with rRom or parental Romero field isolate succumbed with the same kinetics and symptoms, whereas guinea pigs infected with rCan did not exhibit clinical symptoms and were protected against subsequent lethal challenge with the pathogenic Romero strain (53). The ability to manipulate the genome of a genetically defined and phenotypically well characterized rCan raises the possibility of using rCan as a platform to develop polyvalent LAV against multiple HF arenaviruses, and for which master virus seeds can be generated to meet FDA requirements for licensure in the USA. In this regard the development of a tri-segmented arenavirus platform has opened the possibility of rescuing rCan containing 2S and 1 L segments (r3Can), where each of the two S segments can contain a gene of interest (GOI) instead of either GPC or NP (Figure 5). The rationale behind this approach was that physical separation of GP and NP loci into two different S segments poses a strong selective pressure to package and maintain the three (1L+2S) segments to provide all necessary viral proteins. Accordingly, a variety of r3LCMV and r3Can expressing different reporter genes including GFP and CAT have been documented and shown to be genetically very stable (27, 32, 53). These findings support the feasibility of the generation of an r3Can expressing LASV antigens from one of the gene locus, NP or GPC, in each of the two S-segments. Immunization with this type of r3Can would be expected to provide immune mediated protection against both AHF and LF, and thereby representing an attractive polyvalent LAV against diseases caused by the two HF arenaviruses, JUNV and LASV, with the highest impact in human health. It is important to emphasize that compelling data support the safety of an r3Can expressing LASV NP and GP antigens. Preclinical studies at USAMRIID and in Argentina demonstrated the safety, immunogenicity and protective efficacy of Can in both guinea pigs and rhesus macaques. Importantly, clinical studies involving agricultural workers in the JUNV endemic areas have shown Can to be an effective and safe vaccine in humans. We have generated and extensively characterized a molecular clone of Can from cloned cDNA, showing its attenuation and protective efficacy. On the other hand, results from studies with several r3LCMV and r3Can expressing a variety of reporter genes have indicated that the tri-segmented genome structure does not confer arenaviruses with any gain of fitness or virulence with respect to the parental bi-segmented virus. Moreover, results obtained with the reassortant ML29 using different animal models of LASV infection have documented that the gene products, NP and GPC, encoded by the S genome segment do not contribute to LASV virulence.

5.4. Phenotypic and genetic stability of novel HF arenavirus LAV

During production of LAV in cultured cells, as well as multiplication of LAV in vaccine recipients, mutations could accumulate in the genome of the LAV that might increase its viral fitness and thereby influence the virulence of the LAV. Similarly, these mutations might affect critical antigenic structures with the consequent impact on the immunogenicity and efficacy of the LAV. Therefore, phenotypic and genotypic stability, together with the absence of transmission potency, are key properties of LAV to ensure their safety profile (<http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM202439>). The phenotypic and genetic stability of novel HF arenavirus LAV could be assessed by conducting serial passages in different cell substrates, including the cell line planned to be used for vaccine production. Selected serial passages of the LAV candidate can be characterized both genetically and phenotypically. Genetic characterization can be done by determining the consensus genome sequence, as well as by examining the dynamics of the viral quasispecies using Next Generation Sequencing. Phenotypic characterization can be based on determining growth properties in cultured cells and virulence in appropriate animal models of infection of the different LAV passages and compare them with those associated with the LAV master virus seed. Notably, we have found that both rLCMV with altered IGR (51, 52), as well as r3LCMV (32) and r3Can (53) exhibit a high degree of phenotypic and genetic stability.

6. Testing the safety and efficacy of novel HF arenavirus LAV

Infection of guinea pigs with LASV or JUNV recreates accurately many of the features associated with LF and AHF, respectively, in humans. Moreover, the guinea pig model is well established and widely used to test therapeutics and vaccines for HF-causing arenaviruses. The safety of HF arenavirus LAV can be assessed by infecting (i.p.) 8–20 weeks old Hartley guinea pigs with different doses of the LAV candidate, as well as the corresponding parental pathogenic viral strain, and monitor the animals daily for disease symptoms and mortality. Clinical evaluation would include changes in body weight and body temperature. Guinea pigs infected with pathogenic strains of LASV or JUNV will develop fever followed by hypothermia, and significant weight loss and will succumb to infection 14–18 days after infection. The assessment of clinical symptoms should be complemented with hematological and clinical chemistry studies, as well as determination of titers of virus-specific antibodies and infectious virus in serum. Additional studies should include histopathological assessment of main organs and tissues, as well as detection of viral antigen and infectivity, at different times after administration of LAV. To assess LAV induction of protective immune responses, guinea pigs would be immunized with the LAV candidate under investigation, as well as mock vaccinated as control. LAV induced cellular and humoral, including production of neutralizing antibodies, responses will be evaluated on days 30 and 60 post-vaccination and then subjected to a challenge (i.p.) with a lethal dose of the corresponding pathogenic strain and monitored daily for the appearance of clinical symptoms. Serum-transfer experiment could shed light on whether neutralizing antibodies produced in vaccinated animals are, in addition to CMI, required for protection.

Studies in guinea pig can provide valuable data about the safety, immunogenicity and efficacy of HF arenavirus LAV candidates. However, it is highly unlikely that experimental rodents, including the guinea pigs, will be considered as an appropriate model for efficacy trials in humans. Efficacy studies for vaccines to prevent arenaviral HF disease are likely to be conducted under the Animal Rule (21 CFR 601.90 subpart H), established to address cases where human trials would be unfeasible or considered to be unethical. In these cases, the FDA may grant marketing approval to a new vaccine based on data from efficacy studies conducted in well-controlled animal studies that included at least two animal models that recreate the pathophysiology seen in cases of human disease and in which correlates of protection have been defined (54). NHP are the most accurate model of HF arenaviral disease in humans. Both rhesus (*Macaca mulata*) and cynomolgous (*Macaca fascicularis*) monkeys have been used to study LASV pathogenesis and for evaluation of LASV vaccine candidates (14, 55–60). Importantly, recent studies have provided good markers of fatal LF disease in NHP that parallel those previously documented in fatally infected LF patients (61). These studies also showed that early and strong CMI responses were associated with effective control of virus replication and recovery. However, the high cost of efficacy studies in NHP in BSL4 containment and limited availability of rhesus macaques for biomedical research, underscore the value of the common marmoset, *Callithrix jacchus*, as an alternative reliable animal model of LASV infection (20, 62–65), as the relatively small size of marmosets translates to lower cage and feeding costs, and eases handling in a biosafety environment. Accordingly, common marmosets have been successfully used to evaluate safety, immunogenicity, and efficacy of the LASV ML29 vaccine candidate (66). Therefore, LASV infection of marmosets could be the 2nd “small” NHP model to comply with the FDA Animal Rule.

7. EXPERT COMMENTARY

The development of robust reverse genetics systems for HF arenavirus have provided investigators with a novel powerful approach to manipulate the genomes of these pathogens, which has open new avenues for the rapid development of safe and effective LAV against diseases caused by HF arenaviruses. Likewise, the use of reverse genetics approaches to generate mutant viruses of interest combined with cell-based systems and novel animal models of HF arenavirus infection will also facilitate studies aimed at elucidating the molecular bases of attenuation of HF arenaviruses. Some of the main roadblocks faced by the development of LAV against HF arenaviruses relate to the regulatory process required for their approval. The Animal Rule (21 CFR 601.90 subpart H) was designed to facilitate vaccine testing for diseases for which human trials are not feasible due to their rare occurrence. However, LF is a rather common disease in West Africa, which raises the possibility of designing vaccine trials in humans within LF endemic regions. In this regard it is worth noting that the Kenema Government Hospital (KGH) located in the Eastern Province of Sierra Leone, which is considered to have the world’s highest LF incidence, has the adequate infrastructure to successfully conduct vaccine trials with LASV LAV candidates for which the appropriate safety, immunogenicity and efficacy profiles are first established in NHP models of LF disease. The absence of market-driven forces represents a main obstacle for the speed at which the approval process for the testing of HF arenavirus

LAV is pursued in non-endemic countries. The FDA Animal Rule for regulation of “biodefense” biologics was implemented in 2002 but this pathway has yet to produce a FDA-approved vaccine. An interesting contrasting comparison could be made with the LAV Candid 1 against Argentine hemorrhagic fever. Candid 1 was developed in collaboration between Argentine and US scientists, with support from the national Ministry of Health and US Army Medical Research Institute of Infectious Diseases, and after successful clinical trials was registered in 2006 by the National Regulatory Authorities in Argentina. Shortly after in 2007 Candid1 was incorporated into the National Immunization Plan in Argentina to target populations at high risk, which had a significant impact on the magnitude of epidemic outbreaks of AHF. Based on the results of the use of Candid 1 in Argentina, CDC and NIH permit research with JUNV to be conducted in BSL3 containment with the requirement that the personnel involved is vaccinated with Candid 1. The history of the development and use of Candid 1 illustrates a viable framework for the development of other LAV against HF arenaviruses.

8. FIVE-YEAR VIEW

During the next five years it will be possible to gather data to thoroughly assess the safety and efficacy of HF arenavirus LAV based on the concepts of codon deoptimization and gene rearrangement of the viral genome, including manipulation of the IGR sequences, as well as the use of tri-segmented arenavirus genomes expressing additional antigens of interest to facilitate the development of polyvalent LAV. In this regard, the recent outbreak of an epidemic of Ebola in West Africa has raised the interest in the development of polyvalent LAV against LASV and Ebola virus, which are now for the first time circulating within the same geographic regions.

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Reference annotations

* Of interest

** Of considerable interest

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9. KEY ISSUES

- The use of reverse genetics combined with codon deoptimization facilitates the generation of recombinant arenaviruses with optimal features of LAV.
- Manipulation of the IGR sequences present with the L and S genome segments may provide a general molecular strategy for arenavirus attenuation.
- The use of tri-segmented arenavirus genomes can facilitate the generation of polyvalent LAV.
- The use of reverse genetics approaches to generate arenavirus genomes with predetermined mutations combined with the use of cell-based and novel animal model of HF arenavirus infection will contribute to elucidate the molecular bases of HF arenavirus attenuation.
- Future research is required to establish solid correlates of protection against HF arenavirus infection. Detailed comparison of LASV and JUNV infections and associated diseases, and the use of their corresponding live-attenuated versions for immunization purposes, should provide valuable information about both the viral molecular bases of attenuation and parameters of the host immune response responsible for protection.

Figure 1. Arenavirus genome organization and virion structure

Arenaviruses are enveloped viruses with a bi-segmented negative strand (NS) RNA genome. Each viral genome segment uses an ambisense coding strategy to produce two different proteins in opposite direction and separated by a non-coding intergenic region (IGR) that plays critical roles in both control of virus gene expression and production of infectious progeny. The small (S, ca 3.5 kb) segment encodes for the glycoprotein precursor (GPC) and nucleoprotein (NP). Posttranslational processing of GPC by the cellular protease S1P results in the production of mature GP1 and GP2 that decorate the surface of the virus particle. The large (L, ca 7.3 kb) segment encodes for the virus RNA-dependent RNA polymerase (L) and a small RING finger protein, called Z, which has properties of the bona fide matrix proteins (M) found in many enveloped NS RNA viruses

Figure 2. Characterization of CD NP chimeras

A. Schematic representation of CD NP chimeras. Numbers indicate amino acid (aa) regions containing CD (white) and WT (black) NP sequences. **B. Nucleotide and aa changes in CD NP chimeras:** Discrepancies between mutated codons and aa lengths indicated in panel A are due to codons that are already deoptimized in NP WT or that are encoded by methionine. **C. CD NP expression levels:** HEK293T were transfected with pCAGGS expression plasmids encoding WT and CD NP chimeras. At 48 h post-transfection cell lysates were prepared and NP expression levels determined by Western blot using an antibody to the HA tag that was present at the C-terminus of each NP construct. Empty (E) plasmid transfected cells were included as control and GAPDH expression levels were used as a loading control.

Figure 3. Growth properties of rLCMV(IGR/S-S) in cultured cells
BHK-21 cells were infected with either rLCMV WT or rLCMV(IGR/S-S) (moi = 0.01). Virus titers in tissue culture supernatants were determined at the indicated hours post-infection (h p.i.). Data represent means $\pm$ SD of triplicate samples.

Figure 4. Attenuation and protective properties of rLCMV(IGR/S-S)

Six week-old B6 mice were infected with rLCMV WT or rLCMV(IGR/S-S) using 10^3 ffu, i.c. At 28 days p.i. (broken line), mice that survived were subjected to a lethal challenge with WT LCMV (10^3 ffu, i.c.). After the lethal challenge mice were monitored daily for morbidity and mortality.

Figure 5. LCMV/WT versus r3LCMV genomes

Arenaviruses have a bi-segmented negative strand (NS) RNA genome. The S (ca 3.5 kb) segment encodes for the viral nucleoprotein (NP) and glycoprotein precursor (GPC), which is processed into GP1 and GP2. The L (ca 7.1 kb) segment encodes the virus RNA dependent RNA polymerase (L) and a small RING finger protein (Z), which is the counterpart of the matrix (M) structural protein found in many enveloped non-segmented RNA viruses. To generate tri-segmented (1L and 2S) rLCM viruses expressing additional genes of interest (GOI) the S segment is split into two units. Each of the two segments can be altered to replace one of the viral ORF by the GOI. Physical separation of GP and NP into two different S segments poses a strong selective pressure to maintain all recombinant segments necessary to produce infectious and replication-competent viruses. Each r3LCMV can express up to two different GOI, one from the NP locus and the other from the GPC locus. Alternatively, the same GOI can be expressed from both NP and GPC locus.

Attenuation and protective properties of rLCMV/NP-CD

Table 1

Six week-old B6 mice were immunized with 10^5 pfu of rLCMV/NP_{CD1} administered intraperitoneally(i.p.) and 28 days later subjected to a lethal challenge with 10^3 pfu rLCMV/WT using intracranial (i.c.)inoculation). Mice were monitored daily for morbidity and mortality.

Percent Survival (N=8)					
Days post-challenge (i.c. 10^3 PFU; WT LCMV)	6	7	8	12	
	100	100	100	100	100
Immunization(i.p. 10^5 PFU)	NP1	100	100	100	100
	PBS	100	25	0	-