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Rapid Communication

A recombinant Yellow Fever 17D vaccine expressing Lassa virus glycoproteins

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Abstract

The Yellow Fever Vaccine 17D (YFV17D) has been used as a vector for the Lassa virus glycoprotein precursor (LASV-GPC) resulting in construction of YFV17D/LASV-GPC recombinant virus. The virus was replication-competent and processed the LASV-GPC in cell cultures. The recombinant replicated poorly in guinea pigs but still elicited specific antibodies against LASV and YFV17D antigens. A single subcutaneous injection of the recombinant vaccine protected strain 13 guinea pigs against fatal Lassa Fever. This study demonstrates the potential to develop an YFV17D-based bivalent vaccine against two viruses that are endemic in the same area of Africa.

Keywords: Yellow and Lassa Fevers; YFV17D vaccine; Recombinant vaccine

Yellow Fever (YF) and Lassa Fever (LF) are viral hemorrhagic fevers (VHFs) endemic for West and Central Africa. Lassa virus (LASV), a complex of closely related arenaviruses (Salvato et al., 2005), is transmitted from rats (*Mastomys* spp.) to humans by either direct contact or by mucosal exposure (McCormick and Fisher-Hoch, 2002). Among causative agents of VHFs, LASV affects the second largest number of people (after dengue). Based on epidemiological studies in Sierra Leone, Guinea and Nigeria, the "at risk" seronegative population in these countries may be as high as 59 million, with an annual incidence of illness of 3 million, fatalities up to 67 thousand and up to 3 million re-infections (McCormick and Fisher-Hoch, 2002). The sizeable disease burden and the possibility that LASV can be used as an agent of biological warfare make a strong case for vaccine development (Borio et al., 2002; Fisher-Hoch and McCormick, 2004; McCormick and Fisher-Hoch, 2002). Live replication-competent vaccine candidates based on vaccinia (Fisher-Hoch and McCormick, 2004), vesicular stomatitis virus (VSV) (Geisbert et al., 2005) and Mopeia virus (MOPV), a non-pathogenic relative of LASV (Lukashevich et al., 2005; Peters et al., 1987), have been proposed as potential vaccine candidates. However, vector safety concern remains the major obstacle for further development of these vaccines. In contrast to LF, a live attenuated YFV17D vaccine was available for human immunization since 1936. Over the past 70 years, more than 400 million people were immunized with a remarkable record of safety and efficacy (Monath, 2004). A single subcutaneous (s.c.) immunization with YFV17D elicits long-lasting protective antibody and T cell responses. Based on an outstanding record in humans, the YFV17D has been recently used as a vector for the development of vaccines against other flaviviruses (Japaneses encephalitis, dengue, West Nile virus) and against unrelated pathogens (*Plasmodium yoelii*, influenza M-protein, oncogenes) (Barba-Spaeth et al., 2005; Bonaldo et al., 2005; Monath, 2004; Tao et al., 2005). Here, we describe a

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recombinant YFV17D expressing LASV glycoprotein (GPC) and capable of replicating in vivo and protecting guinea pigs against LASV challenge.

Results and discussion

Recently, a stable full-length infectious YFV17D cDNA has been constructed (Bredenbeek et al., 2003). In the present study, we have used this cDNA to construct a YFV17D recombinant that expresses the LASV GPC protein. This protein is the LASV glycoprotein precursor that is cleaved by subtilase SKI-1/S1P into structural GP1 and GP2 glycoproteins (Lenz et al., 2000). A cDNA fragment encompassing the complete sequences encoding LASV GP1 and GP2, but lacking the 58 amino acids of the GPC signal sequence, was fused in frame between the YFV E and NS1 genes (Fig. 1). The construct was designed so that LASV GPC protein would be released from the YFV17D polyprotein by host signalase. In this construct, the COOH-terminal 23 hydrophobic amino acids of the YFV17D E gene were duplicated downstream of the LASV GPC gene to serve as a signal sequence to ensure insertion of the YFV NS1 protein into the endoplasmic reticulum (ER). In vitro-made RNA from the recombinant clone and from the parental YFV17D clone was transfected into BHK-21J cells by electroporation and labeled with ^{3}H-uridine in the presence of actinomycin D. As seen in Fig. 1D, a single RNA was detected in the labeled cells that were transfected with the recombinant YFV17D/LASV-GPC. This RNA migrated more slowly than YFV17D RNA and was not detectable in mock transfected cells.

The recombinant virus replicated in BHK-21 and Vero cells to titers of over 10^{6} PFU/ml and produced plaques that were slightly smaller than those produced by the parental virus, YFV17D (data not shown). To demonstrate the expression of the LASV GPC in cells transfected with YFV17D/LASV-GPC RNA, cells were analyzed by indirect immunofluorescense. Both the wild-type YFV17D and the YFV17D/LASV-GPC transfected cells were positively stained with anti-YFV17D NS1 antibodies showing a perinuclear punctate pattern that is typical for YFV NS1 expression (Fig. 2A). However, only cells transfected with the recombinant YFV17D/LAS-GPC RNA were positively stained with LASV GP1-monoclonal antibodies. The results of the metabolic RNA labeling and immunofluorescense studies demonstrate that the YFV17D/LASV-GPC is replication-competent in cell cultures. The expression of LASV GPC was detected in infected Vero cells after at least 5 passages of the recombinant virus, suggesting that the virus is reasonably stable.

Western blot analysis was performed to determine whether the LASV GPC protein was properly released from the recombinant YFV17D/LASV protein precursor. As shown in Fig. 2B, monoclonal antibodies directed against LASV GP1 or polyclonal antibodies to LASV GP2 detected both GPC and GP1 or GP2 glycoproteins, respectively, in lysates of cells transfected with YF17D/LASV-GPC RNA but not with the parental YFV17D RNA. In addition to GPC and GP1/

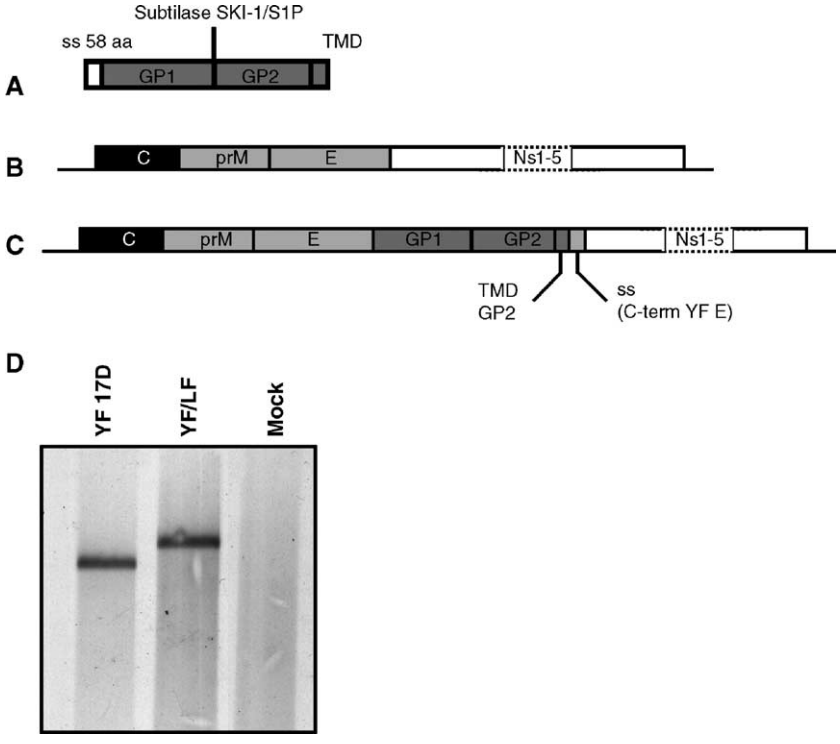

Fig. 1. Schematic representation of YFV17D/LAS-GPC construct. (A) LASV pre-GPC protein; (B) YFV17D genome organization; (C) chimeric YFV17D/LAS-GPC construct. SS, signal sequence; TMD, trans-membrane domain: GP1 and GP2 are final products of Lassa GPC processing which form the mature virion spikes; (D) detection of chimeric YF/LAS RNA in cells transfected with recombinant RNA and labeled with ^{3}H-uridine in the presence of actinomycin D, 18–24 h after electroporation. YFV17D, RNA from cells electroporated with the parental YFV17D RNA; YF/LF, RNA from cells electroporated with recombinant YFV17D/LAS-GPC RNA; Mock, RNA from mock transfected cells.

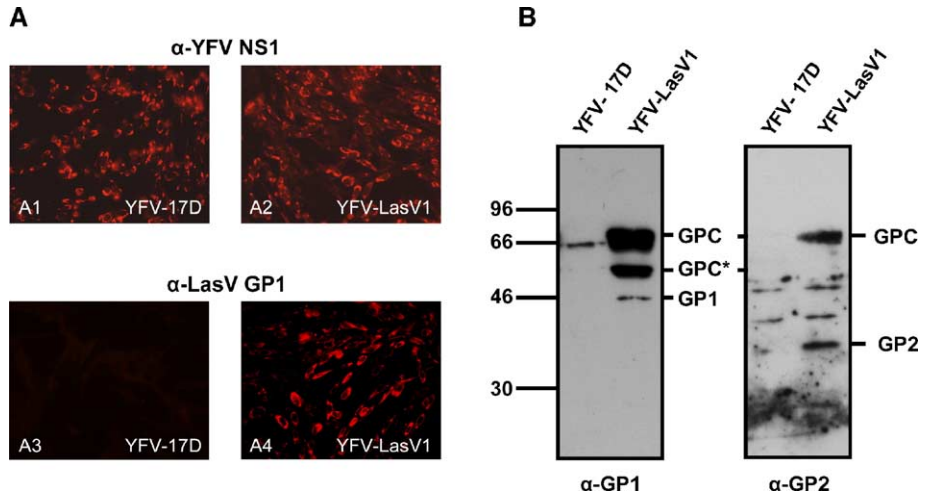

Fig. 2. Expression of LASV glycoproteins in transfected cells. (A) Immunofluorescense staining of YFV NS1 (A1, A2) and LASV GPC (A3, A4) in cells transfected with YFV17D RNA (A1, A3) or YFV17D/LASV-GPC (A2, A4). (B) Western blot analysis of LASV GPC processing in BHK-21 cells. At 24 h after transfection, cells were collected, subjected to SDS-PAGE and proteins were transferred to blots. The blots were probed with LASV GP1- or GP2-specific antibodies. Molecular weight markers in kDa are indicated. Position of the unglycosylated form of GPC is indicated by asterisk.

GP2, a GPC-related protein (labeled as GPC*) was also stained with anti-GP1 monoclonal antibodies in transfected cells. This protein seems to be an unglycosylated form of LASV GPC that is not translocated to the ER (Lenz et al., 2000). These results demonstrate that GPC is properly released from the YFV17D/LASV polyprotein and subsequently cleaved into GP1 and GP2. However, the substantial part of LASV glycoproteins expressed in transfected cells remained unprocessed.

The wild-type YFV infection is primarily viscerotropic in humans and non-human primates. The attenuated YFV17D is not viscerotropic and replicates poorly in tissues of experimental animals (guinea pigs, hamsters, non-human primates). In humans, non-viscerotropism is associated with attenuation and viremia occurred principally between days 4 and 6 and does not exceed 2 $\log_{10}$ PFU/ml (Monath, 2004). LASV infection of guinea pigs and non-human primates is associated with high viremia and the virus replicates very well in target tissues making these animals useful models for human LF (Peters et al., 1987). In LF patients, high viremia ($>1 \times 10^3$ $TCID_{50}$/ml) was associated with high fatality rate and high viremia together with high levels of liver enzymes in plasma (AST > 150 IU/L) carried a risk of death in 78% of LF cases (McCormick and Fisher-Hoch, 2002).

The main goal of the first set of animal experiments was to evaluate the YFV17D/LASV-GPC replication competence and immunogenicity in vivo. The Hartley guinea pigs were inoculated s.c. with 1×10^5 PFU of YFV17D/LAS-GPC and on day 14 all animals were boosted with the same dose of the recombinant virus. Blood and tissue samples were collected from vaccinated animals at different time points for hematology, blood chemistry, RNA extraction, plaque assay, virus isolation and ELISA. As expected, the inoculated animals had no clinical manifestations and all standard measurable blood and chemistry parameters were in normal ranges. In plasma, the recombinant virus was not detectable by plaque assay or by biological amplification (cocultivation in Vero cells). Recombinant viral RNA was not detectable by RT/PCR in 140 μl of plasma extracted on days 4, 10, and 21. However, when RNA samples were prepared from 0.5 ml of total blood, an RT/PCR assay gave a strong positive signal on day 4 after YFV17D/LAS-GPC inoculation (Fig. 3A). Still, blood samples collected on day 10 and 21 were PCR negative (not shown). Viral RNA sequences were only transiently detectable on days 7–14 in spleen and liver (Fig. 3A). Nucleotide sequence analysis of PCR products confirmed their derivation from YFV17D/LAS-GPC. Taken together, these data confirm that recombinant YF17D/LAS-GPC replicated poorly in tissues (blood and the viscera, particularly liver and spleen) of vaccinated guinea pigs. Interestingly, in clinical trials in individuals vaccinated with chimeric YFV17D-based vaccines, viremia levels were even lower than the levels of YFV17D determined to be safe (Monath, 2004). This suggests that insertion of a foreign gene can affect in vivo viral replication and make recombinant YFV17D-based vaccines even safer than the parental vaccine, YFV17D.

Plasma samples collected from vaccinated Hartley guinea pigs were analyzed for the presence of antibodies against YFV17D and LASV antigens prepared as described in Materials and methods. Samples collected during the first 2 weeks after immunization were negative in IgG ELISA (<1:100). After a boost immunization, specific antibodies were detected to both viral antigens, LASV and YFV17D (Fig. 3). These results indicate that, in spite of low level of replication in tissues, the YFV17D/LAS-GPC recombinant virus was able to elicit specific immune responses against the vector, YFV17D, and LASV-GPC. As expected, vaccination of animals with YFV17D ("empty vector" control) did not elicit antibodies against LASV (not shown). Immunogenicity of the recombinant YFV17D/LAS-GPC seems to be lower than the immunogenicity of the another vaccine candidate, attenuated MOP/LAS reassortant, clone ML29 (Lukashevich et al., 2005). This reassortant induced specific IgG ELISA responses detectable on day 14 after a single immunization (1:5000 end-point dilution).

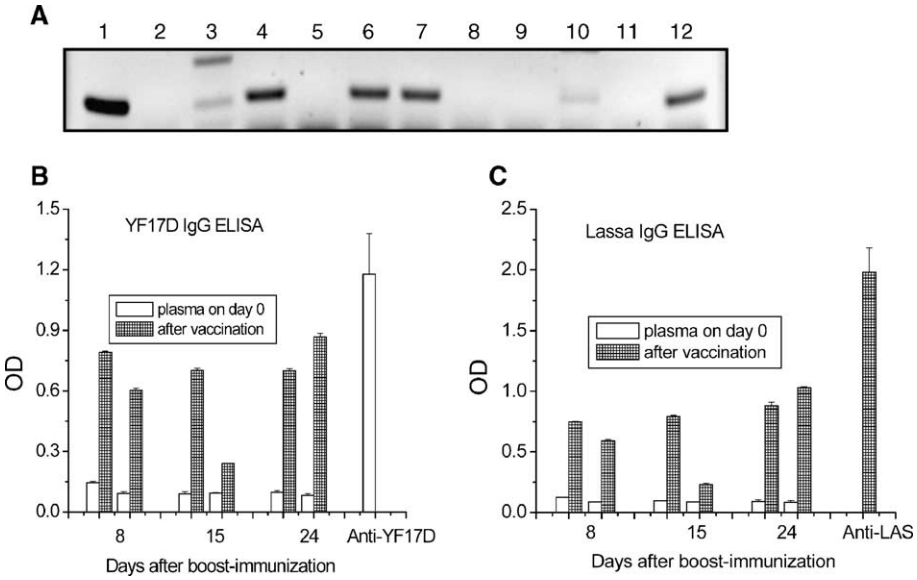

Fig. 3. Replication of YFV17D/LASV-GPC in Hartley guinea pigs. (A) Detection of YFV17D/LASV-GPC sequences in tissues: 1, Positive PCR control (recombinant cDNA); 2, negative PCR control; 3, DNA ladder, 200 and 100 nt; 4, total blood, 4 days after vaccination; 5–8, liver, 4, 7, 10, and 14 days, respectively; 9–12, spleen, 4, 7, 10, and 14 days, respectively. (B, C) Detection of anti-YFV17D and anti-LASV antibodies in plasma of vaccinated animals. LASV and YFV17D antigens were prepared as described in Materials and methods and used in IgG ELISA with plasma samples of 6 guinea pigs. Samples from two animals were collected at indicated time points and used in dilution 1:100. Polyclonal anti-YFV17D mouse antibodies and anti-LASV human serum were used as a positive control in dilution 1:1000.

Outbred Hartley guinea pigs are only partially susceptible to LASV and the infection kills no more than 30% of the animals at doses as high as 2×10^5 PFU/animal (Peters et al., 1987). In contrast, strain 13 guinea pigs are extremely sensitive to the virus and the LD_{50} for Josiah strain is 0.3 PFU (Lukashevich et al., 2005; Peters et al., 1987; Pushko et al., 2001). In the second set of animal experiments, strain 13 guinea pigs were used to evaluate protective efficacy of the recombinant YFV17D/LAS-GPC vaccine. The ideal vaccine for LF should be capable of inducing cell-mediated protection against LASV strains after a single-shot application (Fisher-Hoch and McCormick, 2004). Five strain 13 guinea pigs were s.c. vaccinated with 1×10^5 PFU of YFV17D/LASV-GPC and challenged on day 21 with 1000 PFU of LASV (Josiah). The control group included 5 animals injected with saline (negative control) and challenged with the same dose of LASV. As a positive vaccination control, a reassortant MOP/LAS virus (Lukashevich et al., 2005) was included in these experiments. As seen in Fig. 4, all MOP/LAS-vaccinated guinea pigs survived after LASV challenge and all animals died in the saline-vaccinated group. Four YFV17D/LAS-GPC-vaccinated animals survived after challenge. In this group, one animal died at day 14 after challenge and LASV infection was confirmed by biological and RT/PCR amplification of LASV RNA (not shown).

The YFV17D-based vaccines expressing foreign CTL epitopes (Bonaldo et al., 2005; McAllister et al., 2000; Tao et al., 2005) replicate poorly in animal tissues, and the mechanism of induction of protective T cell responses is not clearly understood. Preferential replication of recombinant viruses in dendritic cells (DCs) at the site of inoculation was proposed to explain the strong immunogenicity of YFV17D-based viruses (Tao et al., 2005). Indeed, it has been shown that DCs infected with YFV17D or YFV17D carrying recombinant epitopes are resistant to cytopathogenicity and effectively process and present antigens to stimulated CD8+T cell (Barba-Spaeth et al., 2005). Stable association of viral epitopes with DCs offers a mechanism for the robust and long-lasting immunity associated with YFV17D-based vaccination.

In our experiments, YFV17D/LAS-GPC vaccination elicited humoral immune responses against YFV17D and LASV antigens. Neutralizing antibodies play the major role in protection against YF (Monath, 2004). In contrast, protection against LASV is associated with strong cellular immune responses in the absence of measurable neutralizing antibodies

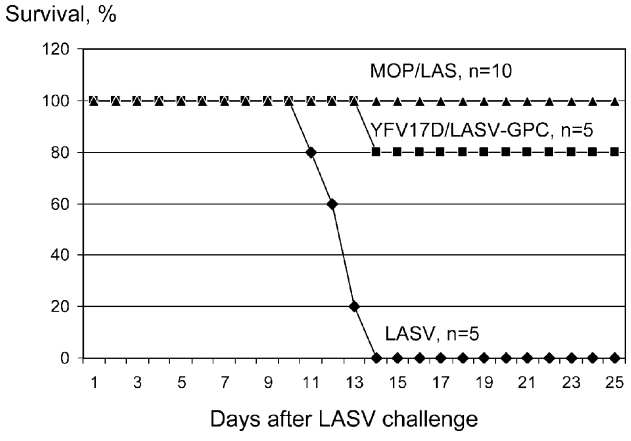

Fig. 4. Challenge experiments in strain 13 guinea pigs. Animals were vaccinated with one subcutaneous injection of MOP/LAS (10^3 PFU) or with YFV17D/LASV-GPC recombinant (10^5 PFU) and challenged subcutaneously with LASV (Josiah), 10^3 PFU per animal. Death or survival past 21 days was set up as an endpoint (Lukashevich et al., 2005; Peters et al., 1987).

(Fisher-Hoch and McCormick, 2004; McCormick and Fisher-Hoch, 2002). In YFV17D/LAS-GPC-vaccinated group, 80% of animals were protected against the fatal challenge. In a positive control group (MOP/LAS), all vaccinated animals were protected against LASV challenge (Fig. 4). Incomplete protection could be explained by differences in vaccine formulation (GPC + NP vs. GPC) and by GPC sequence differences between AV and Josiah strains of LASV (Gunther et al., 2000). The MOP/LAS reassortant contains the LASV nucleocapsid (NP) sequence and the GPC of LASV (Josiah) (Lukashevich et al., 2005). A solid body of literature indicates that both major LASV antigens, GPs and NP, are important for induction of long lasting cross-reactive cell-mediated immunity (Fisher-Hoch and McCormick, 2004; Oldstone et al., 2001; Pushko et al., 2001; Rodriguez-Carreno et al., 2005; ter Meulen et al., 2000). We are considering several approaches to making YFV17D-based constructs expressing LASV NP to enhance the protective potential of recombinant the YFV17D/LASV-GPC vaccine.

Proper processing and presentation of LASV GPC in the YFV17D vector is another issue that has to be analyzed in more detail. Only a fraction of LASV GPC was processed into GP1 and GP2 in the YFV17D/LASV-GPC transfected cells (Fig. 2). The LASV GPC signal peptide is cotranslationally released when GPC is directed to the ER. It has been shown that substitution of the GPC signal peptide with an unrelated signal sequence does not affect translocation of GPC into the ER (Eichler et al., 2003). The cleavage of GPC into GP1 and GP2 is a rather late event in LASV glycoprotein maturation. Since the GPC signal peptide promotes LASV GPC processing by functioning as a *trans*-acting factor mediating GPC cleavage (Eichler et al., 2003), the deletion of the GPC signal sequence is the most likely reason for inefficient cleavage of GPC into GP1 and GP2 in cells infected with the recombinant YFV17D/LAS-GPC virus. Our initial attempts to insert the whole pre-GPC LASV sequence resulted in low titers of recombinant virus and in low levels of LASV GPC expression. It seems that one or two hydrophobic domains of the LASV pre-GPC signal peptide were interfering with the proteolytic processing of the YFV17D polyprotein.

The YFV17D backbone has been used for the development of recombinant vaccines against several flavivirus-mediated diseases like Japanese encephalitis, dengue types 1–4 and West Nile viruses (Monath, 2004). These vaccines elicited protective neutralizing antibodies in rodent and monkey models, passed safety tests and are currently in human trials. Recently, the YFV17D vector was successfully used to design recombinant vaccines for delivery of CTL epitopes against *P. yoelii*, a rodent malaria parasite (Bonaldo et al., 2005; Tao et al., 2005), the immunodominant HLA-A2 M1 epitope of influenza M protein (Barba-Spaeth et al., 2005) and as an attractive therapeutic anticancer vaccine (McAllister et al., 2000). These previous successes and the data presented in this study demonstrate that YFV17D may be a very suitable vector to develop a safe and effective bivalent YF/LF vaccine for Africa.

Materials and methods

The YFV17D/LASV-GPC plasmid was constructed in the background of the full-length YFV17D cDNA clone (Bredenbeek et al., 2003) by fusion PCR mutagenesis. The LASV-GPC gene of the AV strain (Gunther et al., 2000) was amplified by RT/PCR and cloned into pcDNA. The nucleotide sequences of PCR-derived DNA fragments and gene fusions were confirmed by sequencing. The recombinant YFV17D/LASV-GPC plasmid was linearized by *Xho*I and used for in vitro RNA transcription. In vitro RNA transcription, electroporation of the BHK-21J cells, in vivo RNA labeling, preparation of virus stocks, immunofluorescense and plaque assays were previously described (Bredenbeek et al., 2003). For Western blot analysis, transfected cells were lysed at 24 h post-electroporation, lysates were subjected to SDS-PAGE separation and proteins were electroblotted to PDVF membranes (Hybond-P, Amersham, Piscataway, NJ). Monoclonal anti-GP1 and polyclonal rabbit anti-GP2 antibodies (gift from W. Garten, the Institut für Virologie der Phillips-Universität Marburg, Germany) were used to identify the LASV glycoproteins after incubation with an appropriate secondary goat IgG conjugated to alkaline phosphatase using NBT/BCIP (Invitrogen, Carlsbad, CA) as chromogenic substrate.

Sixteen outbred guinea pigs were subcutaneously inoculated with 1×10^5 PFU/0.5 ml of the recombinant YFV17D/LASV-GPC virus and two animals were sacrificed at days 0, 4, 7, 10 and 14 to track the virus distribution in blood and tissues. RNA from plasma and tissue samples was extracted using QIAamp viral minispin and RNeasy kits, respectively (Qiagen Inc., Valencia, CA). RNA from total blood was extracted using a RiboPure isolation kit (Ambion, Inc., Austin, TX). Isolated RNA samples were converted into cDNA (SuperScript III, Invitrogen) and amplified with YFV17D primers (sense: 5′-AATCGAGTTGCTAGGCAATAAACAC; anti-sense: 5′-TCCCTGAGCTTTACGACC AGA). At day 14, six animals were boosted with the same dose of the recombinant virus and plasma samples were collected on days 8, 15 and 24 after to measure antibody responses against YFV17D and LASV-GPC in IgG ELISA. Antigens were prepared from serum-free virus stocks of YFV17D and MOP/LAS (Lukashevich et al., 2005) by ultracentrifugation on sucrose cushion. Concentrated viruses were suspended in carbonate–bicarbonate buffer, briefly sonicated and used to cover wells of microtitration plates (overnight, 4 °C). After blocking, 1:100 dilutions of guinea pig sera were added and incubated for 2 h at room temperature. Peroxidase-labeled goat anti-guinea pig IgG (KPL, Gaithersburg, MD) and substrate solution (Turbo TMB-ELISA, Pierce, Rockford, IL) were used for color development. Challenge experiments were performed in strain 13 guinea pigs purchased from USAMRIID (Fort Detrick, Frederick, MD). Vaccinated animals were challenged within a biosafety level 4 facility at the Southwest Foundation for Biomedical Research (San Antonio, TX) as previously described (Lukashevich et al., 2005). The challenge stock of LASV (Josiah) was obtained from the Centers for Disease Control and Prevention (Atlanta, GA).

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