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UMinho | 2019

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Tese de Doutoramento
Doutoramento em Medicina

Trabalho efetuado sob a orientação do
Professor Doutor Matthias J. Schnell
e do
Professor Doutor António Gil Castro

novembro de 2019

Acknowledgments

"What can be asserted without evidence can be dismissed without evidence"

By Christopher Hitchens

Firstly, I would like to acknowledge Dr. Schnell for your relentless dedication in mentoring me, which in turn allowed me to become the scientist and critical thinker I am now. Your belief and investment in me encouraged to push towards my full potential. I could not wish for a better mentor. I will always cherish those humorous moments that I and the rest of lab shared with you. Also, many thanks for all the Ritter Sport chocolate and the espresso machine, couldn't do it without them!

It is impossible to acknowledge the work done in here without acknowledging all my labmates that accompanied throughout my PhD thesis. Thanks for all the stimulating discussions, all the work and support shared throughout my time in the lab, and finally for making the lab a fun place to be. I would like to finish my acknowledgments to the Schnell lab with a special thanks to Dr. Christoph Wirblich (Chris) who taught me all the technical and theoretical skills required for all the benchwork done, especially in my first year of PhD.

I would like to acknowledge all the faculty at Escola de Medicina in University of Minho that established the MD/PhD program and gave me this opportunity. Thank you for continuing to strive for excellency and in pushing the medical education forward in Portugal.

Finally, to all my friends and family back in Portugal, that, despite being an ocean apart, have continuously supported me throughout these years.

Financial support for this PhD was provided by FCT (Fundação para Ciência e Tecnologia), by the fellowship PD/BD/105847/2014, awarded through the MD/PhD program of University of Minho.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Development of a Lassa/Rabies virus vaccine based on the rabies vector

Lassa fever (LF), is a viral hemorrhagic fever caused by Lassa virus (LASV), for which neither an approved vaccine or effective treatment is available. LASV is an endemic virus in western Africa and a major health and economic burden, causing an estimate 100,000-300,000 infections yearly, with the number of reported infections increasing in the last years. This thesis describes the development of LASSARAB, a dual LF and rabies vaccine based on recombinant rabies vector. Rabies is another equally important disease in Africa that is estimated to cause thousands of deaths every year, despite vaccination being available.

LASSARAB uses a codon optimized version of LASV's glycoprotein (GPC) as its LASV immunogen. After confirming that LASSARAB expresses and incorporates GPC in the virions, LASSARAB's potential as an LF vaccine was tested using several LASSARAB based vaccine candidates: inactivated LASSARAB formulated in PBS only; inactivated LASSARAB formulated with GLA-SE adjuvant (a TLR-4 agonist); live-LASSARAB; and live-LASSARAB-ΔG, a variant of LASSARAB that lacks the Rabies G gene. Inactivated LASSARAB formulations induced a significant GPC specific IgG response above background with LASSARAB+GLA-SE inducing higher IgG titers than LASSARAB alone as well as a lower IgG1/IgG2c ratio. Neither live-LASSARAB vector induced a significant LASV GPC immune response. In LASV challenge studies, in both guinea pig and mouse, inactivated LASSARAB+GLA-SE, significantly protected 80% of the animals against LF disease. Higher titers of anti-GPC IgGs were correlated with protection independently of LASV neutralizing titers. Instead, non-neutralizing LASV GPC-specific antibodies, through antibody-dependent cellular functions (ADCC and ADCP) appear to the main drivers of protection against LF as demonstrated by in *vitro* and *in vivo* studies. Overall, these findings demonstrate an effective inactivated LF and rabies vaccine and suggest a novel correlate of protection for LF. Currently, further LASSARAB immunogenicity studies using NHPs are underway determine its eligibility for clinical phase 1 trials.

Keywords: ADCC; ADCP; arenaviruses; Fc receptor; FcγR; glycoprotein; GPC; Lassa Fever; LASV; non-neutralizing antibodies; rabies; rhabdovirus; vaccine.

Desenvolvimento de uma vacina para febre de Lassa e Raiva baseada no vetor de Raiva

A febre de Lassa (LF), é uma febre hemorrágica viral causada pelo vírus Lassa (LASV), para a qual não existe nem vacina ou tratamento aprovado. O LASV é um vírus presente na África ocidental que incute um pesado encargo na saúde pública regional, e estima-se que causa 100,000 a 300,000 infeções por ano. Esta tese é sobre o desenvolvimento de LASSARAB, uma potencial vacina contra a LF e Raiva baseada num vetor do vírus da Raiva recombinante. A Raiva, é outra doença igualmente importante em África que causa milhares de mortes todos anos apesar de haver vacina disponível.

LASSARAB usa uma versão codon-optimized da glicoproteína de LASV (GPC) como o imunogénio de base contra LASV. Após confirmar que LASSARAB expressa e incorpora o LASV GPC nas partículas virais, o potencial de LASSARAB foi testado através de vários candidatos incluindo: LASSARAB inativado formulado em PBS apenas; LASSARAB inativado formulado com o adjuvante GLA-SE (um agonista TLR4); LASSARAB vivo; e LASSARAB-ΔG vivo, uma variante de LASSARAB que não expressa o gene da glicoproteína de raiva. Ambos os candidatos LASSARAB inativados induziram uma elevada resposta imune contra GPC, com LASSARAB+GLA-SE induzindo níveis de IgGs contra GPC mais elevados assim como um ratio IgG1/IgG2c mais baixo. Em estudos de exposição com LASV em porquinhos da Índia e ratinhos, o LASSARAB+GLA-SE inativo foi capaz de proteger em 80% os animais contra LF. Níveis mais elevados de IgGs específicos para GPC, foram correlacionados com proteção independentemente dos níveis de anticorpos neutralizantes contra LASV. Pelo contrário, IgGs específicos contra GPC não neutralizantes, através de funções celulares dependentes de anticorpos (ADCC e ADCP) parecem ser os principais intervenientes na proteção contra LF. Em conjunto, estes resultados demonstram uma vacina inativada eficaz contra LF e Raiva e sugerem uma correlação de proteção contra LF, baseado na resposta de IgGs específicos contra GPC. Atualmente, estudos em primatas estão a decorrer para testar a imunogenicidade de LASSARAB neste modelo em preparação para clinical phase 1 trials.

Palavras-chave: ADCC; ADCP; anticorpos não neutralizantes; arenavírus; FcγR; Febre de Lassa; glicoproteína; GPC; LASV; rabdovírus; Raiva; receptor Fc; vacina.

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Abbreviations

ADCC – Antibody dependent cellular cytotoxicity
ADCP – Antibody dependent cellular phagocytosis
ALT – Alanine Aminotransferase
APC – Antigen presenting cell
AST – Aspartate Aminotransferase
B6 – C57BL/6 mice
BM – Bone Marrow
BPL – β-Propiolactone
BSL-(n) – Biosafety Level (n)
CEPI - Coalition for Epidemic Preparedness Innovations
cDNA – complementary DNA
CFR – Case fatality rate
coGPC – codon optimized GPC
DC – Dendritic Cell
DIC – Disseminated intravascular coagulation
EBOV – Ebola virus
EGFP – Enhanced green fluorescent protein
ELISA – Enzyme-linked immunosorbent assay
ER – Endoplasmic reticulum
EVD – Ebola hemorrhagic disease
FDA – Food and Drug Administration
GLA-SE – Glucopyranosyl Lipid A in a stable oil-in-water nano-emulsion
GLP – Good laboratory practice
GMP – Good manufacturing practice
GPC – Glycoprotein complex (LASV)
HHD – HLA-A2.1 transgenic mice
HLA – human leukocyte antigen
IFNAR – Interferon-α/β receptor
IFN – Interferon
IgG – Immunoglobulin G
IgM – Immunoglobulin M
IGR – Intergenic Region
IL-(n) – Interleukin
ISG – Interferon stimulated genes
KO – Knockout
L – RABV/LASV polymerase
LASV – Lassa hemorrhagic fever virus
LCMV – Lymphocytic choriomeningitis virus
LF – Lassa hemorrhagic fever
mAb – monoclonal antibody
MHC-I – major histocompatibility complex I
MHC-II – major histocompatibility complex II
MOPV – Mopeia Virus
MP – macrophage
MPLA – monophosphoryl lipid A
mRNA – messenger RNA
MVA – modified Ankara Vaccinia virus
nAb – neutralizing antibody
NHP – Non-human primate
NIAID – National Institute of Allergy and Infectious Diseases
NIH – National institute of Health
NLR – NOD Like receptor
NK cells – Natural Killer Cells
NLR – NOD like receptor
NO – Nitric oxide
NOD – nucleotide-binding oligomerization domain
non-nAb – non-neutralizing antibody
NP – Arenaviridae Nucleoprotein

NW Arenaviruses – New world arenaviruses

ORF – Open reading frame

OW Arenaviruses – Old world arenaviruses

PAMPs – Pathogen associated molecular pattern

PRRs – Pattern recognition receptor

ppVSV – pseudotyped VSV particle

RABV – Rabies virus

RdRP – RNA dependent RNA polymerase

RIG – Retinoic acid inducible gene I

RLR – RIG-I-like receptor

RVG – Rabies Virus Glycoprotein

rVSV – recombinant Indiana vesiculovirus

S1P – Site 1 protease

SSP – stable signal peptide

STAT1 – signal transducer and activator of transcription 1

TLR – Toll like receptor

TNF-α – Tumor necrosis factor alpha

VHF – viral hemorrhagic fever

VLP – virus like particle

VNA – Virus neutralization assay

WHO – World Health Organization

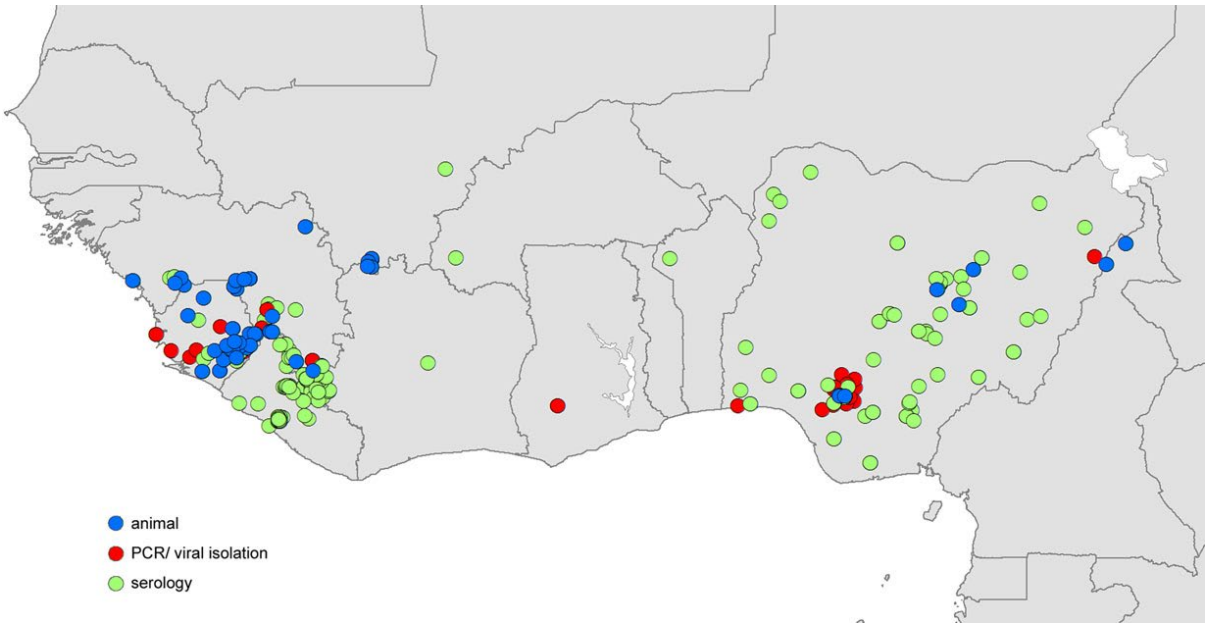
WT – wild type

Chapter 1

Introduction

1.1 Lassa Hemorrhagic Fever: Etiology and epidemiology

Lassa Hemorrhagic Fever (LF) is a viral hemorrhagic fever (VHF) whose etiologic agent is Lassa virus (LASV). LF was first documented and reported in 1969 after a group of three missionary nurses became gravely ill in Lassa, Nigeria, resulting in two deaths[1]. The surviving nurse was evacuated to the US which lead to the subsequent isolation of LASV as the etiologic agent of LF. LASV's lethally and lack of therapy or preventive measures led to its classification as a biosafety level-4 (BSL-4) agent[2,3]. However, unlike other BSL-4 hemorrhagic fevers, LASV was later confirmed to be widely spread throughout West Africa (figure 1). This makes LASV, by far, the single most prevalent BSL-4 virus, infecting an estimated 100,000 to 300,000 humans every year[4]. Of these infections, there is an estimated 5,000 fatal casualties with thousands more suffering severe sequela post recovery (up to a third of the cases), such as neurosensory deafness[5–8]. Indeed, LF is estimated to be one of the leading causes of adult-acquired neurosensory deafness in West Africa[9–12]. As such, the health and socioeconomic burden that LF causes in western African communities cannot be ignored. Thus, with further globalization, climate change and population growth, the probability of LASV transitioning from a local to a global threat is currently increasing[13–18].



Adapted from (13): Mylne, A. Q. et al. Mapping the zoonotic niche of Lassa fever in Africa. Trans R Soc Trop Med Hyg 109, 483–492 (2015)

Figure 1. Epidemiologic map reporting the LASV presence in both human and animal reservoirs. Bue dots are LASV serology detected in animals, red dots are LASV isolated from human cases and green dots represent human serology data indicating previous LASV contact in humans.

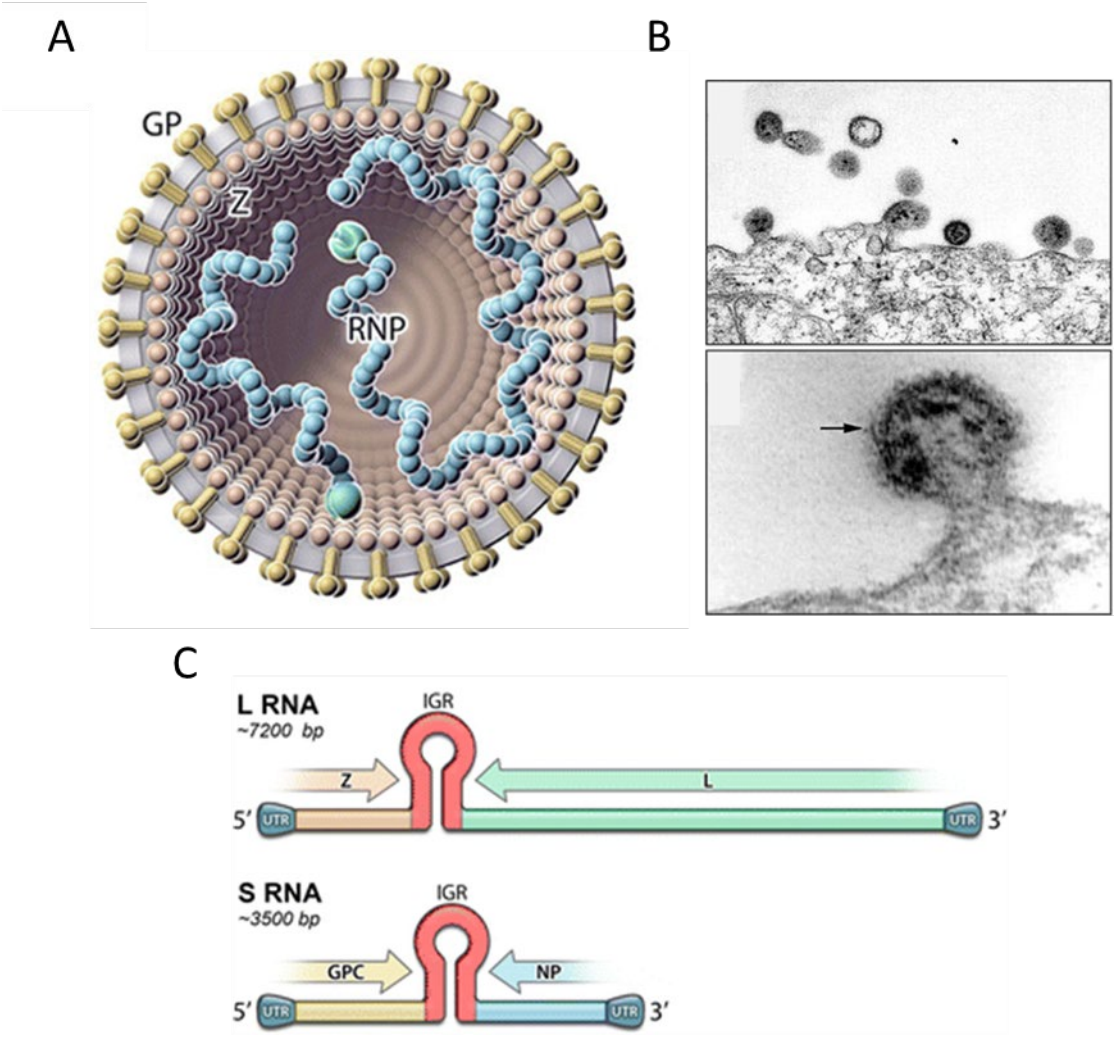
LASV's high prevalence is coupled with the extensive presence of its animal reservoir, *Mastomys natalensis,* or the common african rat[19,20]. *M. natalensis*, bears chronic and productive LASV infection without developing any detrimental effects, continuously shedding virus in its excrements[19–21]. Since *M. natalensis* is commonly associated with human settlements and dwellings, LASV transmission to humans occurs by contacting or inhaling aerosols of contaminated surfaces and materials[22]. Furthermore, due to economic and cultural reasons *M. natalensis* is widely consumed as bushmeat, constituting an important protein source in famine-stricken communities[23]. Indeed, LF cases usually peak following periods of increased *M. natalensis* activity such as the transition to the rainy season[24].

Interestingly, the high number of estimated annual LASV infections (100,000-300,000), contrasts with the relatively much lower estimated numbers that result in human fatalities (5,000)[8,25,26]. The case fatality rate (CFR) based on these numbers significantly differs from LF cases reported in clinical settings, where reported CFR averages 20%[27]. This discrepancy can be partly explained by the fact that a significant amount of infections occurs in individuals that were previously infected, and thus are thought to be protected[28]. As such, as many as 80% of LASV exposures are asymptomatic and go unreported. The remaining 20% of LASV exposures develop into LF which can reach CFR as high as 60%[27]. In the most recent surge of LF, in Nigeria 2018, the CFR was 25%[29,30]. Such discrepancy in fatality rate can be dependent on both the contributing strain (e.g., western strains, found in Mali and Sierra Leone, are reported to be more pathogenic) and the population afflicted (e.g., pregnant women are especially susceptible)[31–33]. It has been postulated that since hospital outbreaks are associated with higher mortality, that human-to-human transmission cases are usually more pathogenic. This was also observed for another VHF, Ebola hemorrhagic fever (EVD), in which human-to-human transmission was associated with a higher viral pathogenicity in the 2013-2016 West African Ebola virus epidemic[34,35]. However, a large-scale sequence analysis performed by a team led by Sabeti (2015) on several LASV isolated strains found no difference in fatality rates between infections from mouse-to-human or human-to-human transmissions. Instead, they found that western LASV strains tended to be more pathogenic[36]. This was later confirmed in the 2018 by Siddle et al, that analyzed LF outbreaks from 2015 to

2018 including the latest 2018 Nigeria outbreak and concluded that human-to-human transmission did not result in higher pathogenicity[29]. They also concluded that the increased number of LF outbreak recently reported in Nigeria 2018 was not due to a spontaneous mutation in LASV that conferred higher pathogenicity, but instead was a result of higher activity and numbers of *M. natalensis*. This higher activity was correlated with an increased average of rainfall and temperature registered in past years.

1.2 Introduction to the *Arenaviridae* family

LASV is a member of the *Arenaviridae* family, a group of viruses that belong to Group V in Baltimore classification, which are ambisense bi-segmented negative sense single-stranded RNA viruses[37]. Morphologically, on electron microscopy (figure 2b), *Arenaviridae* are characterized by having sand like granules of 20-25 nm in size, from which its name was originated (*Arena* translates from latin to sand)[38]. *Arenaviridae* includes the widely spread mouse pathogen LCMV and Junin Virus the etiologic agent of Argentinian hemorrhagic fever. With the recent (2014) discovery of *Arenaviridae* that are capable of infecting reptiles, the *Arenaviridae* order was further subdivided in *Mammarenavirus* and *Reptarenavirus*[39]. Due to geographic barriers and genetic drift *Mammarenavirus* are further divided into Old World *Mammarenavirus*, first discovered and present mainly in the Eastern Hemisphere (Europe, Africa and Asia) and of which LASV is part of, and New World *Mammarenavirus*, such as Junin Virus, which analogously can mostly be found in the Western Hemisphere (Americas)[40]. LCMV is an exception since it is found worldwide, but its genetically classified as an Old World *Mammarenavirus*[41].

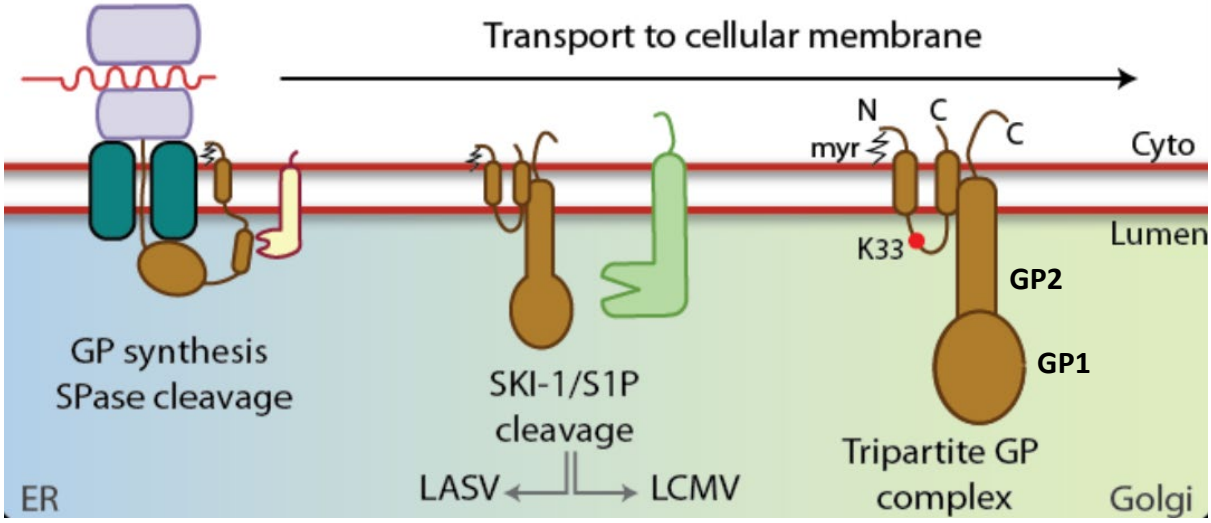


Adapted from (39): Radoshitzky, S. R. et al. Past, present, and future of arenavirus taxonomy. Arch. Virol. 160, 1851–1874 (2015).

Figure 2. *Arenaviridae* morphological and genetic depiction. In (A) a schematic overview of each protein can be observed. In (B) electron micrographs depicting an Arenaviruses virions, both free and budding, with sand like granules easily recognizable. (C) Schematic overview of an Arenavirus genome.

Arenaviruses' genome is encoded within two circular single stranded RNA segments: the ~7.2 Kb segment L (Large) and ~3.4 Kb S segment (Small)[37]. Each segment contains 2 genes in opposite polarity to each other (see figure 2c) and are separated by a non-translatable RNA sequence termed IGR (intergenomic region) that serves as transcription regulator[42]. The Large segment composes of the Z gene (matrix protein) in positive polarity, and the L gene (Viral polymerase) in negative polarity. Meanwhile, the S segment contains the GPC gene (viral envelope glycoprotein) and the RNA binding protein NP (nucleoprotein), which are encoded

in a positive and negative polarity, respectively. Viral mRNA transcription and genome replication are executed by the viral transcription machinery complex that is composed by both L and NP proteins[43,44]. IGR forms a stem-loop structure that serves as a de facto replication termination signal for the viral RNA polymerase[42]. By having 2 genes in the negative sense (L and NP) and 2 genes in the positive sense (Z and GPC), such a strategy enables *Arenaviridae* to elegantly regulate genetic transcription upon entry in the cellular cytoplasm[37,45]. While negative sense genes are quickly transcribed into mRNA and thus firstly translated into their respective proteins, the positive stranded RNA genes require the transcription of a negative sense full genome RNA intermediate that servers as a template for viral mRNA. L and NP, which are required for the initial steps of viral replication, transcription, and interferon antagonism, are produced first, while Z and GPC which are required for viral assembly and budding are produced in later stages of infection (see figure 2c)[46–50]. Indeed, Z not only serves as structural protein for capsid assembly it also functions as an inhibitor of the L-NP transcription/replication complex in high concentrations[51].



Adapted from (53): Burri, D. J., da Palma, J. R., Kunz, S. & Pasquato, A. Envelope glycoprotein of arenaviruses. Viruses 4, 2162–81 (2012).

Figure 3. Lassa Glycoprotein complex (GPC) synthesis.

The glycoprotein complex GPC, a trimeric complex composed of three GP1-GP2 heterodimers and three SSPs (stable signal peptides) at its core, is the viral envelope glycoprotein and is responsible for viral entry after receptor attachment[50,52]. GPC's mRNA is first translated as the polyprotein pre-GPC and traffics to the endoplasmic reticulum (ER) through its C terminal signal peptide (see Figure 3)[53]. In the ER the signal peptide is cleaved, forming pre-GP which

is further processed by site 1 protease (S1P) in the ER into GP1 and GP2[54]. Instead of following the usual degradation path of other signal peptides, the signal peptide is preserved and has critical role in GPC maturation and stability, thus being denominated stable signal peptide (SSP). Throughout the preGP's maturation process, heavy glycosylation occurs in five sites of GP1 and four sites of GP2, which is essential for proper GP1-GP2 heterodimer formation[55,56]. Since GP1-GP2 dimers are not covalently bound, the resulting glycoprotein is relatively unstable. Final maturation into the trimeric glycoprotein complex (GPC) is thought to occur in the Golgi complex where SSP appears to be a catalyzer and stabilizer of GP1-GP2 trimerization[57]. After mature GPC is formed it's trafficked to the cellular surface for viral budding to occur (see figure 2e). Therefore, GPC is exposed in both the virion surface and the cellular membrane. GPC's extensive exposure makes it an attractive target for the immune system. Nevertheless, GPC not only is a relatively unstable protein complex due to the lack of covalent bonding between its subunits, it's also heavily glycosylated (30~40% of its molecular weight)[58,59]. This heavy glycosylation is a hallmark of *Arenaviridae* and is thought to be essential for GPC quaternary structure assembly as well as attachment to its canonical receptor site, α-Dystroglycan. This glycosylation also works as an effective glycan shield that disrupts antibody binding and immune system recognition[60]. Indeed, LASV is described to have more glycosylation than other Arenaviruses[58].

1.3 LASV pathogenesis

As previously stated, LASV infection does not always lead to LF. Although, this can be partly accounted by LASV re-infections in previously exposed individuals, these individuals should still have disease upon the first infection. Therefore, a significant portion of LASV infections in naïve humans are efficiently cleared[8,61]. As previously introduced in chapter 1.1, there is evidence that LASV pathogenicity is linked to geographically restricted strains, with western lineage strains having a higher CFR[36]. However, the increased pathogenicity observed with western strains can also be attributed to genetic differences of the afflicted populations as well as socioeconomic variations[67–69].

Currently, LASV can be subdivide in four lineages (Lineage I-IV) based on genetic variation (see figure 3a)[36,62,63]. Strains belonging to lineages I-III can mostly be isolated from more eastern countries such as Nigeria. Meanwhile, strains belonging to lineage IV, are mostly restricted to Sierra Leone[64]. Strains from lineage IV of LASV, which includes Josiah strain, are often reported to be the most pathogenic and lethal. Indeed, Josiah strain, first isolated in 1976 from a patient in Sierra Leone, has become the prototypical LASV strain owing to its lethality in animal models as well as its availability[65,66]. Recently, based on genetic variation of LASV isolates discovered in Cote d'Ivore and Mali, Manning et al proposed the classification of LASV lineage V, however, as of now, it hasn't been officially recognized[13].

Beyond geographical differences and strain variation, LASV pathogenicity has been tied to the initial immune response post infection, route of infection, and inoculum size[70]. After LASV infection two outcomes are usually observed: 1) mild flu-like symptoms with eventual clearance of the virus by both the innate and adaptive immune system in the first 2 weeks; 2) gradual symptom worsening after day 6-8 with increasing AST/ALT serum levels that in severe cases culminate in hepatic and multiorgan failure, at 10 to 20 days post infection[68,71]. The second outcome is what is classically described as LF.

Most LASV infections are thought to occur through inhalation of aerosolized LASV virions or by mucosal contact with contaminated bodily fluids (eg. blood, saliva etc)[72]. After inoculation,

LASV has been proposed to first replicate in both endothelial cells and dendritic cells (DC)/macrophages (MP) where it then spreads through the lymphatic and blood systems[69,73,74]. *In vitro* experiments have shown that human DC's not only are permeable to productive LASV infection, but LASV infection inhibits DC's activation and maturation into effective antigen presenting cells (APCs)[74]. Recently, Schaeffer et al showed that pathogenic arenaviruses (LASV) are able to activate myeloid DCs but in turn, these DCs cannot activate neighboring T cells in contrast with myeloid DCs infected with non-pathogenic arenaviruses (Mopeia Virus)[75]. Indeed, as disease progresses in lethal LF cases there is infiltration of LASV infected DCs in lymphoid and reticulated organs (e.g. Liver, spleen, kidney)[27,76].

Like other VHF such as Ebola, LASV replication in the endothelia is also a contributing factor to pathogenesis since it leads to increased vascular permeability and dysfunction[71,77]. This in turn culminates in hemorrhage and, in more severe cases, hypovolemic shock[71,78]. However, in contrast with what is observed in other VHFs, the symptoms and hemorrhage are rarely severe enough to cause disseminated intravascular coagulation (DIC). Instead lethal cases of human LF are often accompanied by hepatitis with increased levels of AST/ALT enzymes and extensive liver necrosis (up to 40%). Indeed, LF was initially described as an hepatitis, akin to yellow fever (also common in the area), which, combined with the lack of DIC, has led some authors challenge the categorization of LF as an VHF in the past[2,70,79,80]. The hepatic involvement in LF is thought to be due to the extensive viral replication there occuring[70]. Interestingly, the fully glycosylated form of α-DG, the prototypical viral receptor for OW Arenaviruses and LASV, is expressed at low levels in mature hepatocytes due to downregulation of like-acetylglucosaminyltransferase (LARGE) in these cells. Such observation is inconsistent with the high viral load observed in the liver within fatal cases of LF. In recent years studies by the Kunz and Lukashevich groups proposed that LASV infection of hepatocytes might be through non-canonical receptors such as Axl[73]. This infection induces hepatocyte proliferation as part of a compensatory regenerative liver response, leading to the up-regulation of more proteins that can serve as LASV receptors (such as α-DG, Axl, and DC-SIGN). Nevertheless, McCormick et al (1986) described that the liver damage alone, although present and severe in lethal LF cases, is insufficient to cause hepatic failure and

consequently death[80]. As such, the hepatic impact in LF pathogenesis required further elucidation.

The current consensus in the field, is that LF pathogenesis is through an immunosuppressive mechanism resulting in uncontrolled viral replication and damage which leads to multi-organic failure[68,81]. The fact that LASV infection impedes immature DCs from becoming APCs and activate T cells further corroborates this hypothesis[74,75]. In an NHP study by Baize et al, determining factors for LASV infection survival were associated with inoculum size (higher dosage had, paradoxically, no fatality in contrast with low dosage) and the establishment of an early LASV specific immune response[82]. In survivors, a substantial increase in circulating IFN-γ was detected 3 days post infection something not observed in succumbed animals. Survivors also had higher circulating titers of LASV specific IgGs and a proliferative LASV specific T cell response (total populations of: $CD4^+$, $CD4^+CD25^+$, and $CD8^+CD69^+$) in *ex-vivo* stimulation. In contrast, animals that succumbed to infection had no detectable LASV specific T cell proliferative response in *ex-vivo* stimulation. Instead a marked downregulation of co-stimulatory surface markers such as CD40, CD80 and CD86 in circulating DCs is observed[68,82]. Importantly, these DCs also had decreased secretion levels of type I IFN, IL-6 and TNF-α secretion. LASV specific IgGs were detected at lower titers and 3 days later in animals that succumbed to infection compared survivors. This indicated that LF pathogenesis and lethality in these animals seemed to be linked with a general immunosuppression and inability to control viremia. In human patients with LF, lymphopenia is sometimes observed with accompanying necrosis of secondary lymphoid organs[3,83].

Nevertheless, there is also evidence in which human patients that succumbed to LF had extremely high serum levels of IL-6 and TNF-α, somewhat akin to a "cytokine storm"[84–86]. This would, in contrast, indicate an immunopathological component of the disease. The extensive liver damage associated with neutrophilic infiltrations that are characteristic of LF further corroborates such a theory. Indeed, an immunopathological component of LF like syndrome was observed in two different mouse models infected with LASV[87,88]. In the first instance, the Pinschewer group observed that HHD mice (murine MHC-I KO C57BL/6 mice expressing human MHC-I, HLA-A2.1) were highly susceptible to LASV infection. Instead of quickly clearing

infection, as WT C57BL/6 mice do, these HHD mice displayed extensive vascular leakage with lung and liver parenchymal damage, similar to what is observed in certain human cases of LF[89]. These pathological changes were accompanied by elevated levels of IL-12p40 and nitric oxide synthase (iNOS), which are general markers of a systemic inflammatory condition. However, prior T cell depletion to LASV infection HHD mice rescued this phenotype with no increase in AST/ALT (hepatic damage markers) and no DC/MP activation as measured by iNOS expression and IL-12p40 secretion. Despite the disease rescue presented by mice with T cell depletion, mice were unable to clear LASV infection and developed persistent infection without organ damage. These results indicated that certain LF cases might have an important immunopathological component that is dependent on T cells and possibly the HLA phenotype of the individual. Similar findings were described by the Muñoz-Fontela group using a chimeric bone marrow mouse model. In this study, $IFNAR^{-/-}$ C57BL/6 mice were irradiated to deplete their hematopoietic compartment in the bone marrow and subsequently received a bone marrow transplant from WT C57BL/6 mice thus repopulating their hematopoietic compartment with immune competent cells. These mice were able to replicate key features of LF pathology after LASV infection, which was not observed in either fully WT or $IFNAR^{-/-}$ mice. While WT mice survived LASV exposure with minimal symptom development and full viral clearance, $IFNAR^{-/-}$ survived exposure but failed to resolve LASV infection, developing instead chronic LASV infection with no symptomatic repercussion. In contrast LASV exposure in chimeric mice resulted in high lethality with extensive liver damage and vascular leakage, hallmarks of LF disease. While myeloid cell depletion had no effect in the overall course of the disease in these mice, $CD8^{+}$ T cell depletion rescued this lethal phenotype albeit resulting in protracted LASV infection. Taken together, both these works show compelling evidence of an immunopathological component of LF, albeit in a somewhat contrived system. It's noteworthy to mention that exposure of non-pathogenic Arenaviruses (e.g. Mopeia Virus) in either the HHD or the chimeric mice resulted in quick viral clearance with no pathological consequences.

The apparent multi-etiologic pathogenicity of LASV is a major obstacle for LASV therapeutically development. LASV pathogenicity might not only be tied to strain variation but also to possible genetic susceptibilities (certain HLA types) of the infected individuals. As

one can infer, LASV induced immunosuppression appears to be a critical feature for the establishment of LF pathogenesis. Nonetheless, the extensive histopathological damage caused to the endothelia, liver, and lung in such a short duration seems unlikely to be caused by LASV replication alone[68,70,88]. The T cell dependence of LASV pathogenicity in the mouse models combined with the elevated levels of circulating inflammatory cytokines detected in terminal LF cases indicate that an immunopathological component might be required for certain LF cases. A possible solution to this paradox appears to be the fact that the initial immunosuppression caused by LASV in DCs is responsible for initial widespread viral infection. Thereafter the establishment of replicative repertoires in the target organs (e.g. liver, endothelia) reaches a viral load threshold where immunosuppression is no longer effective. This leads to extensive immune activation and consequent damage of these organs leading to a lethal positive feedback effect. This, however, remains to be elucidated in future studies.

1.4 Lassa Fever animal models

LASV research requires BSL-4 facilities due to its high pathogenicity in humans and lack of effective treatments. These facilities are not only expensive to run but also their availability is scarce. Another major obstacle for LASV research is the lack of established animal models[90]. Small animal models such as mice are resistant to LASV infection without genetic or phenotypic modifications[91]. LASV infection in Guinea pigs is highly lethal however its pathogenicity can be different from human LF depending on the guinea pig strain used[92]. Large animal models, such as NHPs, closely replicate LF pathogenesis observed in humans however their elevated cost and ethical concerns makes their use limited. In the following paragraphs animal models currently in use for LF research will be described.

1.4.1 Murine model

The prevalent use of the mouse model in scientific research provides ample availability of immunological and genetic tools to study LASV infection and its pathogenesis. Nonetheless, rodents are also the viral repertoire of LASV. As such LASV infection of mice through more natural routes (e.g. oral, subcutaneous) usually results in successful viral clearance[93]. LASV is fatal in adult mice if infected through an intracranial route, although the resulting pathogenesis (meningitis) and route of infection itself are vastly different from what happens in humans and will not be explored, but is reviewed here[90]. Another possible outcome that occurs in immunosuppressed/suckling mice is persistent LASV infection without corresponding pathogenicity, as similarly observed with LCMV clone 13[94,95]. The use of mouse model for LF research requires thereof either genetic manipulation, the use of a surrogate LASV virus, or a combination of both. As such, even if reliable replication of LF phenotype is achieved, this might be an artifactual finding due to the use of a contrived system. However, results from these models can provide valuable predictors, indicating whether more expensive and cumbersome animal models should be used[96,97]. In the following sections I'll explore the different approaches that have been described so far.

LASV infection in murine MHC-I KO C57BL/6 mice expressing human MHC-I, HLA-A2.1

In 2010, the Pinschewer group had serendipitously observed that humanized mice (HHD mice) infected with LASV replicated key LF pathogenic features observed in humans[88]. Intravenous (i.v.) injection of 10^6 pfu of LASV (Strain Ba366) in HHD mice caused lethal LF disease that replicated the extensive liver damage observed in humans. Histopathological changes in the spleen, lung and liver were also observed that were analogous to human samples. This model had a 20% lethality rate which mimicks the fatality rate observed in humans with LF in clinical settings. Nevertheless, the lack of uniform pathology are major hurdles for vaccine and therapeutically efficacy studies using this model. Regardless, it remains a valuable alternative small animal model for immune mediated LF pathogenesis research.

LASV infection in STAT1$^{-/-}$ mouse model

Another LF mouse model described is based on highly immunocompromised STAT1$^{-/-}$ mice. After infection with 2 different strains of LASV, mice quickly succumbed to infection in 6 to 7 days with histopathologic findings in the liver and spleen correspondent to LF disease[98]. Curiously, infection with LASV in IFNAR$^{-/-}$ mice, a similarly immunocompromised strain, results in a protracted infection, with little pathology observed. A key advantage for the STAT1$^{-/-}$ model is the uniform lethality induced by LASV infection, however, the exact pathological mechanism was not characterized. Furthermore, the severely compromised immune system in either STAT1$^{-/-}$ or IFNAR$^{-/-}$ mice renders LASV live vaccine testing non-viable while inactivated/DNA vaccinations might not be accurate due to the inherent immunodeficiency[99,100].

The chimeric IFNAR$^{-/-\ B6}$ mouse model

This laborious model consists of an IFNAR$^{-/-}$ with a B6 (C57BL/6) background that is irradiated to decimate its original hematopoietic system[87]. After irradiation, bone marrow (BM) cells from wild-type B6 mice are transferred and allowed to engraft in the recipient mouse BM thus regenerating its hematopoietic system with immunocompetent cells. The resulting mice

are abbreviated as IFNAR$^{-/-B6}$. These mice are highly susceptible to LF disease, which is not the case with the parent WT B6 or IFNAR$^{-/-}$ B6. In this study, irradiation or chimera formation process were also ruled out as the differentiating factor for this differentiation since chimeras injected with BM matching their original background were as resistant as their original strains. LF diseases pathogenesis in the IFNAR$^{-/-B6}$ mice was, as seen with the HHD model, dependent on extensive immune activation and immunopathology (see chapter 1.3). Given that this is a mostly immunocompetent model and LASV infection results in 100% lethality, this is a suitable (albeit a bit contrived) candidate for initial LF vaccine and anti-viral research[97].

Mouse models using LASV surrogate exposures

Surrogate models of LASV challenge have been explored to circumvent the LASV BSL-4 requirements. A surrogate model consists of the use of a viral backbone that is permissible for handling in a lower level of BSL facility (2 or 3) but expresses LASV GPC exclusively, thus, in theory, having a similar tropism as LASV[91]. Several of such systems have been reported in the literature for LASV and other BSL-4 VHFs viruses. In the case of LASV, the use of a delta G LCMV clone 13 backbone that expresses LASV GPC instead of the native G protein has a reported lethality of 60-80% in IFNAR-/- mice, despite little pathogenicity seen in immunocompetent mice[101]. Another, more quantitative system, that has been reported is using a lentiviral backbone that expresses Firefly luciferase pseudotyped with LASV GPC[102]. This system, instead of measuring protection via survival, assays protection as a reduction of light output as read by an *in-vivo* bioluminescence reader, after luciferin is administrated *in-vivo*. This is a safe model that also provides lessened animal discomfort, except for subtract injection, since there is no pathogenesis involved. However, further studies are required to determine if this system can translate into a real pathogenicity study. Finally, the other system that has been used is based on the use of rVSV-GPC in immunocompromised mice. The rVSV-GPC virus is based on a Vesicular stomatitis indiana virus (VSV), a rhabdovirus similar in structure to rabies (RABV), that had its native glycoprotein replaced with LASV GPC[103]. Although developed as a replication competent (live-attenuated) LF vaccine candidate, its high pathogenicity in immunocompromised mice was first reported in a vaccine efficacy study with STAT1-/- mice[100]. After VSV-GPC inoculation, mice quickly succumbing to disease on days

4 and 5 post inoculation. This “LF mouse model” was used again to test the efficacy of LASV GP2 directed mAbs as a potential LF therapeutics[96].

1.4.2 Guinea pig animal models

Guinea pigs have long been considered the gold standard of LF vaccine and antiviral development before moving to NHPs. Their relatively (to NHP) low cost and uniform lethality induced by the Josiah LASV exposure in strain 13 guinea pigs made them a practical model for decades of LF research[65]. There are currently two guinea pig models being used, strain 13 inbred guinea pigs and the Hartley outbred strain guinea pigs. The next paragraphs will cover both models.

Strain 13 inbred guinea pigs

Since the 80’s strain 13 inbred guinea pigs have been used in LASV research[104,105]. This is due to their uniform lethality to the Josiah strain of LASV, the most researched and available strain, and their overall small size in comparison with NHPs. Nevertheless, the clinical features observed by LASV infection in guinea pigs are different from those observed in humans. The predominant symptom is respiratory distress, with lung failure being the etiological causes of death and less impact in the liver compared to humans[92]. Although these symptoms are sometimes observed in human cases they are not the most prominent[106]. Interestingly, outbred Hartley guinea pigs are mostly resistant to the non-adapted Josiah strain[107]. However, the reason for this stark difference between inbreed strain 13 and outbred guinea pigs is still unknown. The disparity observed in clinical symptoms coupled with the strain-specific susceptibility has raised questions if strain 13 is an adequate model. A striking example, was the experimental yellow fever-lassa vaccine, in which ~90% of strain 13 guinea pigs tested were protected against LASV, but this result could not be reproduced in an NHP model[108]. Similar results were observed in a vaccinia based LF experimental vaccine[105,83]. Moreover, strain 13 guinea pigs are not commercially available, thus greatly limiting LASV research in this model.

Outbred Hartley guinea pigs

Given the shortcomings of the strain 13 based LF model, there has been an increasing trend towards using an outbred based guinea pig model, such as Hartley guinea pigs[109,110]. This would not only establish a model based on commercially available guinea pig strain, but it would also account for the impact that different genetic backgrounds might have on vaccination and LASV exposure. As stated above, Hartley guinea pigs are resistant to LASV Josiah strain exposure in contrast to Strain 13 guinea pigs[65]. To circumvent such problem, a guinea pig adapted Josiah strain LASV (GPA-LASV) was developed by Safronetz and colleagues at the intramural research division of the Rocky Mountain Laboratories of the NIAID/NIH[109]. This was accomplished through serial passage of Josiah strain LASV virus in guinea pigs until a uniform lethality was achieved. The pathology caused by GPA-LASV in this model has yet to be further characterized, but it bears clinical similarities to strain 13. Nevertheless, the physiological response to both vaccination and LASV infection appears to be more analogous to that of NHPs and humans[111].

1.4.3 Non-Human primates (NHPs) animal models of Lassa Fever

For FDA approval any drug or vaccine must go through the pre-human clinical trials in an FDA approved animal model. In the case of LF the most closely related animal models are either the rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys[112–114]. Both these NHPs have been extensively used in drug and vaccine development for LF[27]. NHP's immune system have a high degree of similarity to the human immune system with LASV exposure in NHP resulting in an analogous clinical LF. This makes NHPs the *de facto* gold standard for LF research. Nevertheless, their high purchase and upkeep cost as well as ethical concerns keeps their use limited to almost exclusively preclinical trial evaluations, after other, less costly models (murine or guinea pig), have been used. Another, smaller, NHP model is the marmoset[90]. Their smaller size and lower upkeep costs makes them an attractive alternative to the larger NHP models while maintaining their key advantages. There is, however, very few FDA approved vaccines or drugs on the marmoset model thus their use in

the US is still quite limited[115]. Indeed, very few LF studies were published using the marmoset in contrast with high number of publications using either the cynomolgus or rhesus.

1.5 Immune responses to LASV

LASV pathogenesis is closely tied to both the initial innate and adaptive immune responses to the virus. Indeed, failure or delay in mounting an immune response to LASV in the initial phase of infection leads to LF disease. Nevertheless, an immunopathological response might also be the contributing factor for LF, as previously described. As known correlates of protection for a disease are a critical component for vaccine development a more comprehensive understanding of the immune response to LASV should be attained. In this chapter I'll review the literature covering immune responses to LASV and other OW Arenaviruses in animal models and humans in each of the following sections.

1.5.1 Innate immunity response to LASV

In mammals, the innate immune response is the first line of defense against a viral infection. This occurs through the upregulation of interferon (IFN) pathways after activation of pattern recognition receptors (PRRs). PPRs recognize pathogen-associated molecular patterns (PAMPs) that are generated during viral infection and replication[116]. PAMPs include a vast array of different molecular signals such as double-stranded RNA, 5'-triphosphorylated RNA, and other protein/lipid/glycan moieties which activate diverse types of PRRs. The three known families of PRRs are: Toll-Like Receptors (TLRs); NOD-like receptors (NLRs); and retinoic acid-inducible gene RIG-I-like receptors (RLRs)[117]. These different families sense not only different PAMPs, but also activate distinct pathways. However, despite the different pathways, activation of any of these culminate in the up-regulation of type I IFN, amongst other cytokines and proapoptotic factors. Type I IFN upregulation subsequently induces the expression of a panoply of interferon stimulated genes (ISGs), which not only disrupt viral replication but also further induce IFN expression through positive feedback[118]. Upregulation of cytokine expression and secretion is another crucial step of this innate immune response as these will attract and activate local immune cells such as MPs, DCs or T cells[119]. This last step is key for bridging the innate immune response to an adaptive one.

Pathogenic arenaviruses, in contrast with their non-pathogenic counterparts, are thought to inhibit the innate immune system thus hampering an efficient adaptive response[28,82]. Indeed, in the absence of an innate immune response (IFNAR$^{-/-}$ mice), non-pathogenic arenaviruses (Mopeia virus) become lethal[93]. This innate immunity disruption is thought to be mediated by two different proteins, Z and NP[120]. Both these proteins, act as type I IFN antagonists, and are essential for the immunosuppression observed in LASV infection. Specifically, in LASV infected mice and NHPs, successful recovery from disease is accompanied with elevated levels IFN-γ induction early on the infection, but curiously enough not IFN-α[82]. The early increase of IFN-γ is probably correlated with APC activation, which leads to a subsequent successful virus specific adaptive response. In the case of LCMV (clone 13), a low level of type I IFN signaling appears to be the determining factor for protracted infection[121]. This low-level expression induces IL-10 and PD-L1, thus downregulating MHCII presentation and disrupting helper T cell activation[122]. Although not known in LASV infection, in mice exposed to LCMV the IFN-α response appears to be important to contain early viral spread but has no impact in viral clearance while IFN-β is critical for the latter[123]. On the other hand, interferon induction by LCMV also appears to be a determining factor to hamper a specific B cell response development[124–126]. While these data show that a type I IFN response is essential for activation of an adaptive immune response to LASV (or other arenaviruses), the exact ISGs important for initiating an adaptive response are still unknown.

1.5.2 Adaptive cellular immune response to LASV

Although an initial successful innate immune response to LASV is paramount for protection, full protection requires the activation of the adaptive immune response arm. One of the strongest correlates of protection to LASV is the ability to mount a LASV specific T cell response[33]. NHP studies have demonstrated that successful viral clearance was associated with the appearance of LASV specific T cells (all major subtypes)[82]. These T cells were capable of *ex-vivo* proliferation upon LASV antigen stimulation in contrast with T cells isolated from lethal cases of LASV. This is further corroborated with the fact that effective experimental LF vaccines, such as ML-29 or LASV-vaccinia, rely on the induction of LASV specific T cell response[83,127]. Indeed, adoptive transfer of splenocytes from ML-29 immunized mice can

protect naïve mice from i.c. Lassa challenge. In this study it was found that $CD8^+$ T cells were crucial in mediating full protection against disease, while $CD4^+$ T cells were only partially effective. Moreover, in LASV human survivors strong T cell responses are detected after LASV antigen re-stimulation[33]. Despite the evidence supporting the importance of the T cellular response in LF, there have been cases describing it as a key player in its pathogenesis. In Dengue hemorrhagic fever or Ebola viral disease over-activation of virus specific T cells is largely responsible for the pathology observed[78]. Although evidence in LF points to the opposite direction (immunosuppression), two different mouse models have shown important effects of T cell mediated pathology (see section 1.3)[87,128]. This was further corroborated in imported cases of LF in the US, where overactivation of LASV specific T cell seemed responsible for most of pathological damage observed[85,86,89].

1.5.3 Humoral immune response to LASV

The humoral response to LASV has yet a rather elusive role in mediating protection throughout LF research history. In African villages where LASV is endemic there are often reports of locals treating people suspected of having LF with passive sera transfusions from LF survivors[129]. In the 80s, to elucidate the veracity of such reports, Jarhling et al. studied the protective effect of passive sera transfer from LF survivors in either NHPs or guinea pigs that were exposed to LASV[104,112]. Although sera that contained high neutralizing antibody titers against LASV correlated with disease protection, the titer of total IgG levels against LASV infected cells had little to no predictive value. Later in the same decade, McCormick et al. performed in field clinical trials testing the efficacy of the then new antiviral drug ribavirin against passive sera transfer from LF survivors[4]. They found that ribavirin administration early on the course of LASV infection was protective, while passive sera transfer was found to perform poorly in comparison. Although this study established the grounds for the current off-label use of ribavirin in LF patients, humoral response was then deemed unimportant in LF. There were, however, important caveats to such study in the sera used: the transferred sera were not tested for pathogens, thus not assuring the sterility of the sera; the selected sera were purely based on a positive titer for anti-LASV antibodies in ELISA with relatively a low cut off value (1:160); LASV neutralizing antibody titer was not evaluated. Despite these

important caveats, follow up studies using an experimental LF vaccine based on vaccinia found that not only anti-LASV total IgG titers were not predictive of protection but, in the case of an NP based vaccine, higher levels were correlated with disease[83,105]. Furthermore, LF survivors rarely develop LASV neutralizing antibodies and if so, only after a prolonged period of time post-recovery[60,130,3,25]. Thus, until recently, most LF research renegaded the humoral response to a secondary role.

Humoral responses to LASV tend to be highly variable, not only between individuals but also depending on the LASV protein that is targeted[68]. Overall antibody titers against LASV are low in comparison with other viruses and neutralizing antibody activity appears months after infection in few individuals[131]. In terms of LASV protein specificity, the anti-NP response tends to appear faster (within 9 to 12 days post infection) and is stronger than responses to other proteins[132]. For this reason, they are often searched for LF diagnosis and consist of an important marker for disease epidemiological monitoring. On the other hand, anti-GPC responses are weaker and only appear weeks to months after infection[133]. They also have a much lower diagnostic value than anti-NP responses since GPC variability between strains is the highest of all 4 proteins[134,135]. In 2016, the Robert F. Garry group at Tulane isolated and characterized for the first time a large panel of LASV neutralizing and non-neutralizing monoclonal antibodies (mAbs) isolated from LF survivors[131]. They found, by immunoprecipitation pulldown, that most neutralizing antibodies required conformational epitopes that bridged between GP1 and GP2, while weakly binding to either subunit (GP1 or GP2) alone. In posterior preliminary studies using Hartley guinea pig LF model, treatment with selected mAbs was shown to confer full protection against LF disease[110]. This was later confirmed in an NHP study with the three most efficacious mAbs[136]. Although initially protection was projected to be correlated with in *vitro* mAb neutralizing potency, this was not the case. Some of the most potently in *vitro* neutralizing mAbs failed to protect guinea pigs while some weakly in *vitro* neutralizing mAbs conferred protection. This suggested that fc dependent antibody functions, either through antibody dependent cellular cytotoxicity, phagocytosis, or complement, might be a potentially important mechanism of protection against LF. Later studies by Hastie et al (2017), through LASV GPC co-crystallization with a LASV neutralizing mAb, demonstrated that one of the most potently LASV neutralizing

antibodies (37.7H) required quaternary epitopes present in fully conformational GPC, and blocks GPC switch from pre-fusion to a post-fusion conformation thus effectively aborting receptor mediated viral entry[137].

These studies established important grounds that LASV directed antibodies, and by extension, a LASV humoral response are an important correlate of protection. Nevertheless, the LASV GPC antibody and neutralizing antibody response is rarely induced during natural infection as proven in a study by the Pinschewer group[60]. They found that GPC specific antibodies are induced in mice post recombinant LCMV/LASV infection, however these antibodies can't neutralize LASV. This is due to the extensive glycosylation present in GP1 and GP2 effectively forming a glycan shield that hinders epitope binding by GPC specific antibodies. Indeed, these LASV GPC specific IgGs were able to bind and even neutralize when glycan deficient LCMV/LASV mutants were used instead. This suggested that GPC's glycan shield instead of inhibiting the induction of neutralizing antibodies, it reduces the "on-rate" of anti-GPC antibodies. Moreover, since in a natural LASV infection glycan deficient GPC is most likely a byproduct of incomplete GPC maturation and thus non-functional, they can serve as effective "decoy" antigens for LASV GPC directed antibodies. Altogether, only recently has the role of the humoral response in LF protection has been started to be explored, however it has been mostly focused in the role of LASV neutralizing antibodies, which are rarely induced by either immunization or LASV infection. Further research is thus required to understand the role of non-neutralizing antibodies in protection against LF.

1.6 LF vaccine and therapeutic development

There are currently no FDA approved vaccine or antiviral therapy for LF or other Arenavirus caused HF. The off-label use of ribavirin can improve LF prognostic if a high dose is administrated in the first few days after LF symptoms have begun, but has no effect in later stages of disease[32]. Furthermore, its most severe side effect, hemolytic anemia, is often reported to be much more nefarious than what is observed in approved use, due to the higher dosage required, allied with a higher incidence of comorbidities[138]. There is, therefore, a pressing need for either new antiviral agents or vaccine development. Besides the off-label use of ribavirin, another, more promising drug, favipiravir, is currently getting fast tracked by the FDA in clinical trials for LF treatment[97,109,139]. Favipiravir, an RNA dependent RNA polymerase inhibitor, also shows promise for a myriad of other RNA viral infections, such as filoviruses and flaviviruses, given the universality of its molecular target[140]. In terms of antibody-based therapeutics, a cocktail of three different LASV neutralizing antibodies showed 100% protection in NHPs up to 8 days post infection. However, LF diagnosis and treatment in remote or conflict-stricken areas is a difficult task[141]. As such, LF vaccination offers a compelling solution to circumvent such problems. In the next sections some of the experimental LF vaccines will be explored as well as their potential correlates of protection in the respective animal model tested. This is also summarized in table 1.

1.6.1 Modified Vaccinia Ankara expressing LASV antigens

Vaccinia viruses, as the name implies, have been one of the most commonly used recombinant viral vectors used for vaccine purposes[142]. The modified Vaccinia Ankara (MVA) vector is a severely attenuated vaccinia virus strain that was used in smallpox vaccination campaigns in humans. There have been three different LF vaccine candidates based on MVA: 1) A replication competent (live) vaccine based on the MVA vector expressing LASV GPC (MVA-LASV-GPC); 2) a variant of 1) expressing LASV NP (MVA-LASV-NP) instead; 3) The MVA-LASV-VLP (GEO-LM02), which is based on the GeoVax™ MVA VLPs that have limited local replication and express GPC[83,143,144,145]. A high dose of MVA-LASV-GPC (10^9) successfully protects both guinea pigs and NHPs. It also established that for LF protection using the GPC antigen, both the GP1 and GP2 units had to be present in a full GPC conformation. However

further studies were not pursued beyond the initial publications. The MVA-LASV-NP was protective in guinea pig model, however it failed to protect NHPs from LF. The GEO-LM02 is a much more recent experimental VLP based vaccine being developed by GeoVax, that protected mice from a LASV/Mopeia reassortment (ML-29) i.c. challenge after a single i.m. immunization.

1.6.2 Mopeia virus (MOPV) based vaccines

Early studies in 1989 by Fisher-Hoch et al., established that rhesus macaques were protected against LASV if previously immunized with Mopeia virus (MOPV), a non-pathogenic arenavirus that is closely to LASV[144]. To produce a more specific live vaccine against LASV that retained the non-pathogenic features of MOPV, in 2005 Lukashevich et al. screened a reassortment library of MOPV/LASV, produced from co-infected VERO cells, and isolated clone 29 (ML29)[146]. This clone is genetically composed of the S segment of LASV Josiah strain, and the L segment of MOPV (strain An20410). The live vaccine ML29 clone was initially found to be both safe and protective when given in low doses in NHPs (both marmosets and cynomolgus)[147–149]. They also concluded that cellular immune responses were mostly responsible for protection, since a low humoral response was detected, and adoptive transfers from immunized mice protected naïve mice from disease. However, in more recent studies persistent ML29 infection was found in immunocompromised mice and NHPs[99,150]. Furthermore, the attenuation mechanism is still not fully understood, thus greatly limiting its use.

With the advent of recombinant virus recovery, an attenuated MOPV based virus vaccine platform was developed by the Baize group, in which the DEDDH exoribonuclease domain of MOPV NP was disrupted[151]. This domain, conserved in all Arenaviruses, digests ds-RNA thus inhibiting the innate immune response. By abrogating this domain, the safety profile of MOPV (or any arenavirus) can theoretically be improved. This new attenuated MOPV vaccine platform was named $MOPV_{exoN6b}$. In a proof of concept study, $MOPEVAC_{LASV}$ a $MOPV_{exoN6b}$ expressing LASV GPC was shown to be protective against LF with a single immunization in NHPs. Its mechanism of protection is believed to be, as in the case for ML29, to be based on cellular immunity.

1.6.3 Glycoprotein deleted VSV expressing LASV GPC or NP (VSV-GPC/VSV-NP)

Indiana vesicular stomatitis virus (VSV) is an arthropod born rhabdovirus that is minimally pathological in humans[152]. In early 2000s, Feldmann et al., based on the recombinant RABV and VSV recovery techniques developed by Schnell et al. (1994), developed a recombinant VSV live vaccine platform with its native glycoprotein deleted[103]. The recombinant VSV (rVSV) based platform has a good safety profile in immunocompetent humans and can produce strong cellular and humoral responses to foreign antigens it expresses[153]. Since then several experimental VHF vaccines based on rVSV have been developed, being the most clinically advanced the Merck® VSV-ZEBOV vaccine, an EBOV live vaccine that has gone through clinical phase III trials in west Africa[154]. Regarding LASV, two vaccine candidates were developed, expressing either LASV Josiah strain NP or GPC[132]. As observed with previous studies with the MVA platform, the LASV-NP based vaccine does not protect guinea pigs to a significant extent against LF. Meanwhile, the GPC based vaccine, named VSV-LASV, was shown to protect both strain 13 guinea pigs and NHPs against LF with a single dose. It also cross-protected NHPs against heterologous LASV challenge, an important requirement given LASV's strain diversity. Additionally, a yearlong study with Hartley guinea pigs showed that a single dose of 10^6 pfu of VSV-LASV can protect against LF up to a year post immunization[111]. The mechanism of protection is thought to be due to the contribution of both LASV specific humoral and cellular responses. Yet, this vaccine has some important safety considerations such as: high pathogenicity is observed when immunocompromised mice are immunized with VSV-LASV (see chapter 1.4); lack of safety evaluations in a pregnant animal model, since pregnant women are a population particularly stricken by LF[32,155].

1.6.4 A Lassa Fever DNA vaccine

A DNA vaccine expressing either the ORF of LASV NP or GPC have been reported in the literature[156]. As seen for other vaccine platforms, the immune response elicited by LASV GPC is more protective than the response to NP, although few studies with the latter were

reported. After further refinements to DNA vaccine technology as well as the codon optimization of LASV GPC ORF (coGPC), a second generation of LASV GPC DNA vaccine was tested in both guinea pigs and cynomolgus macaques[157,158]. In the case of the cynomolgus macaques, animals were first immunized (prime) by intradermal injection and followed by 2 boosts of intradermal DNA electroporation given four weeks apart. Monkeys that received the LASV DNA vaccine were fully protected against LF with a modest induction of LASV neutralizing antibodies after challenge (although the mock vaccinated NHPs also developed LASV neutralizing antibodies post-challenge). Furthermore, neither adverse reactions at the site of injection nor LASV viremia were detected throughout the study. Meanwhile 50 to 80% of the mock immunized group succumbed to LF while the rest recovered, albeit with severe symptoms.

1.6.5 Other vaccine candidates

Other, less prominent, LF vaccine candidates have also been investigated and provide valuable insight for future LF vaccine development[159]. An important, but ultimately unsuccessful candidate was based on recombinant Yellow Fever 17D platform expressing LASV GPC[160,161]. Although it successfully protected strain 13 guinea pigs against LF, it eventually failed to protect marmosets against LF. This vector also had low genetic stability of the inserted full length ORF of GPC, thus only shorter ORFs such as GP1 or GP2 only could be used. Curiously, this vaccine candidate induced strong LASV specific $CD8^+$ T cell responses. An attempt for an LF vaccine was also made based on irradiated LASV virions, however little to no immune response was detected post immunization and subsequently failed to protect from LF[162]. Finally, two noteworthy vaccine candidates were recently reported, which, despite the lack of standardized LASV challenge studies, have intriguing results. The first is a VLP vaccine based on an alphavirus replicon vaccine platform that uses Venezuelan equine encephalitis TC-83 IND vaccine (VEEV)[163]. Succinctly, VEEV structural genes were replaced with a bicistronic RNA expressing the wild type Josiah (clade IV) LASV GPC ORF and a truncated clade I LASV GP fused with fibritin. This vaccine was able to produce stronger humoral and cellular immunity than ML29 and although not studied, should induce a cross-protective LASV immune response. The second vaccine candidate, a proof of concept study, used a watery-

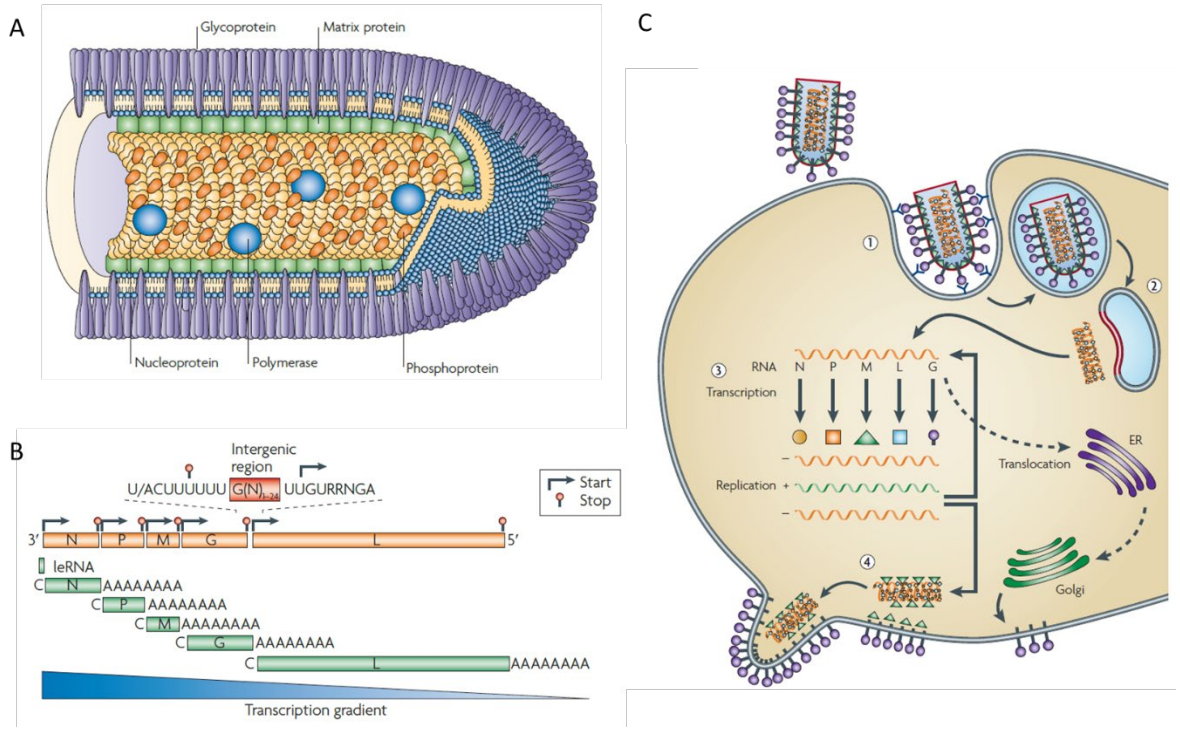
core polymersome (PS) nanocarrier vaccine system that encapsulated truncated soluble LASV GP1 (Josiah)[164]. This PS system can efficiently deliver antigen in lymph nodes and promotes MHCII presentation of its cargo by APCs. They found that, in mice, PS encapsulated GP1, by itself, was able to induce significantly superior humoral responses to LASV in comparison with free antigen. They observed comparable results when free GP1 was used in combination with the TLR-4 agonist monophosphoryl lipid A (MPLA).

Technology/ platform	Vaccine	Live	Prime/ Boost	LASV Antigen used	Efficacy	Immune response tested
Modified Vaccinia Ankara Virus	MVA-LASV-GPC	Yes	No	GPC (Josiah)	Protects NHPs and Guinea Pigs against LASV (Josiah) exposure	Presumably T cell based immunity
	MVA-LASV-NP	Yes	No	NP (Josiah)	Protects Guinea Pigs against LASV (Josiah) exposure. Failed to protect NHPs.	Presumably T cell based immunity
	MVA-LasVLP (GEO-LM02)	Yes	Not specified	Not specified	Protects mice agains i.c. exposure of ML-29	Not specified
Mopeia Virus	ML-29	Yes	No	Josiah S segment (GPC/NP)	Protects NHPs and Guinea Pigs against LASV (Josiah) exposure	T cell based immunity
	$MOPEVAC_{LASV}$	Yes	No	GPC (Josiah)	Protects NHPs against LASV (Josiah) exposure	T cell based immunity
Vesicular Stomatitis Indiana Virus	VSV-LASV	Yes	No	GPC (Josiah)	Protects NHPs against LASV (Josiah) exposure	Presumably cellular and humoral immunity
	VSV-LASV NP	Yes	No	NP (Josiah)	Partially protects NHPs against LASV (Josiah) exposure	Presumably cellular and humoral immunity
CELLECTRA-3P ID-EP	LASV DNA vaccine	No	Yes	Codon-optimized GPC (Josiah)	Protects NHPs against LASV (Josiah) exposure	Presumably cellular immunity
Yellow Fever 17D virus	YFV17D/LASV-GPC	Yes	No	Truncated GP1/GP2 (Josiah)	Protects Guinea Pigs against LASV (Josiah) exposure. Failed to protect NHPs.	T cell based immunity
VEEV (TC-83) Replicon	VEEV-LASV	VLP	No	wtGPC and ΔLGPfib (AF181853 and Josiah strain)	Not tested	T cell based immunity
Polymersome emulsion	PS-LASV GP1	No	Yes	truncated GP1	Not tested	CD4+ T cell and humoral

Table 1. **Overview of previously published experimental LF vaccines.**

1.7 Rabies virus vaccine platform

Rabies encephalitis is a lethal and terrifying disease caused by lyssaviruses, most often by the lyssavirus, Rabies virus (RABV)[165]. Its effects are so devastating that Rabies prevention is one of the first known examples of public health measures, evidence of which can be found in some of the oldest written law codes to be discovered[166]. Rabies was also amongst the earliest diseases for which a successful vaccine was developed[167]. Notwithstanding, Rabies continues to be the cause of an estimated 50,000 deaths annually. This is mostly due to the lack of rabies vaccination coverage for both humans and domestic animals in countries with well-established rabies animal reservoirs. Rabies encephalitis can be prevented if post exposure prophylaxis treatment is administrated early on post infection. Nevertheless, the treatment is expensive and has no effect when rabies disease symptoms arise[168]. Thus, incentives are needed to make rabies vaccination more prevalent.



Adapted from: Schnell M. J. et al. The cell biology of rabies virus: using stealth to reach the brain. Nature Microbiology reviews, vol.8, 51-61 (2009)

Figure 4. Rabies virus schematic overview. In (A) the Rabies virion particle overview of each protein can be observed. In (B) transcription gradient produced by rabies polymerase, foreign genes inserted in the rabies genome are expressed in the same order as the transcription represented. (C) Rabies infection cycle overview. LASV preGP is processed in the ER and Golgi and is trafficked to the membrane. Budding rabies virions expressing LASV GPC should incorporate it alongside with RABV G as exemplified in step 4.

RABV is a non-segmented negative stranded RNA virus belonging to the *Rhabdoviridae* family[165,169]. Its genome, whose size is between 10-11 kbp, contains 5 genes and 1 pseudogene in the following order from 3' to 5': Nucleoprotein (N); phosphoprotein (P); Matrix protein (M); glycoprotein (G); the pseudogene (Ψ); and the largest gene, the RNA dependent RNA polymerase (L). As with other non-segmented negative stranded RNA viruses, upon cellular entry, RABV's 5 genes are first transcribed into 5' capped mRNA by the polymerase complex in a gradient like fashion from the 3' end to the 5' end of the genome[170]. Due to this transcription gradient, the N gene, the closest to 3' end, has the highest number of mRNA transcripts, while inversely the L gene has the least, since it's closest to the 5' end. This is due to the viral RNA dependent RNA polymerase (RdRP) inefficiency in restarting transcription when a Start-Stop signal is encountered, which may result in genome detachment and thus transcription termination. The transcription gradient thus generated can be harnessed in vaccine development. By inserting the desired antigen/gene in rabies, properly flanked by a start-stop signal, a higher or lower expression of the gene of interest can be accomplished. Such a strategy has two advantages: 1) infected cells can transcribe higher amounts of the inserted gene mRNA with, depending on the gene, little compromise to viral production; 2) The extra genetic material and the extra genetic material that the RdRP must process further attenuates the virus by making it slower.

The technology for making recombinant RABV was originally developed and published by Schnell et al. in 1994. This was based on mammalian cell transfection with minigenome plasmids containing the cDNA of the RABV N, P, G, and L genes under the T7 polymerase promoter expressed by a Vaccinia virus. The technique was further refined and currently, recombinant RABV recovery is possible in FDA approved cell lines for vaccine production (e.g. VERO), independent of Vaccinia virus expressing T7[171]. This allows an easy transposition to GLP/GMP facilities, an obligatory prerequisite for preclinical and clinical vaccine trials.

Areas where Rabies are a major public health concern are often overlapped by other emerging viral zoonoses, such as LASV[165,172]. Nevertheless, vaccination logistics in developing countries are more challenging due to either strife, remoteness of human settlements, or substandard health infrastructure[18,173]. The use of a bi/multivalent vaccine that could

combine coverage against several zoonoses would be highly advantageous. Using recombinant virus recovery technology, RABV can be genetically engineered to express other viral antigens and be used as a vaccine[174,175]. Moreover, given that RABV platform is based on an established human vaccine that is not prevalently used in the general population, previous immunity does not hinder its use as a bi-valent vaccine[176]. This platform has thus key advantages for areas where Rabies is still a major public health burden, such as in Africa, since it simultaneously protects against Rabies disease[171,177–180]. Additionally, the RABV platform has been extensively studied and can easily accommodate and express foreign genes[176].Together with the fact that the Rabies vaccine is used worldwide with a remarkable safety record, makes RABV an attractive platform for the development of a LF vaccine.

Aims

The 2013-2016 west Africa Ebola epidemic was a stark reminder of our unpreparedness for rising VHF. As the largest documented outbreaks of LF are currently being reported in Nigeria, Lassa vaccine development remains in preclinical phase. Moreover, most LASV experimental vaccines are based on live vectors. While these were shown to induce lasting and protective immune response, safety concerns remain since their use in pregnant women, an overly sensitive population to LASV, is greatly limited. On the other hand, LF's correlates of protection have yet to be accurately defined, thus further hampering vaccine development.
The present thesis consisted of the development of an LF vaccine based on the RABV vaccine platform, as well as attempts to better define correlates of protection to LF.
The following specific aims were explored:

1. **Design, recover, and characterize Rhabdovirus vectors expressing Lassa Glycoprotein in *vitro.***
2. **Develop immunological tools/systems that allow the study of Lassa glycoprotein immunogenicity.**
3. **Characterize the immune response of several rhabdovirus based Lassa vaccine candidates in the mouse model.**
4. **Test the efficacy of the select candidates in the guinea pig Lassa Fever model.**
5. **Attempt to define the vaccine's correlates of protection.**

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Chapter 2

Experimental work

Chapter 2.1

Tiago Abreu-Mota, Katie R. Hagen, Kurt Cooper, Peter B. Jahrling, Gene Tan, Christoph Wirblich, Reed F. Johnson, and Matthias J. Schnell

Non-neutralizing antibodies elicited by recombinant Lassa-Rabies vaccine are critical for protection against Lassa Fever

Nature Communications ***9****:4223 (2018)*

DOI: 10.1038/s41467-018-06741-w

Non-neutralizing antibodies elicited by recombinant Lassa-Rabies vaccine are critical for protection against Lassa Fever

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Abstract

Lassa fever (LF), caused by Lassa virus (LASV), is a viral hemorrhagic fever for which no approved vaccine or potent antiviral treatment is available. LF is a WHO priority disease and, together with rabies, a major health burden in West Africa. Here we present the development and characterization of an inactivated recombinant LASV and rabies vaccine candidate (LASSARAB) that expresses a codon-optimized LASV glycoprotein (coGPC) and is adjuvanted by a TLR-4 agonist (GLA-SE). LASSARAB elicits lasting humoral response against LASV and RABV in both mouse and guinea pig models, and it protects both guinea pigs and mice against LF. We also demonstrate a previously unexplored role for non-neutralizing LASV GPC-specific antibodies as a major mechanism of protection by LASSARAB against LF through antibody-dependent cellular functions. Overall, these findings demonstrate an effective inactivated LF vaccine and elucidate a novel humoral correlate of protection for LF.

Introduction

Lassa fever (LF) is a viral hemorrhagic fever (VHF) whose etiologic agent is Lassa virus (LASV), a bio-safety level 4 (BSL-4) pathogen. Similar to other VHFs caused by other viruses, such as Ebola virus (EBOV) and Marburg virus (MARV), LF can be highly fatal and no vaccine is currently available[1]. The need to develop vaccines against emerging viral pathogens became starkly apparent during the 2014-2016 West Africa Ebola epidemic[2, 3, 4]. Indeed, reaffirming the urgency and importance of preventive measures, an unprecedented major LF surge, with 25.4% high case fatality rate, is currently unfolding in Nigeria[5]. Unlike most other BSL-4 agents which cause temporally and geographically confined epidemics, LF is believed to be widespread throughout most of West Africa, with an estimated 100,000 to 300,000 humans infected annually[6, 7]. As many as 80% of LF exposures are mildly symptomatic and thus go unreported[6], however, the case fatality rate of LF has been reported to reach as high as 50%[8]. Such discrepancy can be dependent on both the contributing strain and the population afflicted (e.g., pregnant women are especially susceptible)[9, 10]. Even among survivors, LF can cause severe neurosensory sequela; it is a leading cause of viral-induced neurosensory deafness in West Africa[8].

A logistical hurdle for an effective LASV treatment is the often poorly equipped health infrastructure in developing nations such as Guinea or Sierra Leone[11]. While the off-label use of ribavirin seems effective in treating LF, the drug is often accompanied by severe side effects. Coupled with the presence of conflict-stricken regions, the relative remoteness of some human settlements and the widespread presence of LASV's natural reservoir, *Mastomys natalensis* (common African rat), both diagnosis and treatment of LF is a challenging task[12]. With climate change and increasing globalization, the likelihood of LF becoming a global threat increases, thus making development of a vaccine for LASV a high priority.

Unfortunately, undefined correlates of protection for LF have impeded LASV vaccine development. Studies with experimental live vaccines, such as ML29 (a Mopeia-Lassa virus reassortment-based vaccine) and recombinant vaccina virus expressing LASV glycoproteins,

have shown that cellular immunity occurs in the absence of humoral response and successfully protects treated animal model[13, 14]. Additionally, these findings, together with findings on another promising LASV vaccine platform, VSV-LASV, have indicated that either no correlation, or even a negative correlation, exists between LASV humoral response and vaccine efficacy[15, 16]. Nevertheless, it has also been shown in some animal models that cellular immunity may be the source of immunopathology seen in LF[17, 18, 19]. Meanwhile, studies have reported that passive sera transfer therapy from LF survivors protects against disease and death in animal models of LF, supporting the role of humoral response against disease development[20, 21].

LASV's genome, as a member of the *Arenaviridae* family, encodes 4 proteins, including an envelope glycoprotein that is responsible for viral entry[22]. LASV's glycoprotein is expressed as a polyprotein and is cleaved into SSP, GP1, and GP2 to form a mature trimeric glycoprotein complex (GPC) on the surface of host cells and virions[22]. GPC is an appealing immunogen because of the surface exposure of GPC in LASV virions and its crucial function for viral entry[16, 23, 24, 25, 26]. Indeed, human monoclonal antibodies that target GPC and neutralize LASV *in vitro* were recently shown to protect guinea pigs and non-human primates (NHPs) exposed to LASV from disease[25, 27]. However, the efficacy of GPC-specific non-neutralizing mAbs was not investigated and neutralizing potency *in vitro* did not necessarily correlate with protection[25, 26]. Furthermore, the occurrence of neutralizing antibodies (NAbs) against LASV is uncommon in survivors and has been poorly elicited by previous LASV vaccine strategies[28].

Besides direct viral neutralization, antibodies can also lead to effector cell activation and clearance of the viral antigen-expressing cells through antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP)[29]. Through this mechanism, antibodies bound to antigen interact with Fcγ-receptor-bearing immune effector cells, such as macrophages or NK cells, through Fc region cross-linking[29] that triggers clearance of the antigen-expressing cell. As such, ADCC/ADCP are among several mechanisms that bridge the adaptive and innate immune responses. ADCC/ADCP has been shown to be highly relevant for protecting against and clearing several different viruses, including HIV, influenza virus, and EBOV[30, 31, 32, 33, 34].

However, the role of ADCC, ADCP, and other antibody-mediated effector functions in LASV infection and disease outcome has not been investigated.

Here we report the use of a rabies virus (RABV)-based vaccine vector as an inactivated dual vaccine for LASV and RABV. This vaccine, named LASSARAB, expresses a codon-optimized version of LASV GPC (coGPC) in addition to RABV G. LASSARAB elicits lasting humoral response against LASV and RABV in both mouse and guinea pig models, and it protects both against LF. In developing LASSARAB, we also sought to uncover its mechanism of protection, which our results suggest is dependent on a previously uncharacterized antibody-mediated protection of LASV through effector cell functions of GPC-targeted non-neutralizing antibodies (Non-NAbs).

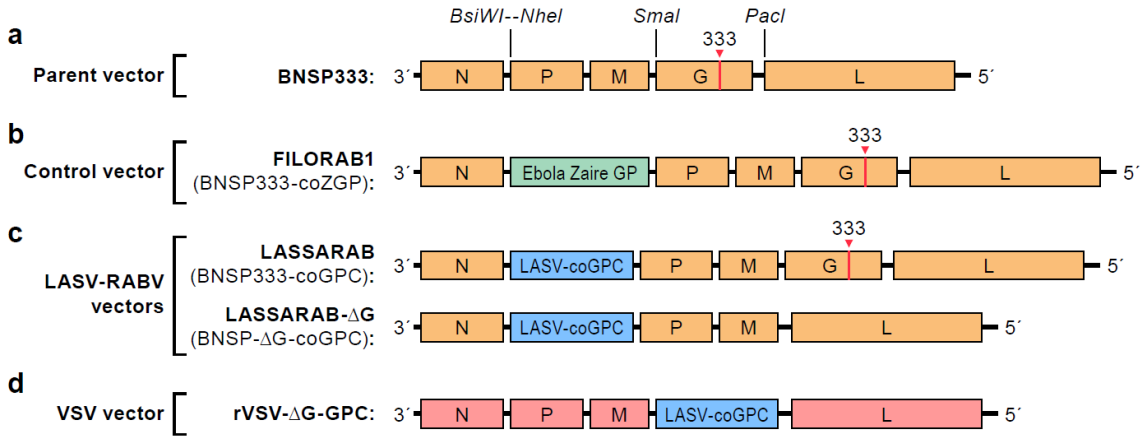

Fig. 1. Diagram of vaccine constructs and controls. BNSP333 is the parental vector and FILORAB1, the control used, is based on BNSP333 with a codon optimized Zaire Ebola Virus Glycoprotein (EBOV GP) inserted between N and P through the BsiWI and NheI restriction digest sites. LASSARAB was generated in a similar manner as FILORAB1, from BNSP333 by cloning a codon optimized version of Lassa virus glycoprotein (LASV GPC) in the BsiWI and NheI restriction digest sites. LASSARAB- Δ G was further generated from LASSARAB by removing the native rabies glycoprotein (G) by using the restriction digest sites SmaI and PacI. rVSV-GPC was generated by replacing the native VSV glycoprotein (G) by LASV GPC at the same sites. rVSV-GPC was created to be used as a control vector and as a scaffold to produce a native LASV GPC antigen for ELISAs (see Methods section).

Generation of rhabdoviral-based vectors expressing LASV GPC

To generate a recombinant RABV-expressing LASV GPC, we used the previously described vector BNSP333[35]. BNSP333 is a modified RABV vaccine strain (SAD B19) with an arginine-to-glutamate change at position 333 of RABV G that further reduces neurotropism and improves its safety profile[35]. A codon-optimized LASV-GPC was cloned into BNSP333 using two unique restriction sites (BsiWI and NheI) that flank a RABV transcription start/stop signal between the RABV N and P genes, and it was designated as LASSARAB (Fig. 1). Utilizing LASSARAB, we also constructed LASSARAB-ΔG by deleting the RABV G. For a control vector, we constructed a recombinant vesicular stomatitis virus (VSV) expressing the same GPC as the RABV vector (rVSV-GPC); similar to LASSARAB-ΔG, it lacks its native glycoprotein (G). In several prior NHP studies, similar rVSV-GPC vectors have been used as live-attenuated (replication-competent) vaccine candidates for LASV with promising results[15, 16]. As an additional control, we used

BNSP333-expressing Ebola GP (FILORAB1), a vaccine extensively characterized by our group[36, 37, 38].

GPC is transported to the cell surface and incorporated into virions

Successful utilization of LASSARAB and LASSARAB-ΔG as vaccines depends on LASV GPC expression at the cell surface membrane. VERO cells were infected at a multiplicity of infection (MOI) of 0.1 or 1, and cell surface expression of LASV GPC and RABV G was evaluated by immunofluorescence and flow cytometry at 48 h post-infection (Fig. 2a and 2b). Immunostaining with antibodies directed against either LASV GPC or RABV G detected both LASV GPC and RABV G cells on the cellular surface of VERO cells infected with LASSARAB (Fig. 2a and 2b panel LASSARAB). In cells infected with FILORAB1, only RABV G was detected on the cell surface as expected (Fig. 2a and 2b, panel FILORAB1) whereas for the LASSARAB-ΔG and rVSV-GPC-infected cells, LASV GPC but not RABV G was detected on the cell surface (Fig. 2b panel LASSARAB-ΔG/rVSV-GPC).

To analyze whether LASV GPC affects RABV growth kinetics, we performed a multi-step growth curve analysis of LASSARAB, LASSARAB-ΔG, and FILORAB1 (Fig. 2c). LASSARAB and FILORAB1 grew similarly and reached titers of 10^8 after 72 h. LASSARAB-ΔG grew to a higher titer than the RABV G-containing construct LASSARAB, indicating that LASV GPC is being functionally expressed. The higher titer achieved by LASSARAB-ΔG might be explained by its shorter genome or its expression of two glycoproteins, or both.

LASSARAB's potential as an inactivated vaccine depends on LASV GPC incorporation in LASSARAB-inactivated virions. As such, sucrose-purified virions from infected VERO cells were analyzed by SDS-PAGE gel, Western blotting, and ELISA (Fig. 2d, 2e, and Supplementary Fig. 1). SDS-PAGE protein stain of purified FILORAB1 (control) and LASSARAB virions showed protein migration in the expected size for the RABV proteins, as can be seen by the FILORAB1 control, as well as proteins consistent with the molecular weight of LASV GP2 (40-38 kDa). LASV GP1 (47-42 kDa) is comigration with RABV P and therefore difficult to detect. However, LASV GP1/GP2 incorporation in LASSARAB was confirmed by Western blot analysis which demonstrated both GP1 (48-42 kDa) and GP2 (40-38 kDa) consistent with their respective molecular sizes (Fig. 2e and Supplementary Fig. 1)[39, 40, 41]. Glycosylation patterns in both GP1 and GP2 similar to previous studies were demonstrated by mobility shift assay using

LASSARAB virions treated with either Endo H or PNGase F in comparison with untreated virions (Supplementary Fig. 1e and 1f)[39, 40, 41]. Finally, to confirm that LASV GPC on inactivated LASSARAB particles was conformationally resent in its pre-fusion state, particles were analyzed by the GPC conformational sensitive mAb 37.7H[26, 42] (Supplementary Fig. 1g).

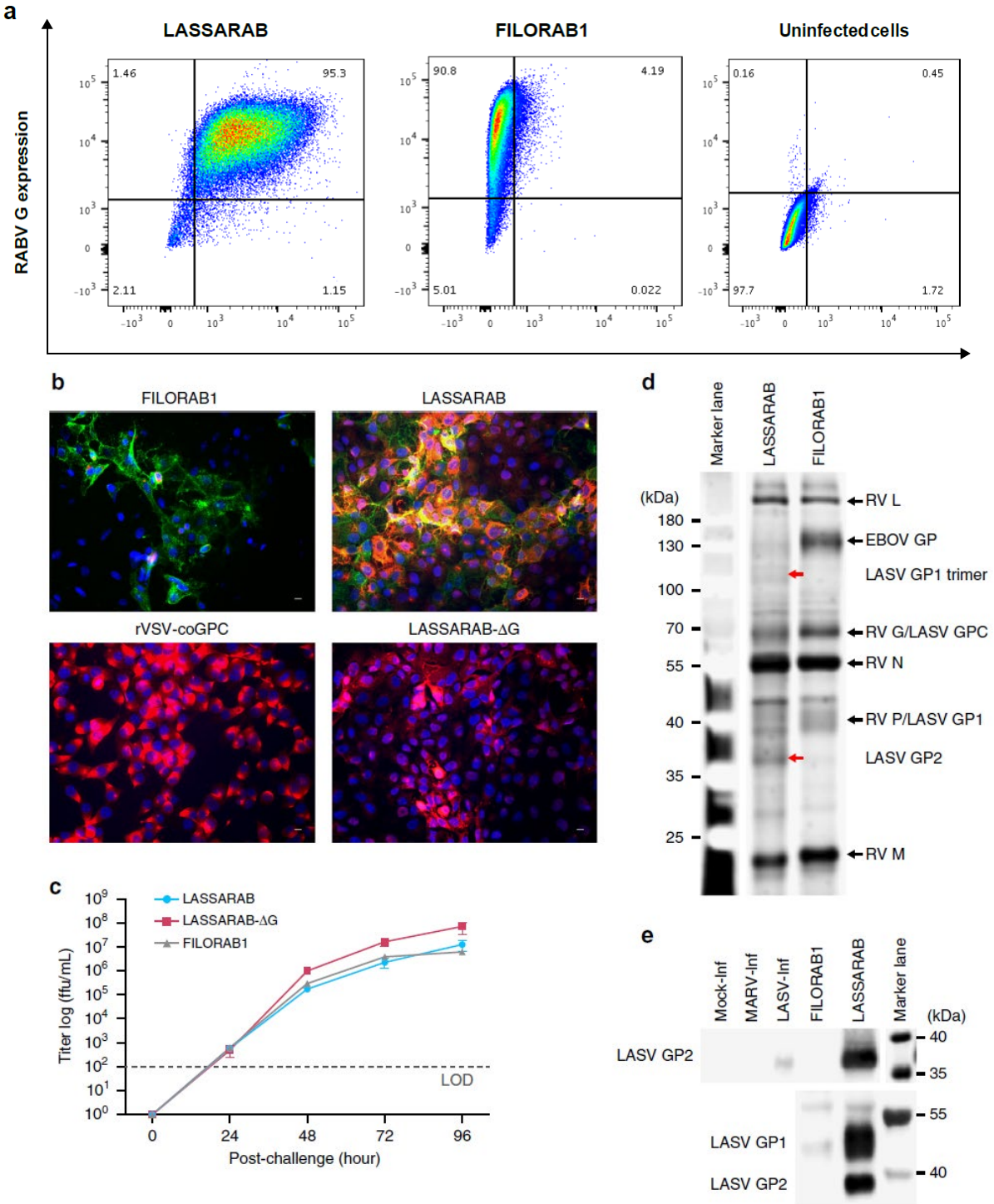

Fig. 2. Evaluation of LASSARAB and LASSARAB-ΔG vectors in cell culture and inactivated virion characterization. (a) LASSARAB, FILORAB1 and uninfected VERO cells were probed for LASV-GPC and RABV-G

expression with 37.7H anti-LASV human mAb and 1C5 anti-RABV G mouse mAb and analyzed by flow cytometry 48 h post infection. (b) VERO cells were infected at a MOI of 0.1 with 4 viruses: FILORAB1, LASSARAB, LASSARAB-ΔG, and rVSV-coGPC. 48 h later (24 h for VSV based vectors) cell surface expression of LASV Glycoprotein (GPC), in red, and RABV Glycoprotein (G), in green, was probed by a α-LASV GPC rabbit polyclonal and a α-RABV G human 4C12 monoclonal, respectively. In LASSARAB infected cells, yellow is observed as the superimposition of LASV GPC surface expression with RABV G. (c) VERO CCL-81 cells were infected with a MOI of 0.01 and media supernatant was collected at 0, 24, 48, 72, and 96 h. Virus titers were measured through foci-forming assay (in Y axis) and plotted through time (X axis). (d&e) LASSARAB and FILORAB1 virions were concentrated through TFF and sucrose purified through ultra-centrifugation. Pellets were resuspended in PBS, BPL inactivated at 1:2000 for 24 h, and 2 μg of each was loaded in a denaturing 10% SDS-PAGE gel. In (d) SYPRO Ruby staining was used. (e and f) LASV GPC incorporation in LASSARAB particles was confirmed by Western Blot with either an anti-LASV GP2 rabbit polyclonal (upper panel) and anti-GPC/GP1/GP2 guinea pig survivor serum (lower panel). Uncropped versions are available in supplementary figures.

LASSARAB is avirulent in mice

Expression and incorporation of LASV GPC in the highly attenuated BNSP333 live vaccine vector might change its tropism and thus increase its pathogenicity. To determine whether this is the case, Swiss Webster mice were inoculated both intranasally (IN) and intraperitoneally (IP) with 10^6 foci-forming units (ffu) of LASSARAB, LASSARAB-ΔG, FILORAB1, or 10^6 plaque-forming units (pfu) rVSV-GPC, or PBS. Animals were monitored for disease (e.g., hunched back, ruffled fur) and changes in weight for 28 days (Fig. 3a). IN exposure with BNSP (RABV group), which has been shown to be pathogenic after IN exposure, was used as a positive control, while FILORAB1 and PBS were used as negative controls because previous studies had demonstrated that they are not virulent[43, 44]. On day 8, RABV-infected animals started to exhibit clinical signs of rabies, particularly weight loss. (Fig. 3a, RABV group). Mice inoculated with LASSARAB or FILORAB1 showed no clinical signs of disease. For the LASSARAB-ΔG IN inoculated group, one mouse died at day 14 without displaying previous clinical signs or weight loss (Fig. 3a, LASSARAB-ΔG group, m2). However, 3 mice from the rVSV-GPC group displayed signs of neurological deficits (Fig. 3a, rVSV-GPC group, m2/4/5); 2 succumbed and 1 survived, indicating pathogenicity after IN inoculation of this vaccine. None of the animals inoculated through the IP route displayed clinical signs of disease.

We further characterized the safety profile of the infectious LASSARAB vaccine by intracranial inoculation (IC) in both adult BALB/c and adult severe combined immunodeficiency (SCID)

mice (3b). Increased pathogenicity was not observed following infections with LASSARAB compared with BNSP333 in either Balb/C or SCID mice (Fig. 3b). Finally, to confirm absent or decreased pathogenicity in a more sensitive model[44], Swiss Webster suckling mice were IC-exposed with LASSARAB or BNSP333 (Fig. 3c). Independent of the virus dose used, LASSARAB or BNSP333 suckling mice started to succumb to the infection by day 7.

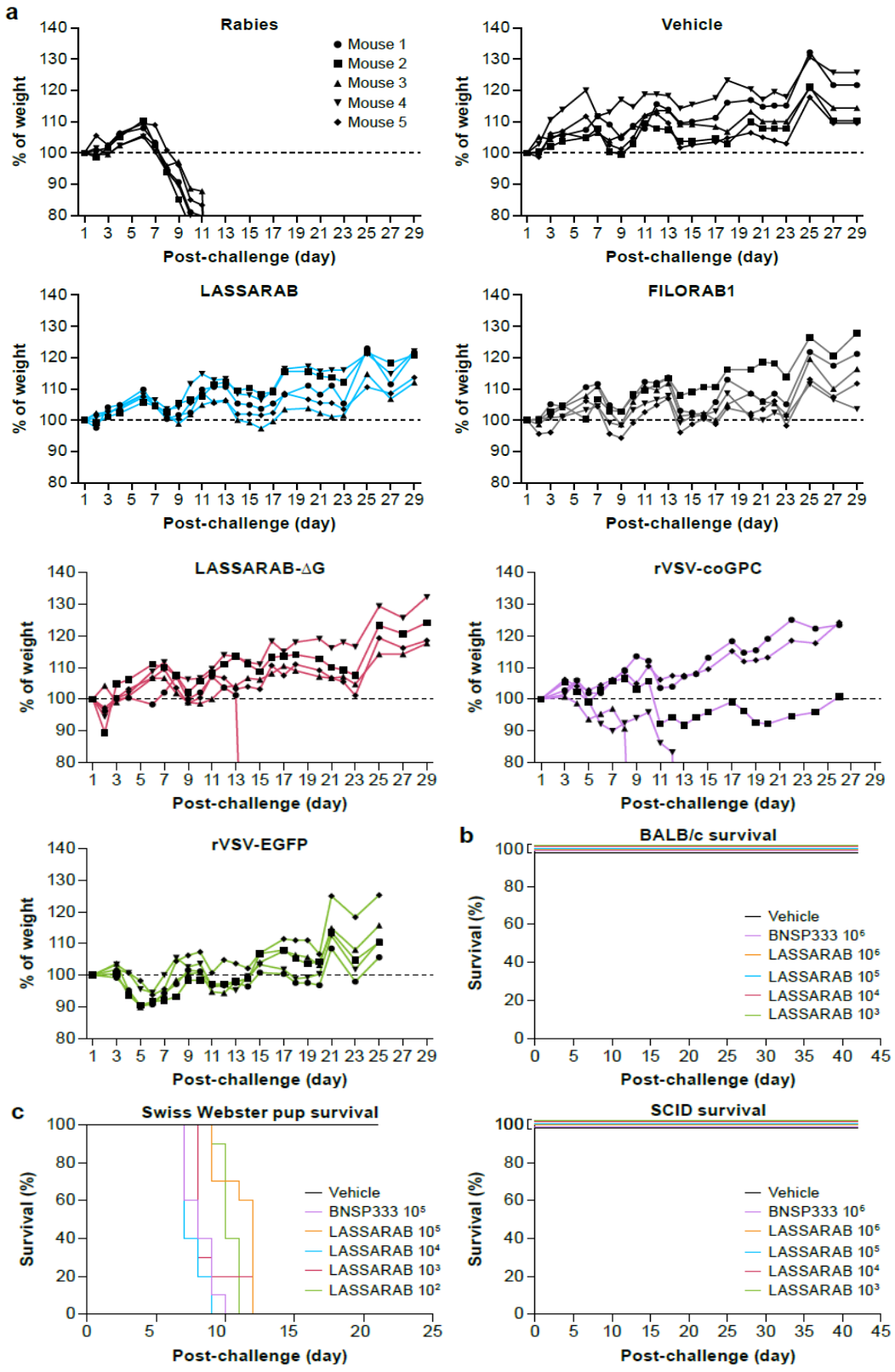

Fig. 3. Evaluation of LASSARAB, LASSARAB- Δ G, and rVSV-GPC pathogenicity. (a) Weight curves of 6- to 8-week-old female Swiss Webster mice that were inoculated intranasally with 10^5 ffu of either LASSARAB, LASSARAB- Δ

G, or rVSV-GPC. As controls, mice were inoculated with the same dosage of either BSNP parent vector (Rabies) *(cont.)* without the 333 mutation in the Rabies G, FILORAB1, rVSV-EGFP, or Mock (PBS). Weight is standardized as percentage of weight loss or gain in comparison with first day of exposure. Rabies virus infected animals developed clinical signs on day 8 with further weight loss until day 11 when endpoint criteria were reached. In LASSARAB-ΔG one mouse died at day 14 without displaying any signs or weight loss. In rVSV-GPC, 3 mice displayed signs of neurological deficit with 2 succumbing and 1 surviving. All other mice showed no signs of pathology. (b) Survival curves of BALB/c or SCID mice that were subjected to intracranially (IC) exposure with either LASSARAB or BNSP333. No signs of disease nor death were observed post- exposure. (c) IC exposure of Swiss Webster suckling mice with either LASSARAB or BNSP333. Suckling mice started succumbing to infection by day 7 in BNSP333 group and survived as long as day 12 in LASSARAB group with none surviving by the end of the study.

Live LASSARAB doesn't induce LASV-specific GPC IgGs.

We first evaluated immunization with replication competent vaccines. All live-attenuated (replication-competent) RABV based vaccines will be referred from now on with an rc- suffix (e.g., rc-LASSARAB). rVSV-GPC is always used as replication competent vaccine. C57BL/6 mice were intramuscularly immunized on day 0 with 10^6 ffu rc-LASSARAB, rc-LASSARAB-ΔG, rc-FILORAB1, or 10^6 pfu of rVSV-GPC. Humoral immune responses were analyzed by a newly developed LASV GPC-specific ELISA bi-weekly until day 42 post-immunization (Supplementary Fig. 1 and 2). By day 14, both rc-FILORAB1- and rc-LASSARAB-immunized mice had high titers of RABV-G-specific total IgG, and by day 28, maximum titers were achieved and were maintained until day 42, as seen previously (Supplementary Fig. 2)[36]. rc-LASSARAB-ΔG and rVSV-GPC immunized mice did not seroconvert to RABV-G. In contrast, LASV GPC-specific titers were detected in rc-LASSARAB-ΔG immunized mice only, and only at low titers on days 28 and 42 (Supplementary Fig. 2). rVSV-GPC had a significant LASV GPC-specific immune response (Fig. 4c, purple line).

Inactivated-LASSARAB virions induce humoral response in mice

We also explored the humoral immunogenicity of inactivated LASSARAB virions. Inactivated LASSARAB or FILORAB1 virions will simply be referred as LASSARAB or FILORAB1. We intramuscularly administered 10 μg of β-propiolactone (BPL)-inactivated LASSARAB or FILORAB1 particles to C57BL/6 mice following the standard three-inoculation RABV vaccination schedule (Fig. 4a). Both vaccines were further tested in 2 different formulations:

either in PBS only (LASSARAB/FILORAB1 groups), or adjuvanted with TLR4 receptor agonist (Glucopyranosil Lipid A) in a stable emulsion (LASSARAB+GLA-SE group)[45]. GLA-SE is a clinical-trial stage adjuvant that has been shown to enhance the breadth and quality of humoral immune responses for FILORAB1 and influenza virus[37, 38, 46]. Blood was collected and the humoral immune response was analyzed periodically until day 42 (Fig. 4 and Supplementary Fig. 2). Analysis of total IgG against LASV GPC by ELISA indicated seroconversion at day 14 by both LASSARAB and LASSARAB+GLA-SE groups; by day 28 both achieved statistical significance in comparison to control groups (Fig. 4b). Since endpoint titers of both inactivated LASSARAB and LASSARAB+GLA-SE had achieved appreciable total IgG responses against LASV GPC, we examined the quality of this humoral response by IgG2c and IgG1 sub-isotype-specific LASV GPC ELISA. IgG1/IgG2c ratios lower than 1.0 indicated an increasing Th1-bias response, which is desirable for antiviral responses. LASSARAB+GLA-SE not only achieved a significantly higher IgG2c response than LASSARAB, but also achieved consistently lower and uniform IgG1/IgG2c ratios (F-test, $p<0.01$), thus decreasing the variability of the immune response between mice (Fig. 4d and 4e).

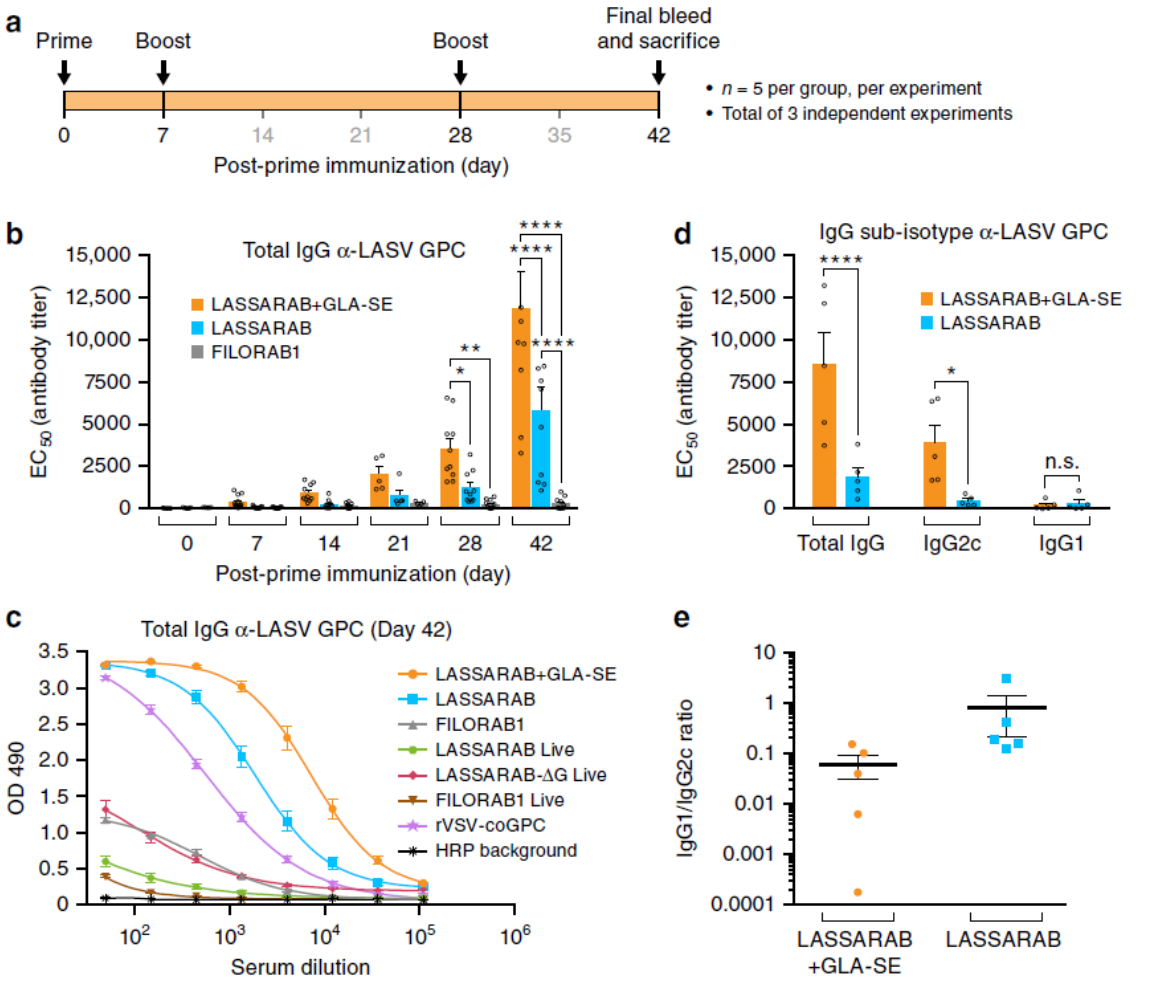

Fig. 4. Analysis of the humoral response towards Lassa virus glycoprotein. C57BL/6 mice were immunized IM in the gastrocnemius muscle with either 10 µg of β-Propiolactone inactivated viral particles in PBS or adjuvanted with 5 µg of GLA, a TLR-4 agonist formulated in 2% of stable emulsion (SE); LASSARAB+GLA-SE, LASSARAB, FILORAB1 groups) and boosted 2 times with the same amount on day 7 and 28 (a). Immunizations with replication-competent viruses were executed with a single time inoculation of 10^6 ffu or pfu virus IM in the gastrocnemius (rc-LASSARAB; rc-FILORAB1 groups and rVSV-GPC). (b) The EC_{50} values (obtained from the 4PL regression ELISA curve) of the total IgG titers against LASV GPC are plotted since day 0 until day 42. Error bars are representative of the standard error mean (SEM) and is calculated from 15 mice per group. Statistical significance was calculated by using 2-way ANOVA – post-hoc Tukey's Honest Significant Difference Test. (c) ELISA of total IgG against LASV GPC of all day 42 groups are shown for all immunized groups. ELISA curves are generated from 4PL regression. Error bars are representative of the SEM of OD 490 values (5 mice per group, in triplicates). (d) Day 35 EC_{50} antibody titer of IgG sub-isotype (IgG2c and IgG1) against LASV GPC of sera from LASSARAB+GLA-SE and LASSARAB group was analyzed. Error bars are the SEM of a total of 5 mice per group and statistical significance by 2-way ANOVA (post-hoc Tukey's Honest Significant Difference Test). (e) The ratios of the respective EC_{50} antibody titers IgG1/IgG2c are plotted and the F test was applied to check for variance difference ($p<0.001$). (****$P<0.0001$; ***$P<0.001$; **$P<0.01$; *$P<0.05$).

LASSARAB does not induce neutralizing antibodies

The development of neutralizing antibodies (NAbs) was investigated for LASSARAB using a pseudotyped VSV *in vitro* assay. This assay utilizes a single round ΔG-rVSV pseudovirus (ppVSV) which expresses both NanoLuc and eGFP as reporter genes[38, 47]. When pVSV pseudotyped with RABV G was used, the sera of either replication-competent or inactivated LASSARAB achieved high NAbs against RABV G (>10,000) compared to negative controls (Fig. 5, RABV). Since RABV G NAbs are a correlate of protection against RABV, these results indicated that LASSARAB is a suitable vaccine against RABV. Protection by RABV NAbs was further confirmed by using the WHO standard (Fig. 5a and 5c) in which values >0.5 IU/ml are considered protective against RABV; every group achieved IU/ml values much higher than 0.5 IU/ml, indicating that the addition of LASV GPC in the RABV backbone did not compromise its ability to generate RABV NAbs. Conversely, when ppVSV was pseudotyped with LASV GPC, we were not able to detect GPC-specific NAbs both in the presence or absence of complement (Supplementary Fig. 3), whereas the control human mAbs (12.1F, 25.10C and 37.7H) exhibited neutralizing activity at similar concentrations as described[26], indicating that our assay was functional (Fig. 5a and 5b).

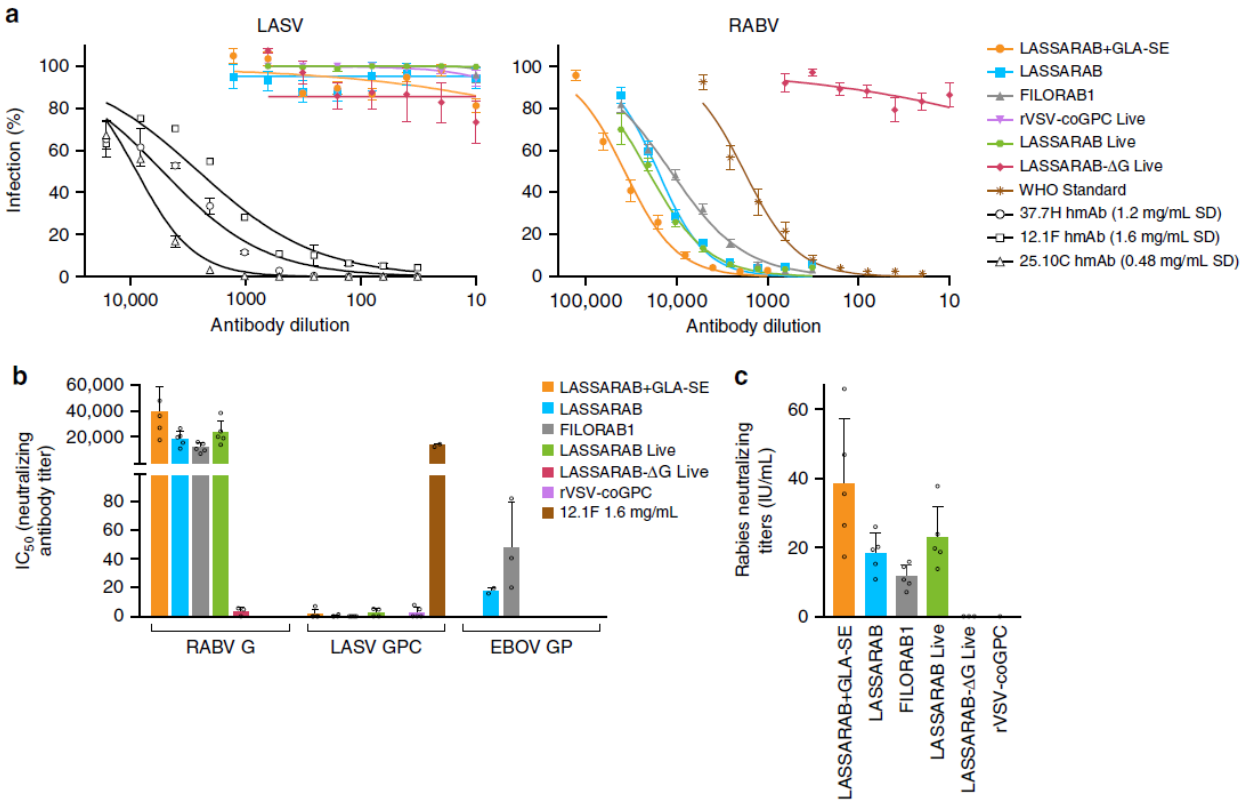

Fig. 5. Virus neutralization antibody titers. Day 42 sera from immunized mice was incubated with pseudotyped rVSV-ΔG-NL-GFP. (A) rVSV-ΔG-NL-GFP was pseudotyped with either RABV-G, LASV-GPC, or EBOV-GP to assay for RABV-G, LASV-GPC, or EBOV-GP NAb titers, respectively. 12.1F, 37.7H and 25.10c are LASV-GPC neutralizing antibodies used as a positive control for LASV-GPC neutralization[26]. Y axis in Fig. 5b represents 50% of inhibitory serum dilution (IC_{50}) titers obtained based on the antibody dilution that has 50% infection percentage of infected cells curves obtained in Fig. 5a. All groups achieved high neutralizing titers against RABV-G except for the groups immunized with virus lacking RABV-G: rc-LASSARAB-ΔG and rVSV-coGPC, as expected. Regarding LASV-GPC pseudotyped VSVs, no immunization achieved appreciable amounts of neutralizing antibodies. Neutralization of LASV GPC pseudotyped viruses with 12.1F, 37.7H, and 25.10C had an average IC50 of 1546 ng/ml, 375 ng/ml, and 69 ng/ml, respectively. (c) Rabies neutralizing titers were calculated by using the IC_{50} values of the WHO sera standard (2 IU/ml) serial diluted with rVSV-ΔG-NL-GFP pseudotyped with RABV G. WHO international units/ml (IU/ml) were then calculated using the following formula: (sample IC_{50} titer)/(WHO standard IC_{50} titer) x 2.0 (WHO IU/ml standard starting dilution). IU/ml from test sera is plotted Y axis. All error bars represented are the SEM of triplicate values of 5 mice per group. (****P<0.0001; ***P<0.001; **P<0.01; *P<0.05).

LASSARAB+GLA-SE is efficacious in guinea pigs

We evaluated LASSARAB vaccine efficacy using outbred Hartley guinea pigs and the guinea pig-adapted LASV[48]. Six groups of 10 Hartley guinea pigs were used (Fig. 6a): 3 groups were immunized with inactivated LASSARAB+GLA-SE particles once (1), twice (2), or three times (3); 2 groups were immunized with replication competent LASSARAB (rc-LASSARAB) or rVSV-GPC; and one group received RabAvert. All groups were challenged 58 days after the primary immunization with 10^4 pfu of the guinea pig adapted LASV Josiah strain. The animals were monitored for viremia and clinical signs were recorded daily up to day 47 post-challenge (Fig. 6b and 6c). Significant protection was observed for animals immunized 3 times with LASSARAB+GLA-SE ($p=0.0019$) or replication competent rVSV-GPC ($p=0.0008$) (Fig. 6b, red and purple lines). Guinea pigs inoculated with rc-LASSARAB or immunized once or twice with LASSARAB+GLA-SE showed no significant protection but a trend toward it. Interestingly, remarkably different clinical signs were observed in the 2 groups that were protected against LASV exposure (Fig. 6c, rVSV-GPC&LASSARAB+GLA-SE (-58, -51, -30) groups). While all animals in rVSV-GPC vaccinated group had an onset of clinical signs by day 12, all but 2 of the LASSARAB+GLA-SE immunized animals were free of clinical signs of disease. Curiously, in endpoint qPCR LASV RNA viremia analysis (Fig. 6d, survivors group), ~20% of surviving animals across all groups (except rc-LASSARAB and RabAvert) had an average of 10^5 LASV RNA copies per ml, indicating that despite being protected, some viremia was still present (Fig. 6d).

Next, we analyzed endpoint NAbs titers by LASV GPC pseudotyped ppVSV (Fig. 6e). The NAb response was highly variable across groups, being present in both survivors and succumbed animals with no significant difference between them ($p=0.18$). These data indicated that either NAbs play a minor role in survival or, in the case of the succumbed animals, develop too late in the infection to play a significant role.

The absence of NAbs against LASV across survivors led us to investigate correlates of protection in surviving guinea pigs by analyzing total IgG levels against LASV GPC in both pre-challenge and post-challenge serum (Fig. 6f and 6g). As shown in Fig. 6f, the groups that were protected against challenge, rVSV-GPC and LASSARAB+GLA-SE (3), had significantly higher titers of LASV GPC specific IgG ($p=0.0001$ and $p<0.0001$, respectively) in the pre-challenge sera when compared to RabAvert group. When post-challenge terminal sera were assayed (Fig.

6g), concentrations of LASV GPC-specific IgG were significantly higher in survivors compared to animals that succumbed ($p<0.0001$). Overall, our data suggests that, in both prior and post-exposure to LASV, higher levels of non-neutralizing LASV GPC-specific IgGs correlate with protection.

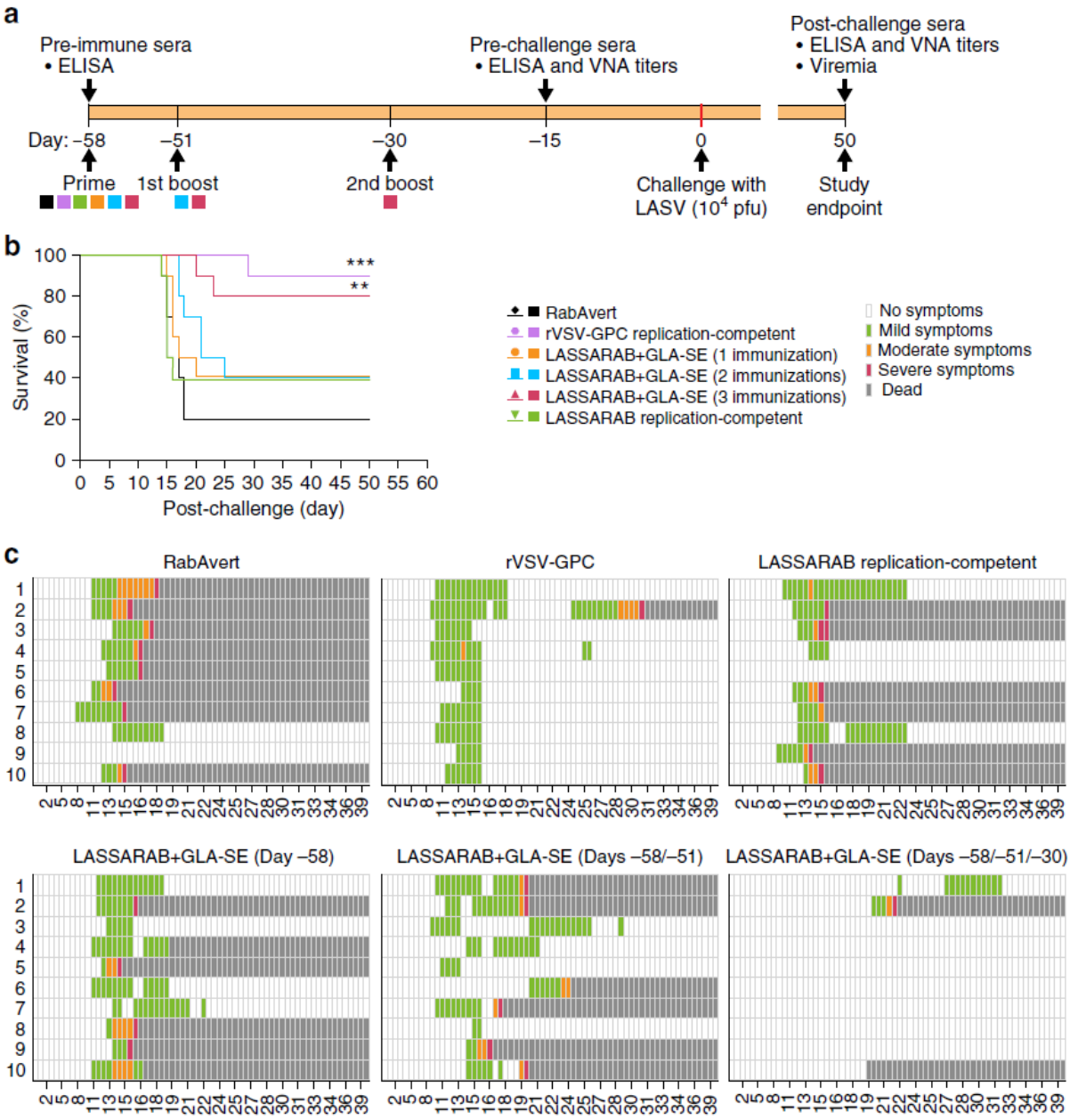

Fig. 6. *(continue in next page)*

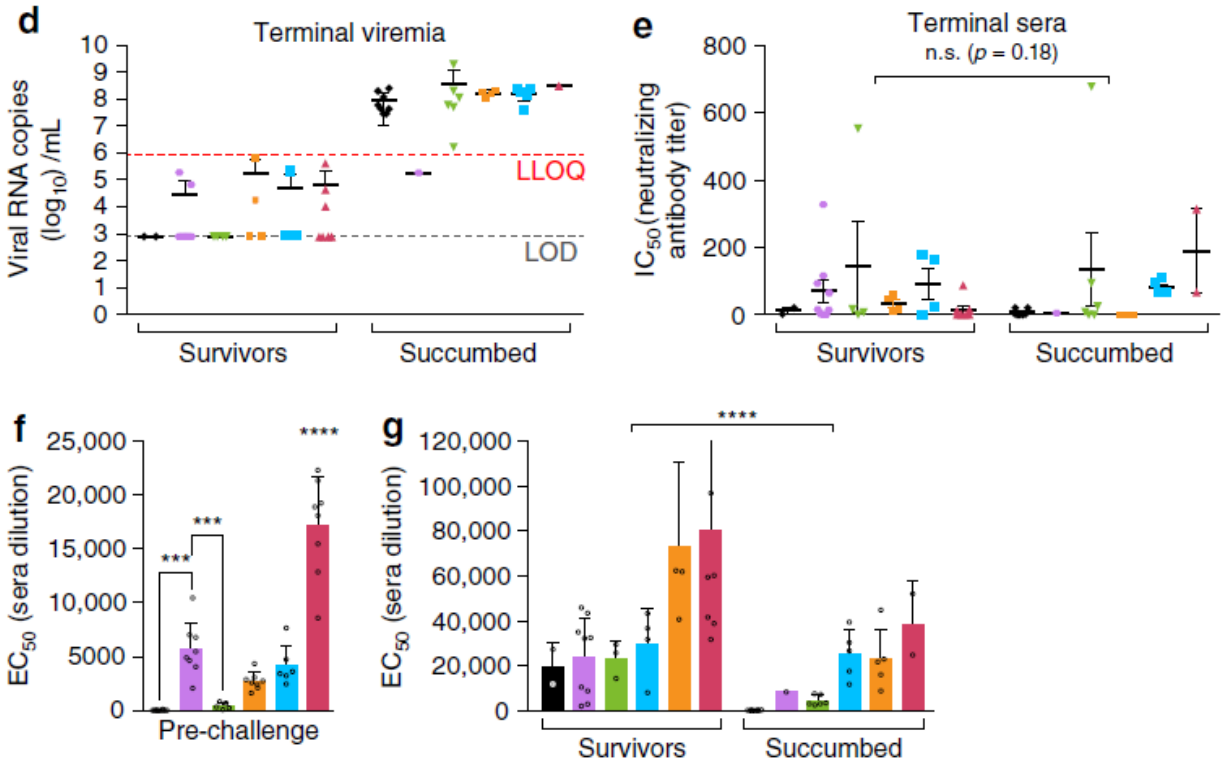

Fig. 6. LASV challenge of outbred Hartley guinea pigs immunized with several vaccine candidates and control. (a) Guinea pigs were immunized with either 2 replication competent vaccines: rVSV-GPC (positive control for survival) and LASSARAB replication-competent at 10^6 ffu by intraperitoneal injection (IP); or inactivated LASSARAB+GLA-SE with different immunization schedules: Day -58 (LASSARAB+GLA-SE (1)), Day -58, Day -51 (LASSARAB+GLA-SE (2)) and Day -58, Day -51, and Day -30 (LASSARAB+GLA-SE (3)). RabAvert was used as mock immunization (negative control). (b) Survival curves post IP exposure with 10^4 pfu guinea pig adapted LASV Josiah strain. Statistical significance is compared against Rabvert group using log-rank (Mantel-Cox) test. (c) Heat plot representing the clinical score information. X axis represents days' post-challenge and Y axis represents the individual animal number. (d) Terminal viremia was plotted using LASV RNA copies/ml in Y axis. Statistical significance was calculated using Kruskal-Wallis one-way ANOVA (not significant). (e) LASV neutralizing antibody titers is reported as the IC_{50} (half maximal inhibitory concentration) of serum dilution. The human mAbs 25.10C, 12.1F, and 37.7H[25, 26] were used as positive LASV neutralization controls. (f) Pre-challenge titers of LASV GPC specific IgG were performed on sera collected on Day -15 prior to challenge by ELISA with LASV GPC antigen and the EC_{50} (50% effective concentration) of serum dilution was plotted in the Y axis. Statistical significance (compared to the RabAvert group) was calculated by using one-way ANOVA (post-hoc test Tukey Honest Significant Difference Test). (g) Post-challenge titers of LASV GPC-specific IgG was performed on sera collected on terminal bleeding of both succumbed animals and survivors (day 50 post challenge) and the EC_{50} of serum dilution is plotted on the Y axis. Statistical significance reported between survivors and succumbed in (e&g) was determined by using two-way ANOVA. All error bars represented are the standard error mean (SEM) of 10 animals per group (in triplicates). (****P<0.0001; ***P<0.001; **P<0.01; *P<0.05).

LASSARAB induced Non-neutralizing antibodies stimulate ADCC

Once we found that a high LASV GPC-specific IgG titer with low or no NAbs correlated with protection in the LASSARAB+GLA-SE group, we determined whether non-neutralizing antibodies (non-NAb) can mediate protection through cell-mediated mechanisms, such as ADCC or ADCP. For this purpose, we used sera from mice immunized twice (on day 0 and day 28) with LASSARAB+GLA-SE (LASSARAB sera) or FILORAB1+GLA-SE (control) (Supplementary Fig. 3). First, we analyzed NK cell-mediated ADCC activity using an *in vitro* assay modified from a previously described rapid and fluorometric antibody-dependent cellular cytotoxicity (RFADCC)[49]. Briefly, we developed a stable 3T3 cell line expressing LASV GPC (3T3-LASV) and used it as target cells, and purified murine C57BL/6 NK cells as effectors, as described in Methods and Supplementary Fig. 3. 3T3-LASV cells were incubated with either LASSARAB sera or control, and different ratios of effector cells to target cells (E:T) were used (Fig. 7a and Supplementary Fig. 3). In the presence of LASSARAB sera, murine NK cells mediated significantly more killing ($p<0.01$) at any E:T compared to controls (Fig. 7a). This effect was reduced to background levels when another 3T3-based cell line expressing an irrelevant viral glycoprotein (3T3-MARV) was used as a target cell (Supplementary Fig. 3).

To determine which antibody isotype is important for ADCC-mediated killing of 3T3-LASV, we isolated IgG from the sera and conducted the assay with 40 µg/ml of either purified IgG or IgG-depleted sera (Fig. 7b and Supplementary Fig. 3). Again, killing of 3T3-LASV was significantly higher in the presence of LASV-specific purified IgG than in the control; in contrast, target cell cytotoxicity was reduced to background levels when IgG-depleted sera were used. Together these findings indicate that ADCC is mediated by the LASV GPC-specific IgG.

Macrophages mediate ADCP after immunization with LASSARAB

To examine whether other antibody-dependent cell-mediated mechanisms are involved in the clearance of LASV, we modified our ADCC assay to test if macrophages are involved in ADCP. As seen for the NK cells, peritoneal C57BL/6 macrophages (IC-21) induced 3T3-LASV cell killing compared to control sera when incubated with LASSARAB sera (Fig.7d and Supplementary Fig. 3). Moreover, we observed that peritoneal BALB/c macrophages (J774A.1) internalized 3T3-LASV cells in the presence of LASSARAB sera, likely through ADCP

(Fig. 7c and 7e). Target cell internalization was confirmed to be dependent upon Fcγ-R activation as macrophages incubated with anti-Fcγ-RIII mAb (but not anti-Fcγ-RI or anti-Fcγ-RIV) abolished 3T3-LASV internalization to background levels (Fig. 7c).

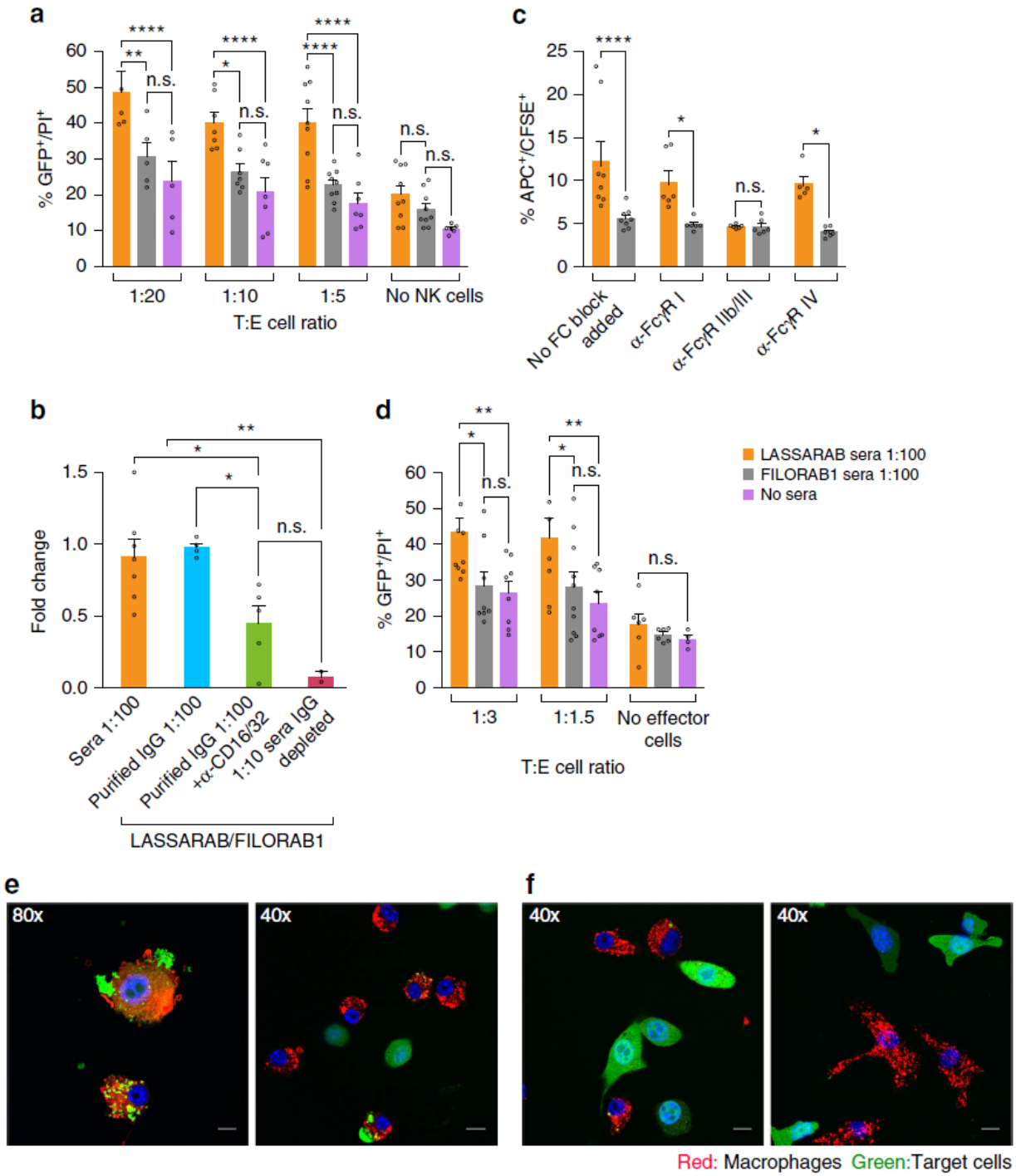

Fig. 7. Evaluation of antibody effector cell functions mediated by murine NK and macrophage cells against 3T3 expressing LASV GPC. Day 42 sera from immunized mice was incubated with 3T3-LASV cells and 30 min either

murine NK or macrophage cells were added and results were analyzed 4 h later by either flow cytometry (Supplementary Fig. 3 and Fig. 7a, 7b, 7c, and 7d) or confocal microscopy (Fig. 7e). Purified murine C57BL/6 NK cells (a) or IC-21 macrophages (d) were added at different Target:Effector cell ratios (T:E) with target cells incubated with either LASSARAB sera (yellow), FILORAB1 sera (grey) or no sera (pink). The Y axis represents the percentage of cellular cytotoxicity based on GFP^+/PI^+ cells (gating strategy and flow plots in Supplementary Fig. 3). (b) To determine which antibody isotype class is important for ADCC, NK cells were added at 1:5 T:E and incubated with either unprocessed sera (sera 1:100 condition), purified IgG (20 µg/ml), or IgG impoverished sera (1:5 dilution) from LASSARAB and FILORAB1 immunized mice. The Y axis represents cytotoxicity fold change of LASSARAB sera or IgG compared to FILORAB1 sera or IgG with same respective conditions. Anti-CD16/32 (Fcγ-RII/III) was also added at 25 µg/ml to confirm that FcγR blockade reduces ADCC activity. (c) and (e&f). To analyze ADCP J774.A1 macrophages were added at 1:5 T:E or 1:1 T:E (confocal) to 3T3-LASV cells incubated with either LASSARAB sera (c&e) or FILORAB1 sera (c&f). In (c) anti-CD16.2 (Fcγ-RIV), anti-CD16/32 (Fcγ-RII/III), and anti-CD64 (Fcγ-RI) were added at 25 µg/ml to check the effect of different FcγR blockade on ADCP activity. All error bars are the SEM of at least 3 independent experiments executed with duplicates. All statistical significance represented was performed through either a one- or two-way ANOVA and using a post-hoc analysis Tukey Honest Significant Difference test. (****P<0.0001; ***P<0.001; **P<0.01; *P<0.05).

Fcγ-receptor function is critical for protection in mice

We also investigated the relevance of antibody cellular effector function (ADCC and ADCP) *in vivo*. Because non-NAb effector function in mice is dependent upon Fcγ receptor engagement, we used Fcγ chain KO mice ($Fc\gamma^{-/-}$)[50] to test whether non-NAb against LASV GPC are as relevant in protection against LF as our previous results suggest. To that end, we developed a surrogate LASV murine model utilizing rVSV-GPC (Supplementary Fig. 4), since LASV is a BSL-4 agent with no established LASV murine model. Because rVSV-GPC expresses LASV GPC as its sole glycoprotein, it should have a similar tropism to LASV, and such approach has been a strategy used elsewhere for other VHF viruses[51, 52, 53]. Mice were made more susceptible to rVSV-GPC by blocking the interferon-α/β receptor (IFNAR) with anti-IFNAR mAb followed by an IP exposure of rVSV-GPC 24 h later[54].

BALB/c (WT) and BALB/c $Fc\gamma^{-/-}$ mice were immunized twice with either LASSARAB+GLA-SE or FILORAB1+GLA-SE (controls) in a total of 4 groups (Fig. 8a). On day 42 post primary immunization, mice were exposed IP with 10^4 pfu of rVSV-GPC and clinical signs and weight were monitored (Fig. 8b and Supplementary Fig. 4). WT LASSARAB immunized mice mostly resisted infection, with 8/10 mice having only transient weight loss (Fig. 8b and

Supplementary Fig. 4, continuous orange line). Meanwhile, all (10/10) of the Fcγ$^{-/-}$ LASSARAB mice quickly lost weight and succumbed to infection by day 5, with some showing signs of hemorrhage (Fig. 8b and Supplementary Fig. 4 dashed orange lines) indicating that Fcγ is essential to control viral infection in LASSARAB immunize mice. In FILORAB1 immunized mice (control), both WT and Fcγ$^{-/-}$ groups had a similar outcome, with 2/5 mice of each group surviving infection until study endpoint (Fig. 8b and Supplementary Fig. 4, grey lines), demonstrating that both WT and Fcγ$^{-/-}$ are equally susceptible to surrogate LASV exposure.

Upon pre-exposure analysis of GPC-specific IgG titers, both WT and Fcγ$^{-/-}$ mice immunized with LASSARAB had significantly higher titers in comparison with FILORAB1 control mice (Fig. 8c and Supplementary Fig. 4c), but no LASV NAbs were detected in neutralization assays (Supplementary Fig. 4b). In post-exposure analysis of LASV NAbs, surviving LASSARAB immunized mice developed little to no neutralizing antibody (Fig. 8d, orange symbols), while one WT FILORAB1 vaccinated mouse developed modest levels of LASV NAbs (Fig. 8d). Overall this data shows that previous LASSARAB immunization is heavily dependent on non-NAb effector function activity *in vivo* for protection against LASV.

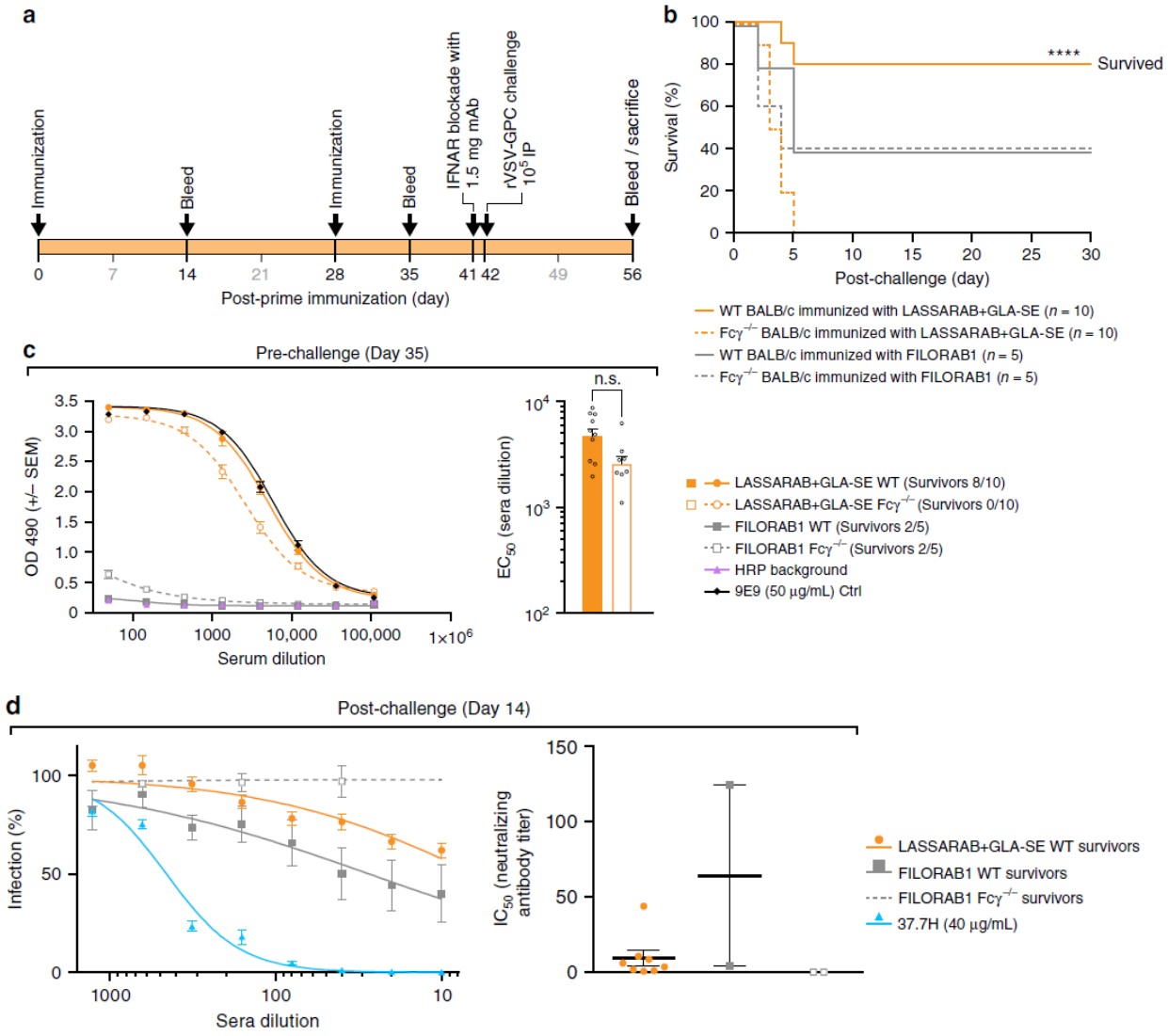

Fig. 8. Evaluation of *in vivo* relevance of non-NAbs LASV GPC specific antibodies induced by LASSARAB+GLA-SE vaccination. (a) 8- to 10-week-old Balb/c (WT) or Balb/c with Fcγ chain KO ($Fc\gamma^{-/-}$) female mice were immunized with 10 µg of inactivated particles of either LASSARAB or FILORAB1 (mock control) on day 0 and boosted on day 28. All 4 groups in total were adjuvanted with 5 µg of GLA in a 2% SE with each vaccination. One day before exposure (day 41) animals were injected with 1.25 mg of anti-Ifnar mAb (MAR1-5A3, Leinco technologies) through intra-peritoneal injection (IP). On day 42, mice were exposed to 10^4 rVSV-GPC virus IP and general health (weights and clinical observation) was recorded until endpoint criteria were reached or end of study (supplemental). (b) Survival curves post-exposure of rVSV-GPC. Significance is compared between the WT LASSARAB vaccinated and the $Fc\gamma^{-/-}$ vaccinated using the log-rank (Mantel-Cox) test. (c) Pre-exposure total IgG titers anti LASV GPC were measured by ELISA on day 35 post-prime and ELISA curves were plotted according to OD490 reading value (Y axis) and serum dilution (X axis). On the right, EC_{50} (half maximal effective concentration) of serum dilution of both LASSARAB groups (WT and $Fc\gamma^{-/-}$) is plotted on Y axis on a log scale; statistical significance was calculated using one-way ANOVA. (d) Virus neutralization assay using pseudotyped VSV-GFP-

NanoLuc with LASV GPC. On the right, percentage of cells infected is plotted against the serum dilution (survivors on day 14 post-exposure) of each respective group. On the right, the IC_{50} (half maximal inhibitory concentration) of serum dilution is plotted individually and significance was calculated using one-way ANOVA. Error bars represent Standard Error Mean (SEM) and include all mice (n=10 per group [WT and KO] in LASSARAB and n=5 per group [WT and KO] in FILORAB1 control) in pre-challenge and survivor mice in post-challenge. (****P<0.0001; ***P<0.001; **P<0.01; *P<0.05).

Discussion

The WHO R&D Blueprint for Action to Prevent Epidemics[55] defines LF as a priority agent for vaccine development. Accordingly, preferred vaccine requirements include: (1) a highly favorable risk-benefit profile suitable for all age groups, (2) practicality for non-emergency/preventive scenarios, (3) at least 90% efficacy in preventing disease, (4) high thermostability, and (5) the possibility of co-administration with other vaccines. LASSARAB appears to be the first inactivated LF vaccine to fulfill most of these requirements as demonstrated in our study and based on previous work done with the same platform for other VHFs[37]. Another advantage to LASSARAB, as an inactivated LF vaccine, is that it could potentially be used in pregnant women and immunosuppressed patients, both of which are major risk groups for LF. In addition to LF, LASSARAB also confers protection to rabies (Fig. 5b), which is a major health burden in Africa[56].

Most LASV vaccine studies have characterized the role of humoral response against LASV as either a secondary mechanism of protection or even detrimental to survival[11]. Such correlations were drawn based on results measuring antibody responses against LASV nucleoprotein (NP) or nonspecific LASV antigens[11, 16, 57]. Although NP is highly immunogenic, it is neither expressed on the surface of cells nor virions. As such, antibodies directed against LASV NP should only have diagnostic value. Meanwhile, GPC has been shown to be the most effective LASV immunogen but, to our knowledge, no attempts were made to correlate GPC-specific humoral response with LASV protection[16, 58, 59, 60]. Thus, as part of LASSARAB characterization, we were compelled to develop a GPC-specific antigen that is expressed in its native conformation (Supplementary Fig. 1). Throughout the development of LASSARAB, we observed that replication-competent LASSARAB and replication-competent LASSARABΔG were poor inducers of GPC-specific antibodies, despite being able to induce RABV protective response (Fig. 4c, 5, and Supplementary Fig. 2). In contrast, when inactivated LASSARAB immunizations were combined with a late boost (day 28 post-prime), high levels of LASV GPC-specific antibodies were induced at later time points, especially when administrated with a TLR-4 agonist (GLA-SE). This contrast might be attributed to the fact that LASV GPC is a poor immunogen[28, 61] and, as such, induction of antibodies against GPC might be dependent on

replication competent vectors that achieve high or persistent viral loads post immunization. Given that inactivated LASSARAB incorporates LASV GPC, it can safely be administrated in higher dosages in a prime/boost regimen and, as such, more antigen might be available to prime follicular B helper T cells and B cell response. The high effectivity of a TLR-4 agonist in inducing higher levels of anti-LASV GPC antibodies with higher quality (IgG2c bias) further corroborates recent findings by Galan-Navarro et al.[61] indicating that inactivated LASV vaccines might benefit of TLR-4 agonists. Nonetheless, no NAbs against LASV pseudotypes were detected in either replication competent or inactivated approaches (Fig. 4, 5 and Supplementary Fig. 3 and 4).

Because it has been the case with vaccines for some other viruses[44, 62, 63], it might be expected that an effective LF vaccine protects through NAbs. Sommerstein et al. has elegantly demonstrated that LASV exposure or immunization in mice does not induce LASV NAbs due to the LASV GPC's glycan shield [28]. Additionally, as recently shown by the important works of Robinson JE et al. and Hastie et al., most potent LASV NAbs (such as 37.7H) require very specific quaternary epitopes bridging LASV GP1 and GP2, making it challenging to elicit through immunization. Interestingly, these NAbs, instead of blocking GPC receptor binding, achieve neutralization by stabilizing LASV GPC in its pre-fusion conformation[25, 26, 42]. The lack of NAbs induced with the several vaccine candidates, either replication competent or inactivated, in our study (Fig. 5a) and in previous published vaccine candidates, further corroborates this expectation[11, 23]. Even after LASV exposure, only a small fraction of human and animal survivors produce NAbs, findings that our study further confirmed (Fig. 6 and 8)[11, 26, 28]. Additionally, we showed that guinea pigs that succumbed to disease also had NAbs, suggesting either that NAbs by themselves play a minor role in protection or that they develop too late during infection to impact outcome. Studies by Mire et al. have recently shown that some LASV NAbs can mediate protection in NHPs and guinea pigs when administrated prophylactically[25, 26]. Although providing evidence that GPC specific mAbs can mediate protection against Lassa Fever, the role of antibody-dependent effector cellular functions was not evaluated and GPC-specific non-NAbs were not used. Furthermore, LASV neutralizing potency *in vitro* did not necessarily correlate with protection[27]. Together with the findings in our study (Fig. 6), this raises the question whether GPC-specific non-NAb play a role in

protection through other mechanisms, such as ADCC, since guinea pig survival post-LASV exposure was correlated with high levels of GPC-specific non-NAb independent of NAb titer.

In several other viruses (e.g., Influenza, LCMV), antibody Fc-FcγR interactions leading to ADCC and ADCP are important for protection, playing a critical role both in viral clearance and in preventing chronic infection regardless of neutralizing ability[29, 30, 32, 34, 64]. Through our *in vitro* studies, we showed that sera from LASSARAB-immunized mice with high GPC-specific antibodies (Supplementary Fig. 4) did not neutralize LASV but elicited significant ADCC and ADCP of 3T3 cells expressing LASV GPC (Fig. 5 and 7). Interestingly, the Fcγ-RIV blockade did not reduce ADCP activity by macrophages (Fig. 7e), despite having a high affinity for IgG2 subclass-dependent ADCP. This suggests that GPC-specific IgG1 might be mediating ADCP[65]; nevertheless, in contrast with IgG2 subclass, GPC-specific IgG1 titers were almost non-existent in the purified IgG used (S3c).

To corroborate the relevance of Fcγ-R effector functions in LASSARAB-induced protection *in vivo,* we used an Fcγ-KO mouse model challenged with surrogate LASV exposure (Fig. 8)[50]. This approach permitted us to dissect the role that LASSARAB induced non-NAb play in protection against surrogate LASV exposure in the context of a similar immunogenic response. Despite similar levels of antibody titers and isotype to both RABV G and LASV GPC as detected by ELISA (Fig. 8c and Supplementary Fig. 5a and b), LASSARAB immunized Fcγ-KO mice quickly succumbed to surrogate LASV exposure, in contrast to the WT mice. However, some differences exist between human and mouse Fcγ-Rs, and future studies using humanized knock-in models would be of interest[66]. Curiously, besides the critical role that Fcγ-R effector functions played in protection against LASV, our results from Fig. 8 indicated (but not significantly) that $Fc\gamma^{-/-}$ mice immunized with LASSARAB seemed more susceptible to surrogate LASV infection than control mice (Fig. 8 and Supplementary Fig. 5). Although based on a contrived model, this makes us question whether, beyond viral clearance, pre-existing GPC-directed non-NAbs might also work as immune regulators in LASV infection.

By the end of our guinea pig exposure study (Fig. 6d), we observed that ~20% to ~40% of survivors had low (below the LOQ) but detectable levels of LASV RNA in the blood 50 days' post-exposure in all groups, except LASSARAB and RabAvert. This has been reported in the literature for LASV in non-human primates[23, 25]. This result suggests a chronic asymptomatic

infection that, after reactivation, may explain some of the late deaths and clinical signs observed in both the LASSARAB+GLA-SE and rVSV-GPC groups. As such, future studies should consider possible LASV chronicity and reactivation.

As a major LF surge unfolds in Nigeria at the time of manuscript preparation, the necessity to fully understand the immunomechanisms of protection of LASV becomes an increasingly important and crucial task for LF vaccine development. Ideally, a LF vaccine should be protective, safe, and confer a long-lasting humoral immunity that can be easily measured and identified as a correlate of protection. As our results demonstrate, LASSARAB induces high LASV GPC-specific IgG titers that correlate with protection prior to LASV exposure, in the absence of LASV NAbs. This could potentially become a LF correlate of protection that would provide easy screening for vaccine efficacy post immunization. Additionally, the finding that GPC-specific non-NAbs play a crucial role in protecting mice against a LASV surrogate exposure suggests that non-NAb cellular effector functions should be further investigated as a correlate of protection in both LF vaccine development and mAb antibody therapy.

Methods

Generation and recovery of Rhabdovirus vaccine vectors

To generate the vaccine vectors LASSARAB, LASSARABΔG, and rVSV-GPC, the ORF of LASV GPC Josiah strain was codon-optimized for mammalian codon-usage and synthetized by GenScript (Genbank, Accession Number MH778559). LASV GPC was cloned between BsiWI and NheI restriction digest sites of BNSP333, generating LASSARAB. LASSARABΔG was generated by removing the RABV glycoprotein (G) from the LASSARAB cDNA using the PacI and SmaI restriction digest sites and subsequent re-ligation after treatment with Klenow Fragment (Promega).

rVSV-GPC was generated by replacing the native VSV G, through MluI and NheI restriction digestion site, with a codon optimized LASV GPC (above) amplified by the PCR primers RLP3 and RLP4 containing the MluI or NheI restriction sites and cloned in cVSV-XN vector[62]. The correct sequence of all the three plasmids were confirmed by sequencing using RP951, RP952, VP5, and VP6 primers.

Recombinant RABV and VSV vaccines were recovered as described previously[67, 68]. Briefly, X-tremeGENE 9 (Sigma-Aldrich®) in Opti-MEM (Gibco®) was used to co-transfect the respective full-length viral cDNA clones along with the plasmids encoding RABV N, P, G, L or VSV N, P, L proteins, and pCAGGs plasmids expressing T7 RNA polymerase in Vero cells in 6-well plates (RABV), or 293T cells in T25 flasks (VSV). The supernatants of RABV transfected cells were harvested after 7 days and after 3 days for VSV. Presence of infectious virus was detected by immunostaining for RABV N with 1:200 dilution of FITC anti-rabies monoclonal globulin (Fujirebio®, product # 800-092) or for virus-induced cytopathic effect (CPE) in the case of VSV.

Request for material

Upon reasonable request all utilized antibodies, plasmids, and viruses are available from the authors pending on an executed MTA as well as biosafety approval of the requesting institution(s).

Cell culture

Vero (ATCC® CCL81™), 293T (ATCC® CRL-3216™), and BSR (available from our laboratory) cells were cultured using DMEM (Corning®) with 5% FBS (Atlanta-Biologicals®) and 1% P/S (Gibco®)[36]. J774.A1 (ATCC® TIB-67™) macrophages, NIH/3T3 (ATCC® CRL-1658™), and their stable cell line derivatives were cultured using DMEM with 10% FBS and 1% P/S. IC-21 (ATCC® TIB-186™) macrophages were cultured using RPMI (Corning®) with 10% FBS and 1% P/S.

Antibodies

Mouse monoclonal antibodies (mAb) anti-LASV GPC (4C8, 9E9, and 5A3) were produced and provided by Dr. Gene Tan (J. Craig Venter Institute, La Jolla, CA). The human mAbs anti-LASV GPC (3.3B, 22.5D, 37.7H, 25.10C and 12.1F) were a generous gift from Dr. Robert Garry (Tulane University)[26]. Rabbit polyclonal antibody (pAb) anti-LASV GPC was generous gift from Dr. Stephan Guenther (Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany). 4C12 human anti-RABV G mAb was a generous gift from Dr. Scott Dessain (Lankenau Institute for Medical Research, Wynnewood, PA)[37, 62, 63].

Viral production and tittering

LASSARAB, LASSARAB-ΔG, FILORAB1, rVSV-GPC, and SPBN viruses were grown and titered on Vero cells. For virus production, Vero cells were cultured with Opti-Pro serum free media supplemented with 1% P/S and 4 mM L-Glutamine (Gibco®) and inoculated with a multiplicity of infection (MOI) of 0.01 of each respective virus. Viruses were harvested up to a total of 6 times with media replacement (Opti-Pro) or until 80% cytopathic effect was detected. Tittering was performed by limiting dilution focus forming assay using RABV N with 1:200 dilution of FITC anti-rabies monoclonal globulin (Fujirebio®; catalogue number: 800-092). rVSV-GPC titers were determined by plaque forming assay using 2% methyl cellulose overlay.

Purification and virus inactivation

To produce inactivated LASSARAB and FILORAB1[38] (kind gift of Drishya Kurup, Thomas Jefferson University) vaccines, viral supernatant were sucrose purified and inactivated[37]. Briefly, viral supernatants were concentrated at least 10x by Amicon® stirred cell concentrator using a 500 kDa exclusion PES membrane (Millipore®) and centrifuged at 110000 g through a 20% sucrose cushion. Virion pellets were resuspended in 1xDPBS (Corning®) containing 2% sucrose and betapropiolactone (BPL) (Sigma-Aldrich®) was added at a 1:2000 dilution for inactivation. Samples were left at 4°C O/N shaking and next day BPL was hydrolized at 37°C for 30 min.

Adjuvant formulations

The Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion adjuvant (GLA-SE) was produced by IDRI[38]. Formulation with inactivated vaccines was conducted prior to injection with a total 5 μg of GLA for mice or 7.5 μg of GLA for guinea pigs in a final v/v 2% SE concentration.

Immunofluorescence

Vero cells were seeded on glass coverslips and infected at an MOI of 0.1 with the respective viruses. 48 h later (24 for VSV constructs), cells were fixed with 2% paraformaldehyde (PFA) and probed with 10 μg/ml anti-RABV G mAb (4C12) and mouse 50 μg/ml of anti-LASV GP2 mAb (9E9). Secondary goat polyclonal antibody (Jackson ImmunoResearch® catalogue numbers: 109-225-088; 115-165-146) anti-human IgG and anti-mouse IgG conjugated with Cy2 and Cy3 dyes, respectively, were used at 4 μg/ml. Slides were mounted with DAPI containing mounting media (VECTASHIELD®) and images were taken with a Zeiss AxioSkop 40 microscope and color channels were compiled using ImageJ software (OSS NIH).

Viruses and ELISA antigen characterization

Virus particles and purified LASV GPC were denatured with Urea Sample Buffer (125 mM Tris-HCl [pH 6.8], 8 M urea, 4% sodium dodecyl sulfate, 50 mM Dithiothreitol, 0.02% bromophenol

blue) at 95°C for 5 min. 2 µg of protein was resolved on a 10% SDS–polyacrylamide gel and stained O/N with SYPRO Ruby (Thermofisher) for total protein analysis. For Western blot analysis SDS-PAGE gel was transferred onto a nitrocellulose membrane in Towbin buffer (192 mM glycine, 25 mm Tris, 20% methanol) then blocked in 5% milk dissolved in PBS-T (0.05% Tween 20) at room temperature for 1 h. Next, the membrane was incubated O/N with either rabbit pAb anti-LASV GPC or 9E9 mAb anti-LASV GP2 at a dilution of 1:1,000 in 5% bovine serum albumin (BSA). Rabies G and P proteins were confirmed with a rabbit anti-G and P polyclonal antibody used at 1:1000[62]. After washing, the blot was incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG diluted (Jackson ImmunoResearch® catalogue numbers: 115-035-146; 111-035-144) at 1:50,000 in 1% milk PBS-T. Proteins were detected with SuperSignal West Dura Chemiluminescent substrate (Pierce®).

Animal studies

(i) **Animals ethics statement.** Mice and guinea pigs used in this study were handled in adherence to both the recommendations described in the Guide for the Care and Use of Laboratory Animals, and the guidelines of the National Institutes of Health and the Office of Animal Welfare. Animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University (TJU) or the National Institutes of Health, National Institute of Allergy and Infectious Diseases, Division of Clinical Research Animal Care and Use Committee for experiments performed at each respective facility. Animal procedures at TJU were conducted under 3% isoflurane/O_2 gas anesthesia. Mice were housed with up to five individuals per cage, under controlled conditions of humidity, temperature, and light (12-h light/12-h dark cycles). Food and water were available ad libitum.

(ii) **Viral pathogenicity evaluation.** Five groups of five 6- to 8-week-old female Swiss Webster mice were either intranasally (IN) or intraperitoneally (IP) infected with 10^5 PFU/FFU of each of the respective viruses diluted in 20 µl phosphate-buffered saline (PBS). Mice were weighed daily and monitored for signs of disease until day

28 post infection. Mice that lost more than 20% weight or showed severe neurological symptoms were humanely euthanized. Intracranical challenge (IC) was performed in 48, 6- to 8-week-old Balb/c mice were anesthetized using isoflurane to effect, followed by IC injection of 10 fold increasing dose of virus from 10^2 to 10^5 FFU of infectious virus. Mice were monitored daily for up to 21 days post-exposure. Mice were euthanized when signs of neurological disease, including tremors, seizure, prostration, and paralysis, were observed using a pre-determined scale of severity. Forty-eight, 3- to 4-day-old Swiss Webster mice were anesthetized by hypothermia followed by IC injection of 10-fold increasing dose of virus from 10^2 to 10^5 ffu of infectious virus. Mice were monitored daily for signs of neurological disease and euthanized when signs developed or at 10 days post-exposure.

(iii) **Humoral immunogenicity evaluation in mouse model.** Five groups of five 6- to 8-week-old female C57BL/6 mice were immunized intramuscularly (IM) with 10^6 PFU/FFU of live virus diluted in PBS or with 10 µg BPL-inactivated virus (3 doses at 0, 7, and 28 days) formulated in either PBS or GLA-SE adjuvant (see Fig. 4 and adjuvant formulation below). All IM immunizations were performed by administering 50 µl of live or BPL-inactivated virus into each hind leg muscle. For serum collection, retro-orbital bleeds were performed under isoflurane anesthesia on days 0, 7, 14, 21, 28, and 35, with the final bleed on day 42 or 63.

(iv) **LASV challenge on outbred Hartley guinea pigs.** Six groups of 10 Hartley guinea pigs with PinPorts for blood withdrawal (Charles River Laboratory) were vaccinated as follows: Group 1: Mock (PBS), Group 2 rVSV-GPC 10^7 FFU, Group 3 RABV-LASV-GPC 10^7 FFU, Group 4 RABV-LASV-GPC (30 µg) + GLA-SE (7.5 µg) on day -58 of virus exposure, Group 5 RABV-LASV-GPC (30 µg) + GLA-SE (7.5 µg) on days -58 and -51 of virus exposure, Group 6 RABV-LASV-GPC (30 µg) + GLA-SE (7.5 µg) on days -58, -51 and -30 of virus exposure. All subjects were challenged with 10,000 PFU of guinea pig adapted LASV (GPa-LASV(IRF0205); L segment GenBank KY425651.1; S segment GenBank KY425650.1) by IP route[48]. Subjects were monitored at least once daily throughout the experiment and at least twice daily following virus exposure until clinical signs of disease abated. Blood withdrawals

were performed at days -65, -58, -51, -30, 0, 16 and study end at day 42 post-exposure. All LASV experiments were performed in a bio-safety level 4 environment and subjects were anesthetized using isoflurane/O_2 gas anesthesia for all procedures. Clinical scoring to determine euthanasia was based on the appearance of one of the following clinical changes: change in skin and mucous membrane color, unthrifty appearance, unresponsiveniess, agonal breathing, paralysis, head tilt, persistent scratching, tremors. Subjects that met endpoint criteria and subjects that survived to study end, day 42, were humanely euthanized and a complete necropsy was performed.

(v) **In *vitro* antibody-dependent cellular cytotoxicity and phagocytosis (ADCC/ADCP) evaluation.** The sera used for these assays was collected on day 42 from 2 groups of 5 mice each IM immunized with 10 µg BPL-inactivated LASSARAB or FILORAB1 (2 doses: at day 0 and at day 28) formulated with GLA-SE adjuvant. Serum collected from individual mice was pooled and heat inactivated for 30 min at 56°C. For IgG purification, serum was run through a protein G high performance Spintrap column (GE Healthcare).

(vi) **Surrogate LASV challenge on mouse model.** Four groups of either Balb/C or Fcγ knockout Balb/C (Balb/C $Fc\gamma^{-/-}$ generously donated by Dr. Jeffrey V. Ravetch, Rockefeller University) were IM immunized with 10 µg BPL-inactivated LASSARAB or FILORAB1 (2 doses: at day 0 and at day 28) formulated with GLA-SE adjuvant and sera were collected on day 0 or day 35 post-immunization. On day 41, mice were injected IP with 1.25 mg of mouse anti-IFAR1 mAb clone: MAR1-5A3 (Leinco Technologies, catalogue number: I-401). On day 42, mice were injected with 10^4 pfu of rVSV-GPC diluted in PBS. rVSV-GPC was previously confirmed to be pathogenic in immunosuppressed mice by titering the virus to the least amount that causes 100% lethality on naïve Balb/C mice. Health and weight were monitored daily. Mice were sacrificed when: 1) weight loss reached >20% or 2) if severe clinical signs of disease were observed. Terminal bleeding was collected upon sacrifice when possible.

Enzyme-linked immunosorbent assay (ELISA)

Individual mouse or guinea pig serum was analyzed by ELISA for the presence of IgG specific to LASV GPC, RABV G, and EBOV GP. Antigens were resuspended in coating buffer (50 mM Na_2CO_3 [pH 9.6]) at a concentration of 500 ng/ml and then plated in 96-well immulon 4 HBX plates (Nunc®) at 100 µl in each well and incubated O/N for 4°C. Plates were then washed three times with PBS-T (0.05% Tween 20 in 1× PBS), blocked for 1 h (5% milk in PBS-T), washed three times with PBS-T, and then incubated O/N at 4°C with three-fold serial dilutions of sera or control mAb (starting at either 1:50 or 1:150 dillution) in PBS containing 0.5% BSA. Next, plates were washed three times, followed by the addition of either horseradish peroxidase (HRP) conjugated goat anti-mouse: IgG (H+L), Fc specific (heavy chain), IgG2c, IgG2a, and IgG1; or goat anti-guinea pig Fc-specific (heavy chain) secondary antibody at 1:10,000 dilution in PBS-T (Jackson ImmunoResearch® catalogue numbers: 115-035-146; 115-035-071; 115-035-205; 115-035-206; 115-035-208; 106-035-008). After incubation for 2 h at RT, plates were washed three times with PBS-T, and 200 µl of o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich) was added and left incubating for exactly 15 min. The reaction was stopped by adding 50 µl of 3M H2SO4. Optical density was determined at 490 nm (OD490). ELISA data was analyzed with GraphPad Prism 7 using a sigmoidal nonlinear fit (4PL regression curve) model to determine the half maximal Effective Concentration (EC50) serum or antibody titer.

Generation and production of ELISA antigens

RABV G antigen were generated as described[36]. Briefly, RABV G and LASV GPC antigen were generated by infecting BSR cells with either rVSV-GPC (for LASV GPC antigen) or SPBN (RABV G antigen) in Opti-Pro SFM (Gibco®). Viral supernatants were concentrated and purified as described in virus purification methods section (see above). Viral pellets were then resuspended in TEN buffer (100 mM NaCl, 100 mM Tris, 10 mM EDTA pH7.6) containing 2% OGP (Octyl β-D-glucopyranoside) detergent and incubated for 30 min while shaking at RT. Mixture was then centrifuged at 3000 g, and the supernatant was collected and further centrifuged at 250000 g for 90 min. Supernatant was collected and analyzed by SDS-PAGE and WB for LASV GP1 and GP2 presence (see above).

Virus Neutralization Assay

Virus neutralization assay (VNA) was conducted based on a modified VSV based VNA[38], by generating a single round VSV pseudotype reporter virus (ppVSV-NL-GFP) expressing nano-luciferase (NanoLuc® Promega) and GFP.

i) **Generation of VSV pseudovirons (ppVSV)**. To generate ppVSV, the cDNA plasmid backbone of rVSV-GPC was digested with MluI and NheI restriction enzymes to remove the LASV GPC glycoprotein and insert the NanoLuc ORF (Promega). To enable GPC expression, the EGFP ORF plus a VSV start stop signal were inserted in XhoI and NheI cloning sites. Viruses were recovered as described above and further propagated on BSR cell line expressing VSV-G. To pseudotype ppVSV-NL-GFP with either LASV GPC, RABV G, or EBOV GP, 293T cells were transfected with pCAGGS plasmid encoding either LASV GPC (Josiah strain), RABV G (SAD-B19 strain), or EBOV GP (Mayinga strain), respectively, using X-tremeGENE 9 (Sigma-Aldrich) as a transfection reagent. 24 h post transfection, pVSV-NL-GFP was added to the cells at an MOI of 1 and viral supernatant was collected 24 and 48 h later.

ii) **Virus neutralization assay (VNA)**. For VNA using animal sera (mouse or guinea pig), the serum was heat inactivated at 56°C for 30 min to ensure complement deactivation. Next, heat inactivated serum was diluted 2 fold starting at 1:10 dilution (1:100 in RABV-G pseudotyped assays) in Opti-MEM (Gibco), and 10^4 ppVSV-NL-GFP particles were added to each dilution series. Control mAbs (12.1F, 25.10C, 37.7H, and 9E9, see Antibodies section above) and WHO international standard sera was added starting at 30 µg/ml and 2 UI/ml, respectively. The sera/antibody+virus mix was incubated for 2 h at 34°C with 5% CO_2 and transferred to a previously seeded monolayer of Vero cells in a 96 well plate and further incubated for 2 h at 34°C with 5% CO_2. Next, the virus/serum mix was replaced by complete DMEM media. At 18–22 h later, cells were lysed with passive lysis buffer (Promega) and transferred to an opaque white 96-well plate, with NanoLuc®

substrate (Promega) added following the manufacturer's recommendations. Relative luminescence units were normalized to 100% infectivity signal as measured by no sera control (maximum signal). Half maximal inhibition (IC_{50}) values were calculated by GraphPad® Prism 7 using a sigmoidal nonlinear fit model (4PL regression curve). Values that were above 100% infectivity were converted to 100%.

RT-PCR analysis for LASV viral loads.

See also [70]. 200ul of whole blood was lysed for RNA extraction using Trizol LS at a 3:1 vol:vol ratio. RNA samples were then extracted using the QIAMP Viral RNA Mini Kit (QIAGEN) and eluted in 50µl Buffer AVE (QIAGEN). 5uL of extracted RNA per reaction was added to 2X Master Mix with Superscript III Platinum One Step qRT-PCR kit (In*vitro*gen) with final concentrations of 1µM forward primer (5'CCACCATYTTRTGCATRTGCCA), 1µM reverse primer (5'GCACATGTNTCHTAYAGYATGGAYCA) and 0.1µM probe (FAM_AARTGGGGYCCDATGATGTGYCCWTT). Cycling conditions were 45°C for 15min for reverse transcription, 95°C for 2min, followed by PCR amplification for 45 cycles at 95°C for 15s, then 60°C for 30s on an ABI 7500 real-time PCR system (Applied Biosystem®). In-*vitro* transcribed RNA was used as the standard. The LASV sequence from 3255 to 3726 (Genbank accession number: KY425634.1) was cloned under a T7 promoter in vector pCMV6-AC. The fragment was linearized and 1ug of DNA was used in the in-*vitro* transcription reaction using the MEGAscript T7 transcription kit (Ambion). RNA copy number was calculated and 1:10 dilutions were made to provide a standard from $9\log_{10}$ viral RNA copies to $1\log_{10}$ viral RNA copies. Quantification was performed by CT analysis (Applied Biosystem®)..

In *vitro* antibody-dependent cellular cytotoxicity and phagocytosis (ADCC/ADCP) evaluation

i) **Target cell generation for ADCC and ADCP.** Target cell generation (3T3-LASV GPC) was achieved by transducing 3T3 cells with MSCV vector based on pMIGII (a

generous gift of Dr. Jianke Zhang, Thomas Jefferson University) in which the LASV-GPC ORF was amplified by MP3 and MP4 primers (Supplementary Table 1) and added between the EcorI and XhoI restriction digest sites thus generating MSCV-GPC-IRES-GFP. Briefly, MSCV-GPC-IRES-GFP was co-transfected with a pCAGGS-VSV G with Xtreme-Gene 9 in a Gryphon packaging cell line (Allele Biotechnology) and infective retroviral virions were harvested 48 h post transfection. Next, low passage 3T3 murine cell line was transduced with viral supernatant and 8 µg/ml of polybrene and centrifuged at 800xg for 30 min. After 72 h, 3T3 cells were enriched by GFP expression through BD FACSAria II™. Confirmation of LASV GPC expression was done by immunofluorescence by using 50 µl/ml of 9E9 mAb and by FACS using 10 µg/ml of 4C8 mAb. Control target cell line (3T3-MARV) was generated through similar methods but with a Marburg virus GP (Angola strain) expressing MSCV (kind gift from Rohan Keshwara, Thomas Jefferson University).

ii) **Murine NK cell (effector cells) isolation and purification.** Mouse splenocytes obtained from naïve C57BL/6 mouse spleens were made in a single cell suspension through mechanical methods and strained through a 35 µm mesh. Then, the mouse NK Cell Isolation Kit II (MACS-Miltenyi Biotec) was used following the manufacturer's protocol. Purified murine NK cells were collected in RPMI (10% FBS, 50 mM βME, 5 IU/ml of mIL-2 (Biolegend), and 2 ng/ml of mIL-15 (Biolegend) and used immediately for ADCC at either 1:5, 1:10, or 1:20 target to effector cell ratio (see below). Remaining NK cells were stained with 1:200 dilutions of anti-CD3, NK1.1, CD335 (NKp46), CD32/16 markers (BioLegend, catalogue number: 100221; 108709; 137611; 101323), and by 1:1000 dilution of Zombie® UV viability dye (BioLegend) and characterized by flow cytometry (BD LSRFortessa) to confirm NK cell purity and Fcγ-Receptor III expression[29].

iii) **Macrophage effector cells.** IC-21 or J774A.1 macrophages were cultured as per above. At 24 h before an ADCC or ADCP assay, macrophages were scraped in a single cell suspension, centrifuged at 200 g and resuspended in sterile cell culture PBS. For ADCP assays the internal cellular dye CellTrace® Far Red (In*vitro*gen®) was

added following the manufacturer's recommendations. Macrophages were ressuspended in serum free cell culture media containing 5 ng/ml of mGM-CSF (cell signaling technology) and used in the following day for ADCC/ADCP assays and phenotypical analysis. To confirm macrophage phenotype and expression of all Fcγ-receptors[29], macrophages were stained with 1:200 dilution of F4/80, CD64, CD32/16, and CD16.2 fluorophore conjugated antibodies (BioLegend, catalogue numbers: 101323; 139303; 123115; 149513) and characterized by flow cytometry, (BD LSRFortessa).

iv) **ADCC/ADCP assays.** Either 1:100 of heat inactivated sera from immunized mice (see immunizations section), 40 µg/ml of purified IgG from the sera (see immunizations section) or 40 µg/ml of control mAbs (4C8, 9E9 and 5A3) were added to previously seeded $2x10^4$ 3T3-LASV GPC target cells or control target cells and incubated for 30 min at 37°C in 5% CO2. For Fcγ receptor blockade 100 µg/ml of either anti-CD64, CD32/16, or CD16.2 (BioLegend) was added to effector cells (see above) for 30 min. Next, effector cells were added to target cells at different effector to target cell ratios and incubated for 4 h. Target cells were then dissociated from the plate with Cellstripper® solution (Corning), washed, and resuspended in 200 µl of FACS buffer (5% FBS in PBS) with 30 µg/ml of propidium iodine (PI) viability dye. Cells were then immediately analysed by flow cytometry (BD LSRFortessa).

v) **ADCP confocal microscopy analysis.** For confocal analysis ADCP assay was conducted in the same conditions as described above but adapted for later microscopy analysis. Briefly, 3T3-LASV GPC target cells were seeded in glass cover-slips and incubated with the respective sera conditions, and then J774A.1 macrophages previously stained with 1:1000 CellTrace® Far Red (see above) were added at a 1:1 Target to effector cell ratio to allow easy visualization. After 4 h, coverslips were washed and mounted in slides with DAPI containing mounting

media (VECTASHIELD) and allowed to solidify O/N. Next, day samples were analyzed in a Nikon confocal microscope and further compiled through ImageJ software.

vi) **Gating strategy and ADCC and ADCP analysis.** All flow cytometry data was collected using the FACSDiva (BD) software. Laser voltage settings were adjusted for each analysis by running single color controls. For ADCC analysis, cells were first gated for size using the side scatter (SS) and forward scatter (FS) and selecting the 3T3 population (Supplementary Fig. 3). Next, using the histogram function GFP^+ cells were gated and based on this gate a total of 5000 GFP^+ events were captured. Due to size variability, ADCP analysis was performed by excluding PI^+ events and collecting a total of 10000 APC^+ events (macrophages). For data analysis FlowJo 10 (BD) software was used. The percentage of cytotoxicity (ADCC) was measured by the percentage of PI^+ cells of the total GFP^+ population after size gating. Since PI is a continuous dye in apoptotic cells[71], PI^+ histogram gating was based by defining a 10% PI^+ population gate on the control 3T3-LASV GPC cells (no effector cells and sera) as the background. ADCP percentage was measured by measuring the percentage of GFP^+/APC^+ of the total APC^+ population. After defining gating strategy on control cells all gating was applied uniformly to all samples.

Statistical Analysis

All statistical analysis was performed by using the Graphpad 7 (Prism). To determine the statistical test to be used the population was first analyzed to check whether it followed a normal distribution (Gaussian curve) by applying a D'Agostino-Pearson omnibus normality test. If so a parametric two-tailed T test was used for comparison within 2 groups. For grouped analysis, a one-way ANOVA or two-way ANOVA test was used and a post-Hoc analysis using either Sidak or Tukey Honest significant Difference Test with a 95% confidence interval to test significance within groups. Non-parametric tests were used if the population did not follow a normal distribution (indicated in the figure legends).

Data availability

All relevant data are available from the corresponding author upon reasonable request. Sequences of the LASV challenge virus, LASV GPC are available at GenBank as listed above.

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END NOTES

Acknowledgments

This work was supported in part by NIH grants R01 AI105204 to M.J.S, by the Jefferson Vaccine Center, by the Portuguese Foundation for Science and Technology fellowship PD/BD/105847/2014 and by the MD/PhD program from Escola de Medicina, Universidade do Minho (to T.A.-M.).

This work was also funded in part through the NIAID Division of Intramural Research and the NIAID Division of Clinical Research, Battelle Memorial Institute's prime contract with the U.S. National Institute of Allergy and Infectious Diseases (NIAID) under Contract No. HHSN272200700016I and an NIH grant in aid to R.S.B. (AI110700). K.R.H., J.K.B., and M.R.H. performed this work as employees of Battelle Memorial Institute. Subcontractors to Battelle Memorial Institute who performed this work are: SC., DT., E.P., and J.D., all employees of Tunnell Government Services, Inc.; M.L., an employee of Lovelace Respiratory Research Institute; and C.B., and P.S., employees of MedRelief, and D.L., an employee of Charles River Laboratories.

We thank Jennifer Wilson (Thomas Jefferson University, Philadelphia, PA) for critical reading and editing of the manuscript.

Authors distributions

T.A.-M. designed and performed experiments, analyzed data, and wrote the paper; K.R.H, K.C., G.T. and C.W. designed and performed experiments; R. F.J. and M.J.S designed experiments, analyzed data, and cowrote the paper. P.B.J. analyzed data and the edited paper.

Conflict of interest statement

T.A.-M, P.B.J., and M.J.S are inventors on the U.S. Provisional Patent Application No. 62/691,413 Title: NON-NEUTRALIZING ANTIBODIES ELICITED BY RECOMBINANT LASSA-RABIES VACCINE ARE CRITICAL FOR PROTECTION AGAINST LASSA FEVER

Chapter 2.2

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Supplementary data for: Non-neutralizing antibodies elicited by recombinant Lassa-Rabies vaccine are critical for protection against Lassa Fever

Nature Communications ***9****:4223 (2018)*

DOI: 10.1038/s41467-018-06741-w

Introduction

This chapter mostly covers the more technical, assay development part of chapter 2.1, as such for further theoretical background please see Chapter 1 and Chapter's 2.1 introduction section. Part of this work was published in Nature Communications 9:4223 (2018) DOI: 10.1038/s41467-018-06741-w. Further data produced during the development of these assays will also be presented but was not included in the publication since it was either produced after the publication or due to brevity being required. Additional data includes the LASV GPC specific T cell response (S8 figure) as well as a LASV GPC specific mouse mAb panel (S7 figure). Methods for this data are briefly described in the respective figure legend and the references are similar to the ones listed in chapter 2.1.

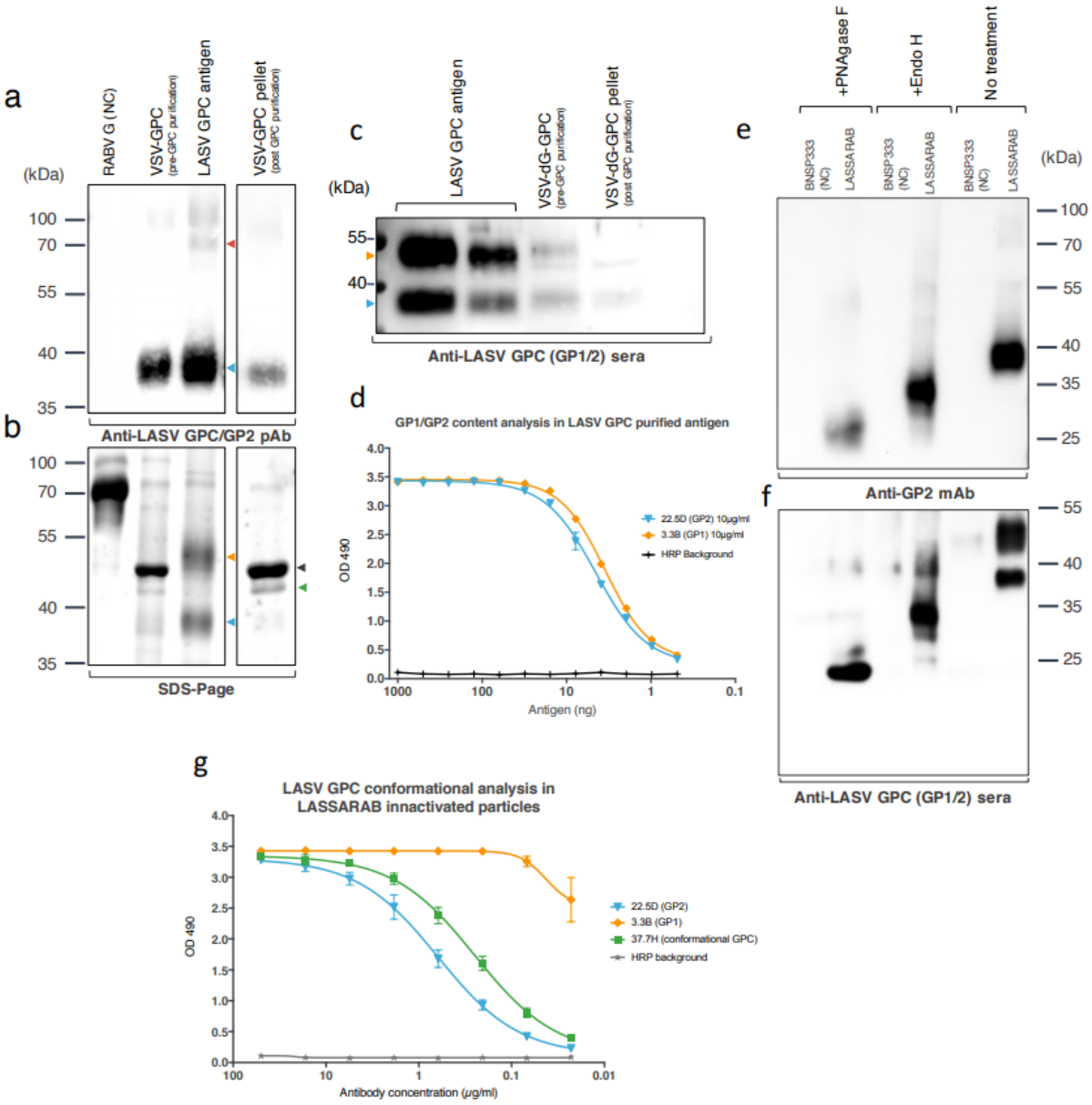
Results

Confirmation of incorporation of GPC in LASSARAB and purity of GPC antigen for ELISA (supplement to results in figure 2 and 4, chapter 2.1)

In supplemental figure 1 (a-d), the confirmation of GPC purification from rVSV-GPC is shown as evidenced by western blot analysis of both GP2 and GP1+GP2 in (a) and (c) respectively. The purity of LASV GPC antigen in comparison with the original rVSV-GPC is also confirmed by a SYPRO-Ruby stain of an SDS-PAGE gel in (b). Antigen quality control is finally assured by ELISA analysis of GP1 and GP2 relative content using 3.3B and 22.5D mAbs respectively. These mAbs recognized the linear epitopes of GP1 (in the case of 3.3B) and GP2, (in the case of 22.5D), and were recovered from LF human survivors by the Dr. Robert F. Garry lab[26].

Panels (e-g) are further confirmation of the incorporation of conformational LASV GPC and its correct glycosylation in LASSARAB particles. In panel (e,f) LASSARAB particles were digested with either Endo H or PNAgase F and probed for GP2 or GP1/GP2 respectively by western blot. The expected shift of GP2 with Endo H (from around 40 kDa to 30 kDa) and PNAgase F (to 23 kDa) confirms that the expected glycosylation is present in LASSARAB particles.

Moreover, in panel (g), an ELISA using LASSARAB as antigen shows that the GPC in LASSARAB particles is fully conformational since it's recognized by the 37.7H mAb which recognizes the quaternary structure of LASV GPC[26,42].



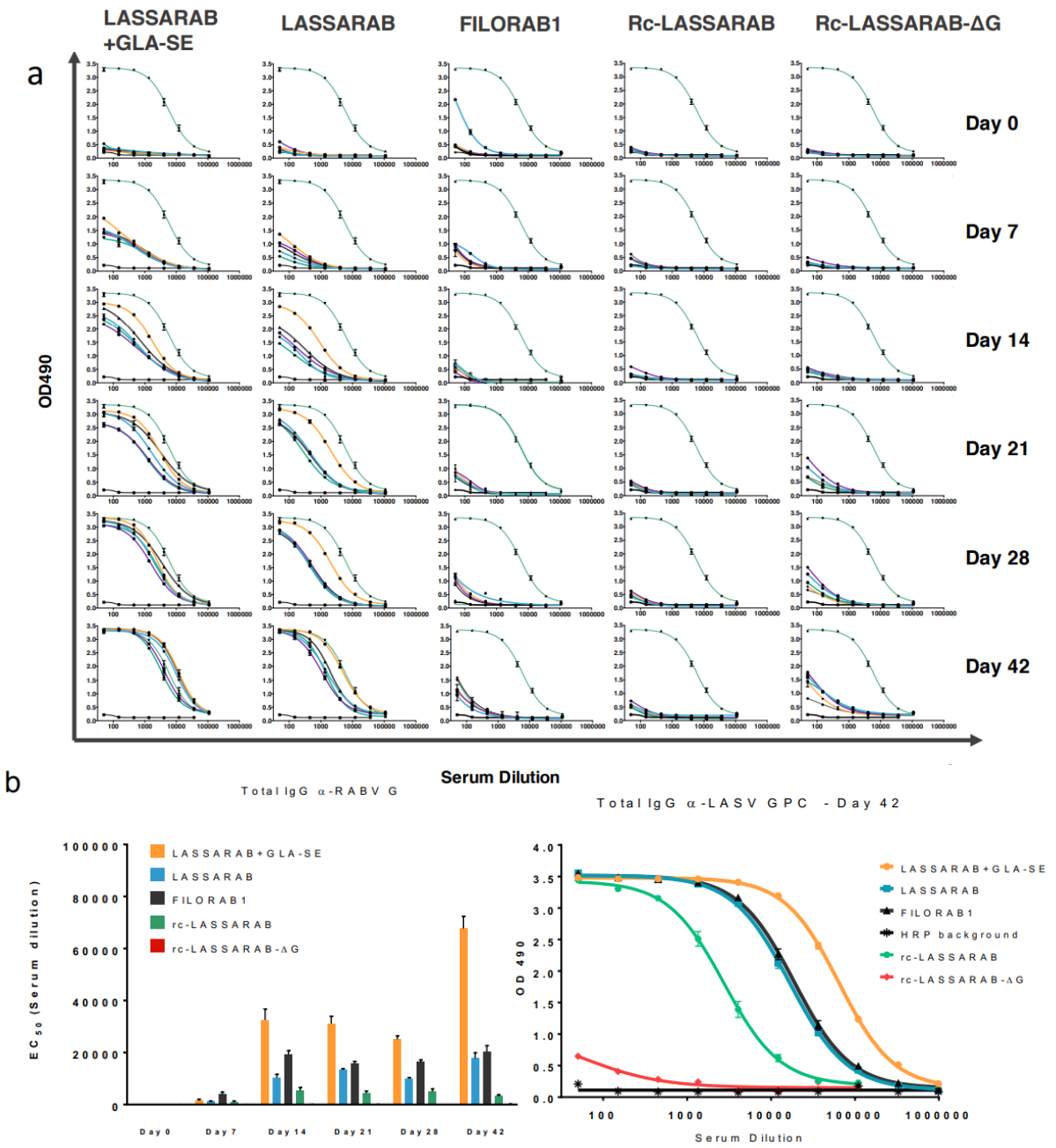
Supplementary Figure 1. Qualitative and quantitative analysis of LASV GPC content in both ELISA GPC antigen and LASSARAB inactivated virions. (a-d) Analysis of ELISA LASV GPC antigen. rVSV-GPC purified particles (before and after LASV GPC enrichment – lane 2 and 4 respectively) and LASV GPC antigen (lane 3) were analyzed by both western blot (a) and SDS-PAGE gel stained with SYPRO ruby (b). Westen blot on (c) was probed with anti-LASV sera of survivor guinea pigs to detect both LASV GP1 and GP2. 2 μ g of protein (measured by BCA) were loaded into each lane. Lane RABV G (a&b), purified RABV G antigen was loaded as a control. In lane VSV-GPC (a-c), 2 µg of sucrose cushion purified rVSV-GPC virions were loaded (pre-GPC purification). On (a-c), LASV GPC

antigen lane represents 1 of the different supernatant fractions of GPC purified antigen (lipid fraction of rVSV-GPC). In (a-c) lane VSV-GPC pellet the non-lipid fraction of rVSV-GPC was loaded to confirm LASV GPC depletion. Both LASV GP1 (45 kDa-orange triangle) and LASV GP2 (38 kDa-blue triangle) can be both observed on LASV GPC antigen lane on (b) and in both LASV GPC antigen and VSV-GPC lanes on (c). In addition to LASV GP2 in (a) a 75 kDa band corresponding to LASV GPC (red triangle) is detected. An enhancement of signal for LASV GP1 and GP2 is observed from VSV-GPC lane to LASV GPC antigen lane accompanied by the disappearance (b) of VSV N and P (black and green triangles respectively) confirming LASV GPC purification. (d) LASV GP1 and GP2 presence in the antigen was further confirmed by ELISA with antigen coated at different concentrations and probed with GP2 specific 22.5D and GP1 specific 3.3B44 .

In panels (e-g) LASSARAB incorporation of LASV GPC in inactivated particles was evaluated through western blot (e&f) and ELISA (g). Glycosylation pattern was also characterized through mobility shift assay by treating LASSARAB virions with both Endo H and PNAgase F (respective lanes). Both GP2 and GP1 have their respective reported sizes (38 kDa and 47-42 kDa respectively) in non-treated conditions (e&f – no treatment lane). Upon Endo H treatment GP2 migrates to around 32 kDa (e) and GP1 shifts to a band spanning from 45 kDa to 35 kDa (f), both consistent with previous reports thereby confirming a similar glycosylation pattern as previously reported. PNAgase F treatment further shifts both GP1 and GP2 to a lower molecular size between 25 to 20 kDa. Of note, LASSARAB treatment with Endo H and PNAgase F resulted in extensive GPC aggregation with a molecular size higher than 180 kDa (shown in uncropped western blot in SD) thus diminishing the signal in western blot analysis. In (g) inactivated LASSARAB virions were coated in ELISA plates and probed for LASV GP1 (3.3B), GP2 (22.5D) and GPC (37.7H – conformational quaternary GPC-B antibody) to further confirm LASSARAB's LASV GPC conformational integrity post BPL inactivation. Error bars are representative of the standard error mean (SEM) of 3 replicates.

Both replication competent and inactivated LASSARAB induce equivalent RABV G IgG titers to the FILORAB1 control groups (supplement to results in figure 4, chapter 2.1)

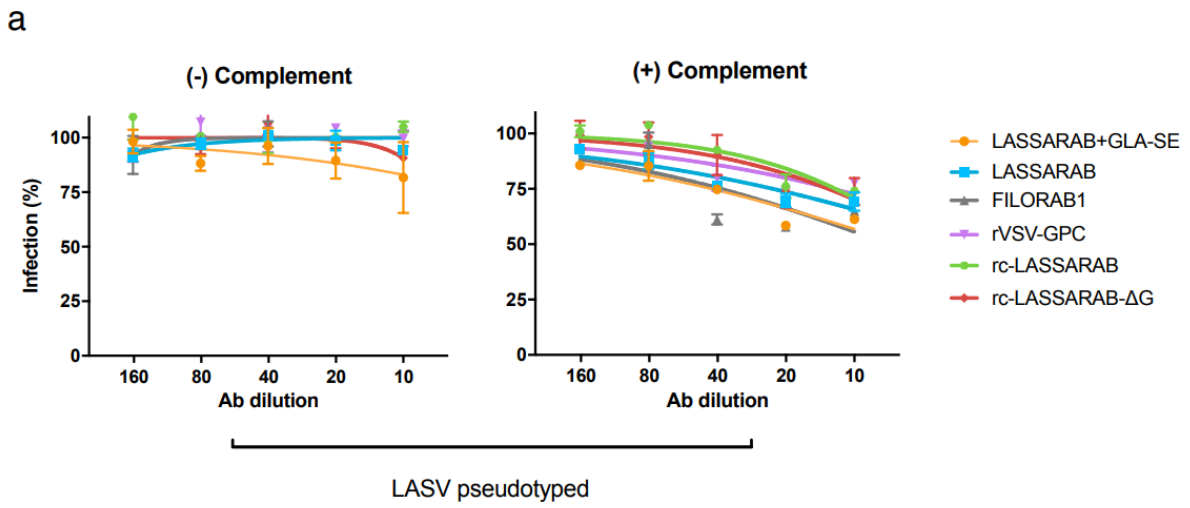
Rabies is an equally important pathogen in Africa for which vaccine coverage is lacking. As shown in supplemental figure 2 LASSARAB is confirmed to induce high titers of RABV G IgG in mice, equivalent to both inactivated and replication competent FILORAB1 which has been shown to be protective against Rabies. Furthermore, despite the fact that replication competent LASSARAB fails to induce LASV GPC IgG titers, it can induce RABV G IgG titers to similar amounts as replication competent FILORAB1 thus confirming that the virus has a significant level of immunogenicity.



Supplementary Figure 2. IgG analysis of LASV GPC and RABV G specific IgG of mice immunized with either Replication-competent LASSARAB or LASSARAB inactivated virions (see figure 4 for experiment outline). (a) Analysis overtime of LASV GPC specific IgG at day 0, 7, 14, 21, 28 and 42. Inactivated LASSARAB+GLA-SE and LASSARAB seroconverted to LASV GPC by day 14. Replication-competent LASSARAB-ΔG had a late seroconversion at day 21. FILORAB1 immunized mice were used as a negative control. (b) At the left the average of EC50 values for RABV G specific IgG titers is plotted over time. All groups, except the replication-competent LASSARAB-ΔG, seroconverted to RABV G by Day 7 and reached maximum titers by Day 14 that were maintained until day 28. On day 42 titers increased after a day 28 boost for inactivated vaccines (LASSARAB+GLA-SE, LASSARAB and FILORAB1 groups). On the right ELISA curves derived from serum dilution are plotted for the indicated groups. Each symbol is the average OD490 value of individual mouse sera of each respective group. Error bars are representative of the standard error mean (SEM) of 5 mice.

The addition of complement does not increase LASV neutralizing antibody potential in sera of LASSARAB immunized mice (supplement to results in figure 5, chapter 2.1)

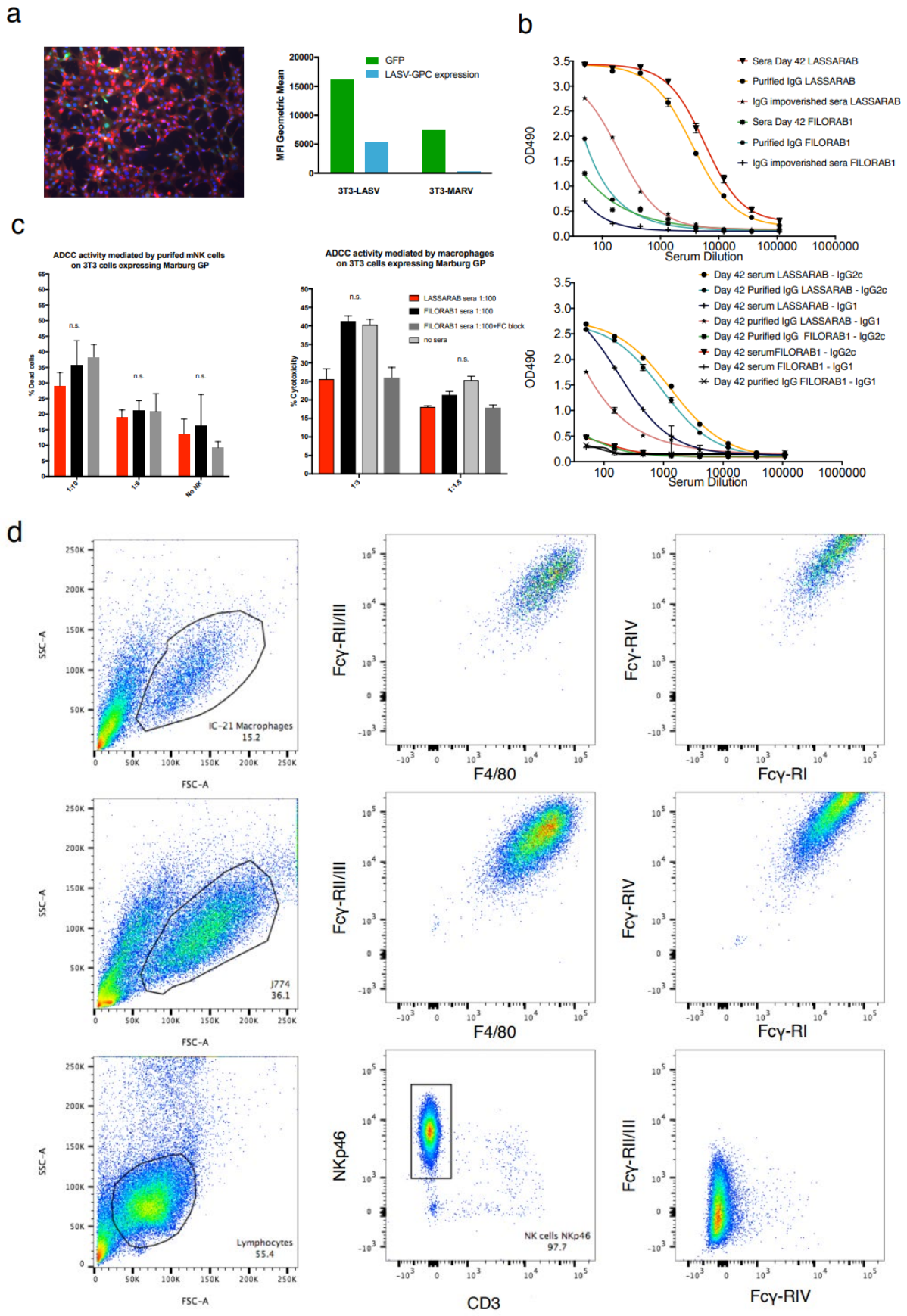
The addition of 10% complement in VNAs has been suggested as a method of increasing the sensitivity of virus neutralizing antibody titers. Since sera from LASSARAB immunized mice did not induce appreciable levels of LASV neutralizing antibody titers a variation of the pseudotyped VSV-NanoLuc assay using 10% complement was performed. As seen in figure S3, although the addition of complement increased the amount of neutralized virus in lower dilutions of sera it also increased background neutralization to similar levels thus maintaining a similar signal to noise ratio.



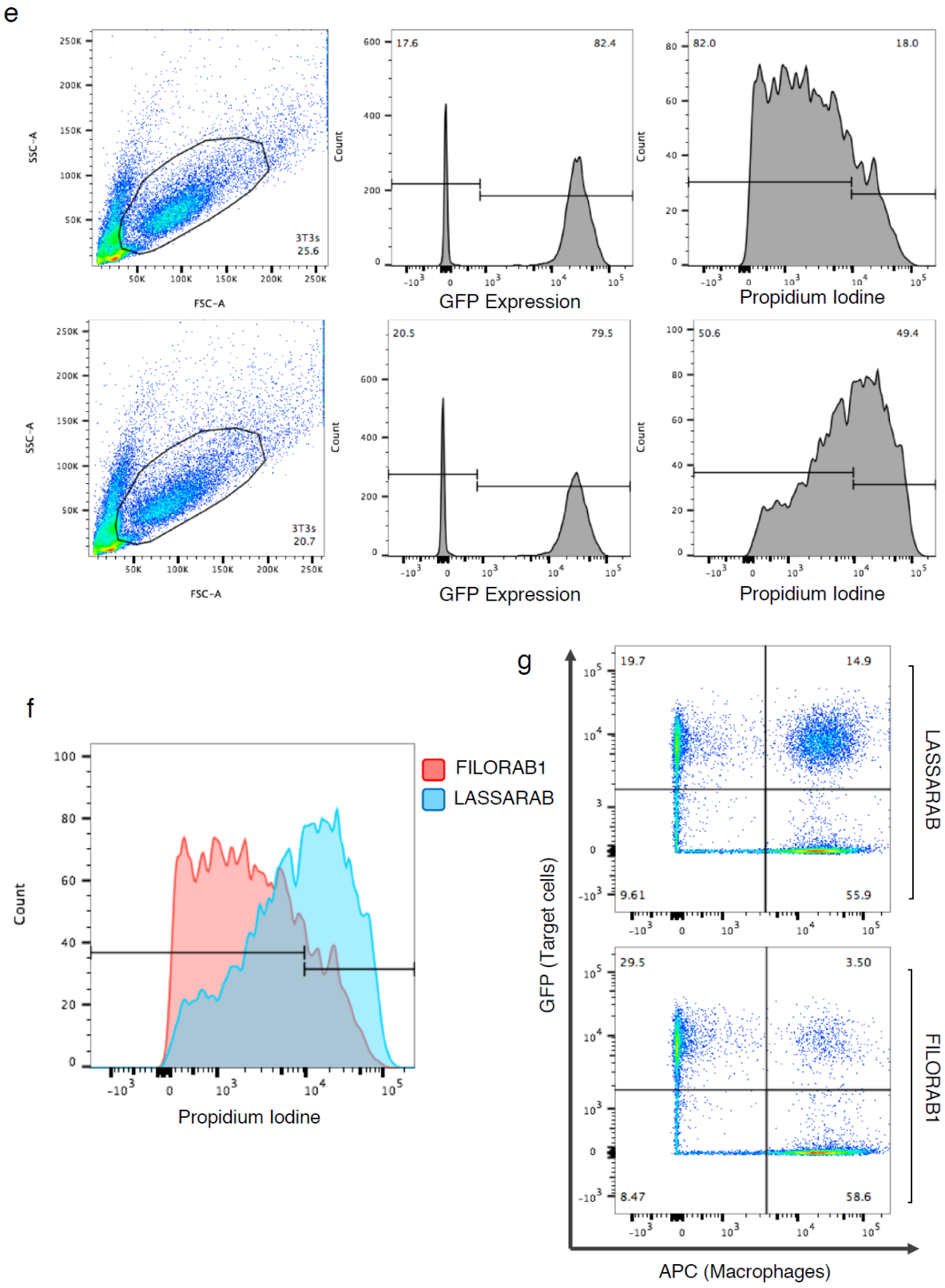
Supplementary Figure 3. Effect of addition of 10% complement on LASV neutralization in the pseudotyped LASV GPC ppVSV-NanoLuc assay. (a) Complement addition increased neutralization at lower antibody dilutions in all groups including FILORAB1 (negative control) group. Since the background neutralization was higher in the samples in which complement, was added complement addition was not further pursued in this pseudotyped virus neutralization assay. Error bars are representative of the standard error mean (SEM) of 5 mice.

Development of a flowcytometry based ADCC/ADCP assay for LASV IgG (supplement to results in figure 7, chapter 2.1)

To measure ADCC/ADCP activity potential in sera of LASSARAB immunized mice, a flowcytometry based ADCC assay was developed. Firstly, the target cell line was established using the immortalized NIH-3T3 mouse fibroblasts. These cells were then transduced with MSCV expressing LASV GPC and GFP and subsequently sorted for GFP signal. The resulting cells were then grown and confirmed to be expressing LASV GPC on their cellular surface by immunofluorescence and flowcytometry (figure S4, a). Simultaneously, B6 mice were immunized on day 0 and day 28 with LASSARAB+GLA-SE or control group and sera was collected on day 42. Then, IgG was subsequently purified through a G protein spin trap column and eluted in a similar volume of PBS. Both unpurified sera and purified IgG was confirmed to be have high titers against LASV GPC in ELISA (figure S4, b). In (c) it was confirmed that no ADCC background activity against Marburg virus GP expressing 3T3s (generated through similar methods) was being induced, in contrast with LASV GPC expressing 3T3s (see figure 7 in chapter 2.1). To further confirm the results of figure 7 the effector cells (both purified NK mouse cells and macrophages) were probed for the expression of FcγRI-IV and for population purity (figure S4, d). In the case of the IC-21 and J774 macrophages both cell lines were pure, as it can be confirmed by the uniform expression of F4/80 macrophage marker as expected. Both these cell lines also express the full gamut of FcγRI-IV as the flow charts indicate and as previously described in the literature. In the case of mouse NK cells, these were purified from splenocytes by negative selection through MACS (magnetic-activated cell sorting) and purity was confirmed to be high (97.7%) as observed by the expression of NKp46 (bottom panel of d). These murine NK cells were also confirmed to be solely expressing FγRIII as expected. For the generation of the graphs plotted in figure 7 chapter 2.1, panels e and f detail the gating strategy used to define cytotoxicity levels in the 3T3-LASV target cells. In panel g, the gating strategy used for ADCP percentage shown in figure 7 chapter 2.1 is likewise plotted.



Supplementary Figure 4. *(continued in next page)*

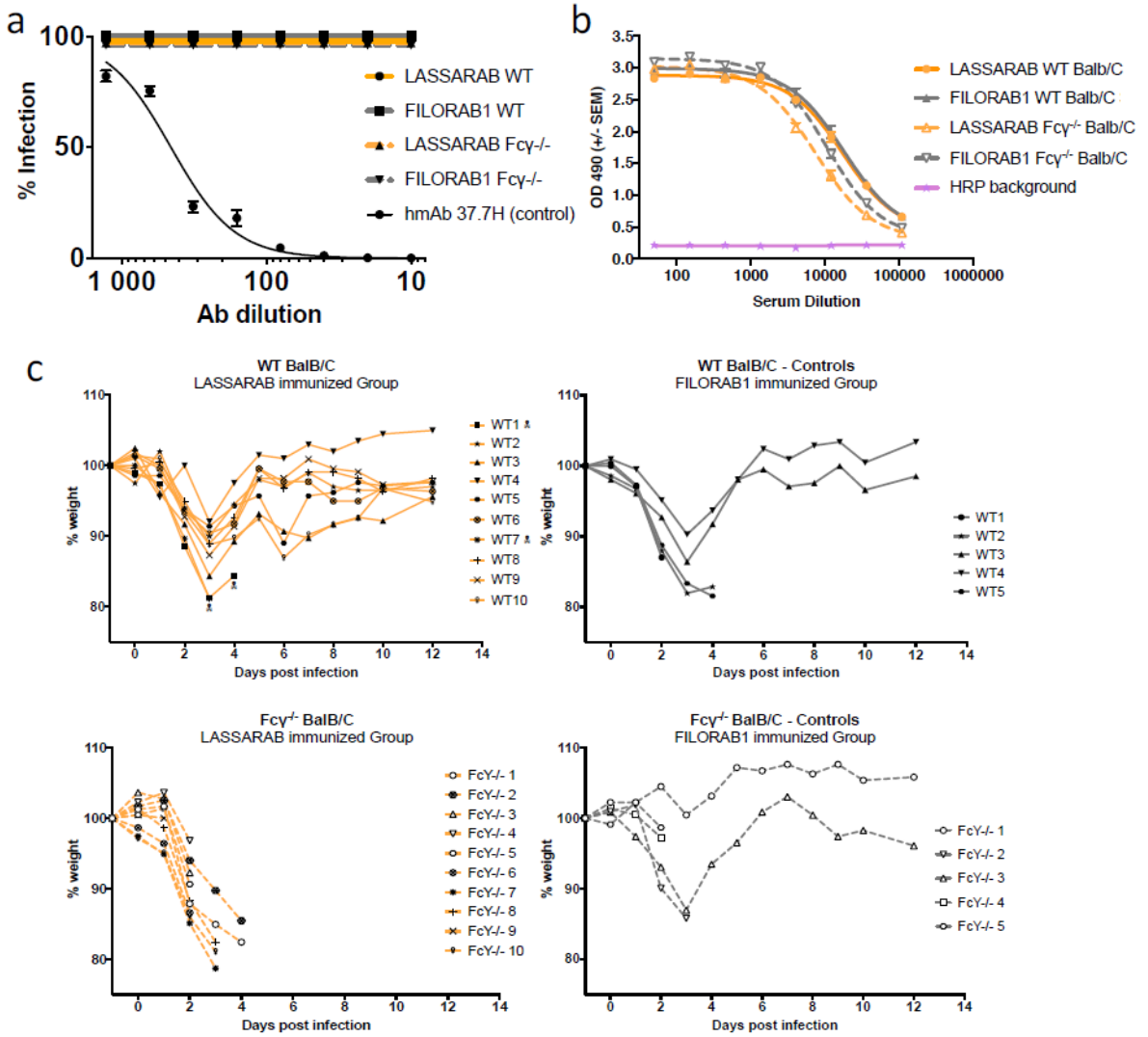


Supplementary Figure 4.

Supplementary Figure 4. ADCC and ADCP assay development (Figure 7). (a) LASV GPC expression in 3T3-LASV target cells was confirmed by IF (left) and Flow cytometry (right). The geometric mean of medium fluorescence intensity (MFI) was plotted in the Y axis. 3T3- MARV were used as a negative control for LASV GPC expression. (b) Day 42 total sera, IgG purified (in PBS buffer) and IgG impoverished sera from B57BL/6 mice immunized with 10 µg of inactivated LASSARAB+GLA-SE and FILORAB1+GLA-SE on day 0 and day 28 was assayed for anti-LASV GPC IgG titers. On the left graph ELISA curves are shown for total GPC specific IgG titers. On the left GPC specific IgG2c and IgG1 titers are shown. (c) ADCC and ADCP activity mediated by mNK cells and macrophages respectively against 3T3 cells expressing Marburg virus glycoprotein. No significant difference was found. (d) Characterization of macrophage and murine NK effector cells used for ADCC and ADCP assays. Top 2 rows of flow plots show gating strategy for the characterization of IC-21 and J774A.1 macrophage effector cells. Both macrophage cell lines have F4/80+ staining and high expression of all Fcγ-R as expected for macrophages. Bottom row shows gating strategy for murine NK cell characterization. Murine NK cells were purified from C57BL/6 mouse splenocytes using a murine NK cell isolation kit (Miltenyi Biotec). After purification murine NK cells (NKp46+/CD3- population) comprise 97.7% of effector cells used in the assays thus excluding potential ADCC by other effector cells (Figure 7a&b). (e) ADCC percentage gating strategy on 3T3-LASV target cells (Figure 7a, b and d). Percentage of cytotoxicity was calculated from the percentage of GFP+ (3T3-LASV) cells and Propidium iodine+ (PI). Top row is a representative flow plot of FILORAB1 sera incubated condition (control). Bottom row is a representative flow plot of LASSARAB sera incubated condition (control). (f) Overlapping PI histograms of 3T3-LASV cells incubated with either LASSARAB sera (blue) or FILORAB1 incubated sera (red) showing cytotoxicity differential. (g) ADCP flow cytometry-based analysis (Figure 7c). After dead cell exclusion by viability dye, top right quadrant represents the percentage of ADCP mediated by macrophages by analyzing the percentage of GFP+ (target cells) and APC+ (Macrophages) and was plotted in (Figure 7c). In (g), top graph is a representative plot of 3T3-LASV cells incubated with LASSARAB sera, and bottom graph is a representative plot of 3T3-LASV cells incubated with FILORAB1 sera. Error bars are representative of the standard error mean (SEM) of at least 3 independent replicate experiments.

Fcγ KO mice and WT mice have similar levels of RABV G IgG titers (supplement to results in figure 8, chapter 2.1)

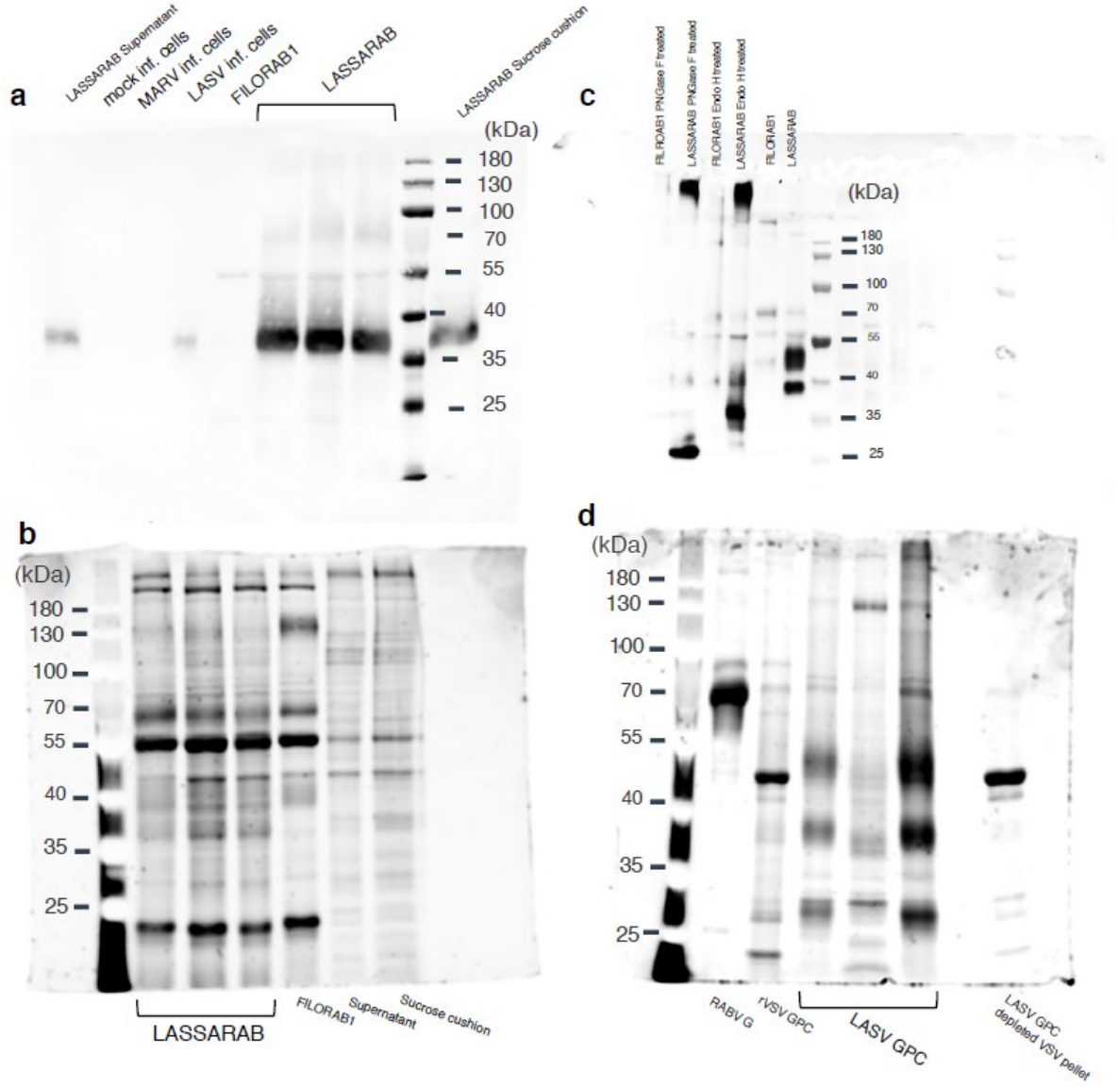
Since Fcγ KO mice could have a potentially different response to vaccination RABV G titers were also checked across all groups (both FILORAB1 and LASSARAB) immunized mice. As panel b shows, all mice have similar levels of RABV G IgG independently of Fcγ, albeit slightly lower levels in the Fcγ KO mice as equally observed for LASV IgG titers. None of LASSARAB mice had LASV virus neutralizing antibody titers before challenge as well (panel a). Moreover, all mice were infected post VSV-LASV exposure as evident by the uniform weight loss across all groups, and while WT LASSARAB immunized mice mostly recovered after challenge, Fcγ KO mice immunized with LASSARAB all succumbed to infection (panel c).



Supplementary Figure 5. In vivo importance of Fcγ-R functions for protection against surrogate LASV exposure (Main Figure 8). BALB/c or BALB/c Fcγ-/- mice were immunized with 10 µg of inactivated LASSARAB+GLA-SE and FILORAB1+GLA-SE on day 0 and day 28. (a&b) Pre-exposure (Day 35 post-immunization) analysis of neutralizing antibodies (NAbs) against LASV pseudotypes and anti-RABV G IgG titers. (a) no LASV NAbs were detected in any group prior to challenge thus excluding prior NAb protection. (b) The degree of response to RABV G is similar between all mice in all groups indicating that the lack of Fcγ does not compromise humoral response to vaccination as seen for LASV GPC antigen in the LASSARAB groups. (c) Mouse weight was recorded post surrogate LASV exposure. WT LASSARAB mice mostly resisted infection, with 2 mice dying without showing severe signs of disease. Fcγ-/- mice immunized with LASSARAB all succumbed to infection, with some showing severe clinical signs before weight endpoint criteria was reached. Control mice from both WT and Fcγ-/- groups all showed mild to severe signs of infection except for the FcY-/- 1 mouse (open circle dashed grey line), and 40% managed to recover from disease. All error bars are the standard error mean (SEM) of a total 20 mice for LASSARAB groups and 10 mice for FILORAB1 groups.

Uncropped western blots and SDS-PAGE gels pictures used for figure 2 and supplemental figure 1

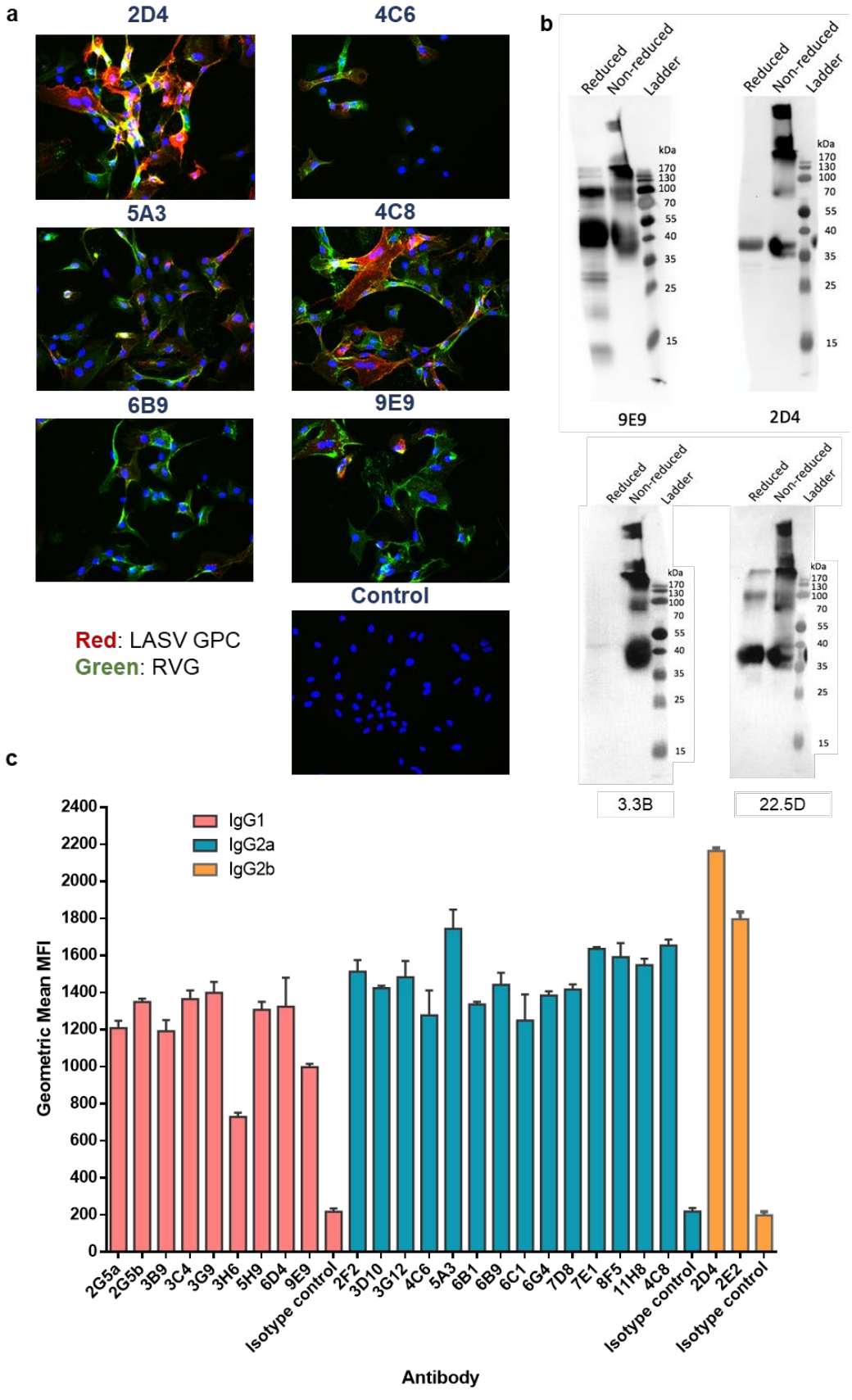
For concerns of data transparency, all cropped western blot and SDS-PAGE pictures used in this thesis are hereby displayed fully uncropped including the molecular ladder.



Supplementary Figure 6. Uncropped gels used to prepare Figure 2 and Supplementary Figure 1. a) Uncropped figure 2, panel e. b) Uncropped figure 2, panel d. c) Uncropped figure 2, panel f, and Supplementary Figure 1, panel f. d) Uncropped figure used in Supplementary Figure 1, panel b.

Development of a panel of LASV mAbs for assay development

Upon the start of my PhD, the number of LASV GPC mAbs previously described was limited. Moreover, none were commercially available. This compelled us to establish a collaboration with Dr. Gene Tan for the development of a panel of LASV GPC specific mAbs. These mAbs were developed by DNA vaccination of mice using pCAGGS plasmid cassette containing the ORF of the codon optimized LASV GPC. These mice were later boosted with purified GPC antigen. After hybridization, a total of 25 mAbs were isolated. They were all confirmed to be specific for LASV GPC (supplemental figure 8) with some being GP1 or GP2 directed as confirmed by western blot. Most these mAbs recognized conformational GPC however as evidenced by the lack of western blot reactivity, in contrast with immunofluorescence and flowcytometry. None of these mAbs were able to neutralize LASV GPC in vitro (data not shown). A pilot assay for probing for ADCC activity also failed to identify potential candidates for further study. Alongside the fact that an excellent work by the Garry lab was published using human LASV GPC specific mAbs isolated from LF survivors, no further studies employing these mAbs were pursued[26]. Of notice, 9E9 (GP1 specific), 2D4 (GP2 specific) and 4C8 (conformational LASV mAb) are going to be used for LASSARAB quality control production assays for further GLP/GMP certification.

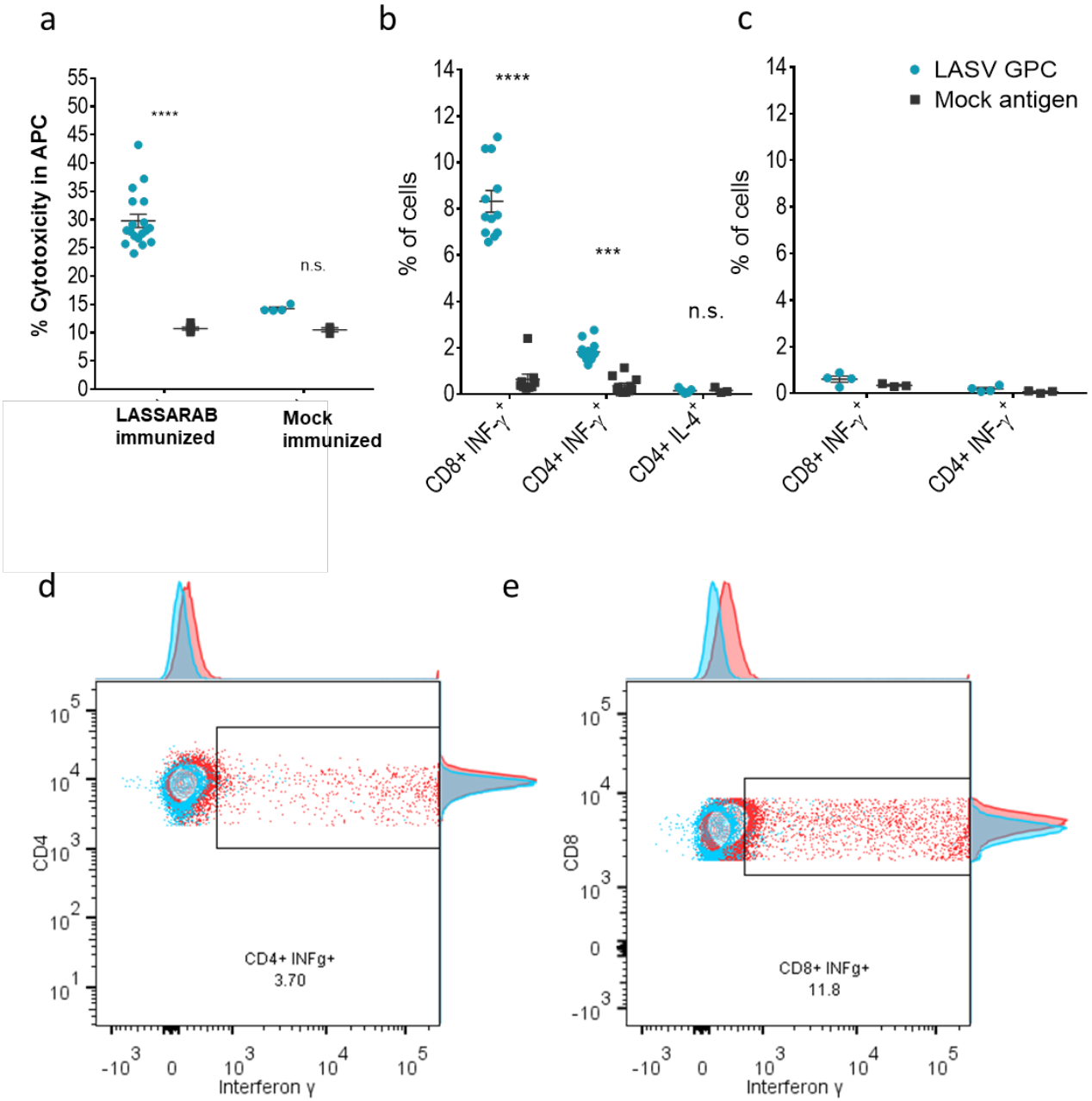


Supplementary Figure 7. Characterization of the mouse GPC monoclonal antibodies. (a) representative immunofluorescence pictures of six LASV specific mAbs. A previously seeded monolayer of VERO cells on

coverslips was infected with LASSARAB at an MOI of 0.1 and virus was left to grow for 48h. Thereafter coverslips were stained with the respective LASV specific antibodies (red) and 4C12 RABV G specific human mAb (green). (b) from the panel of mAb existing, only 2D4 and 9E9 reacted in western blot. As comparison with the previously published human mAbs (below), 2D4 reacts with a band size consistent with GP2 while 9E9 reacts with a band size consistent with GP1. (c) previously infected VERO cells with LASSARAB were detached from the plate with cellstripper®, incubated with the respective mAbs and isotype control and thereafter stained with cy3 conjugated secondary mAb. Samples were then analyzed by flowcytometry.

Development of a LASV GPC specific T cell assay for evaluation of LASV cellular responses

The focus of this work was to establish the role of antibody mediated protection against LF disease. However, as described in chapter 1, cellular LASV immune response has often been hailed as the most relevant mechanism of protection against LF. Although this thesis focused in dissecting a role for an antibody based immune protection against LF, one could not disregard the vital role that cellular response equally plays. This compelled me to establish a LASV GPC specific T cell assay (Supplemental figure 8). Since LASV GPC peptide is not available, and MHCII/MHCI LASV GPC restricted epitopes are yet to be fully identified, an alternative approach was used by using JAWSII mouse dendritic cells. These cells were transduced with MSCV-GPC (similar to 3T3s, see above) and sorted for GFP. LASV GPC expression was confirmed by immunofluorescence (not shown). These cells, by expressing LASV GPC, should also be presenting LASV GPC peptides by MHCI and MHCII and thus have a potential to activate LASV specific CD4+ and CD8+. Thus, isolated splenocytes from previously LASSARAB immunized mice (and controls) were incubated with these JAWS-LASV cells for 18 hours. The percentage of LASV specific T cells was then defined by the percentage of either INF-γ expressing CD4+ or CD8+ cells over the total amount of their respective populations (panel b, supplemental figure 8). In LASSARAB immunized mice, there was a higher percentage of both CD4+ and CD8+ T cells. Moreover, a higher CTL activity was equally detected in LASV GPC expressing JAWSII cells compared to mock antigen when incubated with splenocytes LASSARAB immunized mice (panel a). This assay can be easily adapted towards monkey and human studies and can be a valuable tool for further development of the LASSARAB vaccine and LF study in general.



Supplementary Figure 8. Cellular immunity induced by three immunizations with LASSARAB+GLA-SE after 60 days post last boost. Splenocytes were isolated from mice previously immunized with either LASSARAB or FILORAB1 (mock immunized condition), 60 days after the last boost of either the vaccines. and homogenized to a single cell suspension filtered through a 70 µm nylon cell strainer and counted. Next $1x10^6$ splenocytes were transferred to a u-bottom 96 well plates with $1x10^4$ of previously seeded "feeder cells". These feeder cells consist of JAWSII dendritic cells which express either LASV GPC (blue) or mock antigen (empty vector GFP) (black). The splenocytes and JAWS were then co-cultured for 16 hours in splenocyte media. Thereafter Golgiplug® or Gogistop® was added to each condition (1:1000) and left incubating for 4 hours more. Media was then removed, and cells were then detached with cellstripper®, stained for viability with zombie® UV, and surface stained to CD4, CD8, CD62L, CD3 and CD11b (BioLegend®). Cells were then fixed and permeabilized with

Cytofix/cytoperm (BD®) and intracellular stained for INF-γ and IL-4. Samples were then analysed by flowcytometry and analysed through flowjo X (BD®) and statistics and graphs were elaborated in prism® 7 software. The 2-way ANOVA statistical test with Tukey HSD post-hoc analysis was performed to find statistical significance between samples.

(a) Cytotoxic T lymphocyte activity was measured through the percentage of dead JAWSII (zombie+/GFP+/CD11b+ events). Gating strategy was similar to the one employed in Figure 7 in Chapter 2.1. Splenocytes from LASSARAB immunized mice induced more cytotoxicity in LASV GPC expressing JAWSII (blue dots) than in mock antigen expressing JAWSII (black squares). When splenocytes from mock immunized mice were used, no difference was detected between LASV expressing JAWS or mock (GFP only) expressing JAWS.

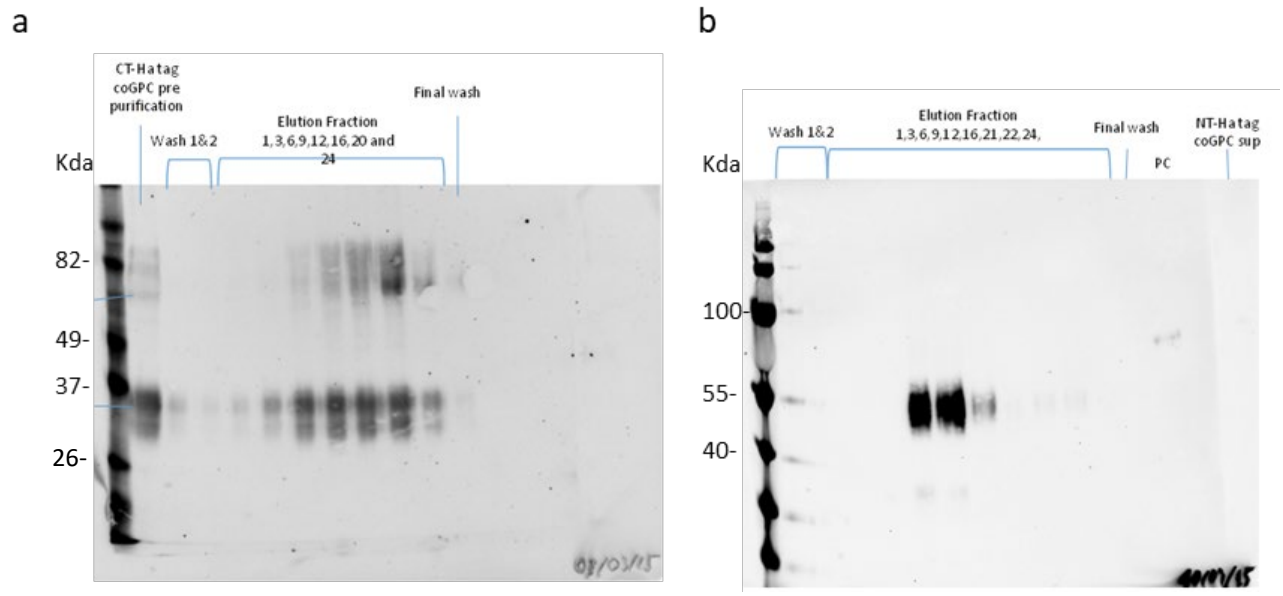
(b) Splenocytes from mice immunized with LASSARAB+GLA-SE. Analysis of the percentage of T cells (either $CD8^+CD3^+$ or $CD4^+CD3^+$) producing interferon-γ in response to LASV GPC antigen in comparison with mock. LASV GPC expressing JAWSII induced a higher T cell response both cytotoxitic T cells (CD8+) and T helper cells (CD4+) than mock antigen. Percentage gating is shown in (d) and (e).

(c) Splenocytes from control mice immunized with FILORAB1. Analysis of the percentage of T cells (either $CD8^+CD3^+$ or $CD4^+CD3^+$) producing interferon-γ in response to LASV GPC antigen in comparison with mock. No difference in response was observed between LASV expressing JAWS or mock JAWS

(d) & (e) Overlapping populations of either CD4+INF-γ+ cells (d) or CD8+INF-γ+ cells (e). Red dots and histogram represent the splenocytes that were cultured with LASV GPC expressing JAWSII. Blue dots represent splenocytes that were cultured with mock antigen expressing JAWSII. Splenocyte populations here depicted are derived from LASSARAB+GLA-SE immunized mice.

Initial development of LASV GPC ELISA antigen

For the development of a LASV GPC antigen for ELISA, the initial strategy was by engineering a LASV GPC deleted of its transmembrane domain with an HA tag either in the CT or in the NT. Purification of this antigen was then achieved through anti-HA column (see supplemental figure 9). Although great purity was obtained the subsequent results in test ELISAS revealed that depending whether CT or NT purified LASV GPC was used, different results were obtained with the same polyclonal sera. The inconsistency of these results inferred that the GPC being obtained was not conformationally stable and thus compelled us to develop an alternative method of GPC purification based on stripping the bilipid layer of VSV-GPC virions (see supplemental figure 1 above).



Supplementary Figure 9. N terminus or C terminus HA tagged LASV GPC antigen. (a) CT HA tagged GPC pre and post purification through an anti-HA column. Western blot was probed with an anti-HA antibody, and, as it can be observed, several band sizes are present, with the lower bands having between 38-34 kDa (corresponding to GP 2 monomers) while the higher bands are between 90-70 kDa (corresponding to dimer or trimeric forms of GP2/GP1 or GP2 only). The Different band sizes in the same size range might represent differential glycosylation patterns. (b) NT HA tagged GPC pre and post purification through an anti-HA column. Western blot was probed with an anti-HA antibody. In contrast with CT GPC, NT GPC can be detected in a band smear from 42 kDa to 56 kDa. This is consistent with the size of GP1 and with its heavy glycosylation pattern.

Table of primers used in the construction of the virus vectors used

This table contains the sequence of the primers used for the construction of the vectors used for the most important works and experiments of this thesis. More primers were used and developed, and their sequence can be given upon request.

Construct	Forward Primer	Sequence 5' to 3'	Reverse Primer	Sequence 5' to 3'
LASSARAB	RP951	GGAGGTCGACTAAAGAGATCTC ACATAC	RP952	TTCTTCAGCCATCTCAAGATCGGC CAGAC
rVSV-GPC	RLP3	CGATCTGTTTACGCGTGCCGCC ACCATGGGACAG	RLP4	GAAGAATCTGGCTAGCTCAGCGTT TCCACTTGACT
rVSV-NL-EGFP	VP11	AGATATCACGCTCGAATGGTGA GCAAGGGCGAGG	VP12	GAAGAATCTGGCTAGTTACTTGTAC AGCTCGTCCATGCC
rVSV-NL-EGFP	VP9	CGATCTGTTTACGCGATGGTCTT CACACTCGAAGATTTCG	VP10	GGATTTGAGGCGGCCTTACGCCAG AATGCGTTCGC
MSCV-GPC-GFP	MP3	CCGGAATTAGATCTCTCGAGATG GGACAGATCGTGA	MP4	AGTGGAAACGCTGAGCTAGCGAAT TCCGCCCCCCCC
rVSV-GPC	VP5	GCGTGGGTCCTGGATTCTAT	VP6	ATCGAGGGAATCGGAAGAGAAT

Supplementary Table 1. Primers used for constructing and sequencing the plasmid vectors used.

Chapter 3

Discussion, conclusions, and future works

The threat that VHFs and other zoonoses pose to public health is becoming more apparent as globalization and further climate change take place[1–3]. Indeed, since the beginning of my medical training, in 2009, previously overlooked viruses such as Ebola and Zika rose to international prominence and became common lexicon in countless languages. LF has been a recognizable public health and social burden in West African countries since 1970s, as one can infer from both academic and popular literature[4,5]. However, only recently it has captured increased funding and stirred interest, partly because of the 2013-2016 west African Ebola epidemic. In Nigeria alone, since the start of 2019, there have been 537 confirmed cases of LF, 122 of which were lethal[6]. Furthermore, Nigeria has extensive areas where case reporting and clinical treatment are scarcely covered due to either strife or sheer remoteness[7], a problem common to other western African countries as well. Thus, LF cases are likely to be underreported. As previously introduced in Chapter 1, LASV is the causative agent of LF. The ubiquitous presence of LASV in west Africa coupled with the fact that LASV's reservoir is one of the most prevalent mammal families (Muridae) on the planet makes LF an important global concern[8–10].

International agencies such as the World Health Organization (WHO) and the newly formed public-private Coalition for Epidemic Preparedness Innovations (CEPI) have categorized Lassa as a priority agent for vaccine development[11]. CEPI calculated that the total cost of vaccine development for 11 different lesser known zoonoses (including LASV) is a fraction of the projected economic burden if a single one of them became global[12]. Since its inception, CEPI has made LASV its highest priority agent and recently awarded $36 million to fast track LF vaccine development. Meanwhile, the WHO has published an R&D Blueprint for Action to Prevent Epidemics for LF vaccine that states the preferred LF vaccine requirements[13]. These include: (1) a highly favorable risk-benefit profile suitable for all age groups, (2) practicality for non-emergency/preventive scenarios, (3) at least 90% efficacy in preventing disease, (4) high thermostability, and (5) the possibility of co-administration with other vaccines. LASSARAB was designed with these requirements in mind and fulfils most of them. Moreover, as an inactivated LF vaccine, LASSARAB can potentially be used in pregnant women and immunosuppressed patients, both of which are major risk groups for LF[14,15]. In addition to LF, LASSARAB also confers protection against Rabies, another neglected zoonosis in western Africa[16,17].

Vaccination campaigns in developing countries located in tropical areas have the added logistical struggle of maintaining a cold chain supply. A vaccine with a high degree of thermostability could forgo the need for a cold chain, thus bypassing this logistical deterrent. Unpublished results from our lab show that lyophilized FILORAB1, an Ebola vaccine based on the same RABV platform, has a high degree of thermostability (up to 56°C for 2 weeks or 37°C for 6 months) while still maintaining efficacy against RABV challenge (JID, submitted). If equivalent results are observed with lyophilized LASSARAB, this thermostability could diminish or remove altogether the dependence on a cold chain. Finally, LASSARAB appears to have minimal adverse interactions when co-administration with other vaccines as preliminary results in an ongoing NHP study show.

3.1 Rabies expression and incorporation of Lassa virus glycoprotein

Arenaviridae, including LASV, are relatively simple viruses composed of four genes only (see chapter 1.2)[18]. Of these four genes, the NP is the most abundantly translated protein. NP is fundamental for viral RNA transcription, replication, and packing while simultaneously serving as a type I INF signaling antagonist[19–21]. Thus, mutations that impair any of these functions are highly detrimental or even fatal for viral replication. This makes NP a highly conserved protein amongst LASV strains. Its abundance, critical role, and genetic homogeneity makes it an attractive immunogen for a potential LASV vaccine. Indeed, NP is also a highly immunogenic protein, since sera obtained from LASV infected humans and other animals contain high titers of antibodies against LASV NP. Additionally, several predicted immunodominant MHC class I epitopes have been identified for LASV NP and hence suggest usefulness in an epitope-based LF vaccine[22]. Nevertheless, there has been little success in NP-based LF vaccines despite NP's high immunogenicity[23–25]. Of note, since NP is an internal protein, vaccine efficacy would presumably require a cellular immune response.

Of the remaining proteins, LASV's GPC appears to be the most attractive candidate for an LF vaccine based on the rabies platform[26]. By binding to α-Dystroglycan and other non-canonical receptors, GPC enables LASV to fuse with a target host cell membrane, enter the cell, and subsequently replicate. The specificity with which GPC binds to its receptor is also the

determining factor for LASV's tropism and consequential pathogenesis. Moreover, GPC's crucial location on the membrane exposes it to antibody binding on both the virion surface and cellular surface (of infected cells). GPC's appeal as the basis for a LF vaccine is further reinforced by experimental data: promising experimental LF vaccine candidates use GPC as its immunogen[27]. This formed the rationale for designing the GPC-expressing rabies LF vaccine candidates: LASSARAB and LASSARAB-ΔG. The GPC gene from LASV Josiah strain was chosen given its extensive use in LASV research[27]. It was codon-optimized (co) for expression in human cells as previous studies by Kurup et al, reported that codon-optimization increased expression and incorporation of foreign glycoproteins in rabies virions[28]. To create the vaccine cDNA, the open reading frame (ORF) of coGPC was cloned between the N and P genes of the BNSP333 rabies vector flanked by a RABV start-stop signal sequence (seven thymines in tandem in the positive sense) and recovered using reverse genetics[29].

Cells infected with LASSARAB expressed LASV GPC comparably to rVSV-GPC, as observed in figure 2 in chapter 2.1. Curiously, upon close observation, single cells do not always express Rabies virus glycoprotein (RABV G) and LASV GPC simultaneously. This might be due to different transport speed of each glycoprotein, thus leading to a temporal component to membrane enrichment of either slower glycoprotein. A potential concern is the loss of GPC by LASSARAB since this protein is redundant and therefore not essential for viral spread. However, such loss of GPC at the genomic level was never observed. On the contrary, LASSARAB virions collected from later harvests had a higher percentage of GPC than RVG (data not shown). Overall, LASV GPC does not appear to be detrimental for rabies growth and virion production. Remarkably, LASSARAB-ΔG, an alternative vector based on LASSARAB but lacking the RABV G, replicates exclusively through LASV GPC thus corroborating such. LASSARAB-ΔG also grew to higher titers than either LASSARAB or FILORAB1. This might be due to the smaller (hence faster) genome as well as the fact that it only has a single glycoprotein thus diminishing the chances for glycoprotein fusion interference. Nevertheless, to fully confirm GPC genetic stability in future experiments, GPC gene deep sequencing could be performed on several LASSARAB passages.

The efficacy of LASSARAB-ΔG's replication demonstrates that RABV can express and depend on functional LASV GPC. This complements results shown in the western blot of figure 2 (chapter 2) where LASV GPC (both GP1 and GP2 subunits) was shown to be incorporated into

LASSARAB virions. Moreover, the LASV GPC incorporated into BPL-inactivated LASSARAB virions maintains its conformation, given that the LASV mAb 37.7H (figure S1, Chapter 2.2) can bind to it, as shown in ELISA. The 37.7H mAb was shown in crystallization studies to be reactive to GP1 and GP2 subunits across different GP dimers in conformational GPC[30,31]. Overall, LASSARAB not only efficiently expresses LASV GPC but also incorporates it in budding virions, thus enabling its use as an inactivated vaccine.

However to ensure that the quantity and quality of GPC content in LASSARAB remains equal in lot-to-lot production a more sophisticated quality control (QC) assay is required[32]. Recently, a myriad of human LASV GPC mAbs were characterized and are currently available[30]. Moreover, an additional 26 mouse LASV mAbs were developed during the PhD (see figure S7 in chapter 2.2). From these, one or two mAbs can be selected, based on their epitope (ideally a conformational epitope that requires GP1 and GP2), for the development of an ELISA-based QC for LASSARAB. The readout of such ELISA can then be converted to (GPC) ELISA units/ml (with the use of a GPC antigen standard) and will provide a more robust lot to lot QC evaluation. This can later be correlated with the minimum effective dosage required for a LF protective immunity to be induced.

3.2 Characterization of the immune response to several Lassa fever vaccine candidates in a murine model

The immune correlates of protection in LF are still poorly defined[33]. Currently, the majority of approved vaccines have settled on sera antibody titers as correlates, either total IgG (determined by ELISA) or viral neutralizing titers (determined by VNA)[34]. Alternatively, vaccine correlates of protection can measure cellular immunity, by assessing either interferon production by T cells or T cell proliferation in response to the desired antigen.

As introduced in chapter 1.5, the most efficacious protective response to LASV has long been considered to occur through cellular immunity[35]. The rationale behind cellular immunity was based on several findings: 1) NHP studies using the experimental LF vaccine LASV-Vaccinia found that higher IgG titers were inversely correlated with survival[36]; 2) Human sera transfer

studies from LASV-immune individuals did not significantly protected against disease[37]; 3) some of the most effective experimental LF vaccines induced low LASV specific IgG titers while inducing strong T cell responses[27]; 4) in other *Arenaviridae* (e.g. LCMV), cellular immunity in mice has been associated with efficient viral clearance[38]. As such, LASV's humoral response has been a backwater subject until recently. Despite mounting evidence in favor of cellular immunity's implication in protecting against LF, there are important caveats to such correlations. The dominant role classically attributed to cellular immunity is contrasted with the inferior performance in NP-based LF vaccines, despite HLA2 immunodominant epitopes known to exist in this protein[22]. Thus, it begs the question if cellular immunity is enough for a LF disease protection.

In most studies, the LASV-specific humoral response has been analyzed using either whole LASV virions or NP protein as the antigen source. LASV NP induces high antibody titers against itself after infection. NP specific IgG titers are thus valuable for LASV infection diagnosis in both active and convalescent LF cases[39]. However, NP is an internal protein with minute presence in the extracellular space[20]. Therefore, NP-directed IgGs most probably have no role in viral clearance[40]. This is contrasted with GPC, the viral envelope glycoprotein, which is constantly exposed through the virus replication cycle, either in the virion membrane or the cellular surface[41]. Therefore, antibodies directed to GPC can theoretically prevent virus fusion (neutralizing antibodies) or function as beacons (through their Fc portion) for other immune cells when bound to GPC expressed in the membrane of infected cells. GPC directed antibodies can also potentially delay viral budding through steric hinderance[42].

3.2.1 Development of a LASV GPC specific ELISA

When this project was started in 2015, there was sparse literature covering GPC directed antibodies induced by either vaccination or infection by LASV. To have a further understanding of how and if GPC directed antibodies played a role in LF disease protection, we developed a GPC antigen for studying the immune response to GPC by ELISA. Originally, the goal was to engineer a secreted form of LASV GPC (without the transmembrane domain) with an HA tag in either the C terminal or the N terminal (necessary for purification). Although

successful purification was achieved (see figure S9), the yield was low and, more importantly, variable results were obtained in the ELISA depending on whether GPC was tagged with HA on either the C or N terminals. In close analysis of Figure S9, it appears that C terminal-tagged GPC was enriched in the GP2 subunit while the N terminal-tagged GPC was in enriched in the GP1 subunit. In retrospect, since the signal sequence peptide (SSP) from GPC was replaced with a human SSP (that of κ chain IgG) for higher secretory efficiency, this might have been detrimental to the structural stability of GPC[43,44]. Indeed, the SSP has been shown to be a cornerstone protein for *Arenaviridae* GPC. Thus, the previous results with HA-tagged GPC drove us in search of an alternative method for the generation of GPC antigen for ELISA development. Previous attempts to purify a soluble RABV G using a similar HA tag method had also proven futile; in that case, HA-tagged RABV G precipitated almost immediately after purification[45]. Therefore, RABV G antigen for ELISA was purified instead by stripping G from the lipid membrane of RABV virions using a gentle detergent (see methods in chapter 2.1). The resulting micelle fraction was further separated by ultracentrifugation from the remaining proteins. While this purification method cannot completely remove endogenous proteins, they are a minute fraction and the G benefits from being maintained in a conformationally correct state by the detergent. A similar approach was thus attempted with LASV GPC. For this purpose, a glycoprotein deficient VSV expressing coGPC was designed and recovered (rVSV-coGPC), similar to a LF vaccine previously developed and tested by the Feldmann group (see chapter 1.5)[25,46]. The GPC antigen resulting from this approach was both adequately pure and conformationally similar to native GPC (see figure S1 in chapter 2.2).

3.2.2 LASSARAB pathogenicity evaluation

The RABV G in BNSP333 is mutated to attenuate the vector. Since LASSARAB adds a fully functional viral glycoprotein (LASV GPC) to the vector, such attenuation might be reverted by either tropism change or gain of function (e.g. neurovirulence)[47]. This prompted us to test LASSARAB live vectors in stringent challenge conditions, by intranasal (i.n.) and intracranial (i.c.) inoculation in both immunocompromised and immunocompetent mice. No reversion of the attenuation was observed. On the contrary, suckling mice inoculated i.c. with LASSARAB

survived longer on average than those inoculated with the parent vector BNSP333 (see figure 3, chapter 2.1).

3.2.3 LASSARAB humoral immunogenicity in mice

We next sought to test LASSARAB for its immunogenicity *in vivo*. A series of replication-competent (live) vectors and inactivated LASSARAB-based vaccines were tested in C57BL/6 mice: live LASSARAB, LASSARABΔG, and rVSV-coGPC; BPL-inactivated LASSARAB with or without the adjuvant GLA-SE (figure 4, chapter 2.1). As control, mice were immunized with either inactivated FILORAB1 with GLA-SE adjuvant or live FILORAB1. The humoral response to each vaccine was evaluated by ELISA, using the newly developed GPC antigen. Interestingly, neither live LASSARAB nor LASSARAB-ΔG induced a LASV GPC-specific immune response significantly higher than the signal detected for control (FILORAB1) immunized animals. Given that the RABV G response induced by live LASSARAB was equivalent to live FILORAB1, it can be assumed that live LASSARAB successfully infected the mice and replicated in the tissues comparably to FILORAB1. However, it didn't induce a significant GPC-specific response. On the other hand, inactivated LASSARAB induced a modest but significantly higher titer of GPC-specific IgGs, especially at later timepoints. With the addition of GLA-SE, a TLR4 agonist, a significant increase in GPC specific IgGs was detected[48,49]. This result is in accordance with a recent publication showing that LASV GP1 complexed with a different TLR-4 agonist increased the breadth of antibody response against LASV GPC[50]. The fact that an inactivated vaccine induced a higher response than a live one is indeed interesting. A possible hypothesis is that the low immunogenicity intrinsic to LASV GPC is compounded with rapid clearance of the replication-competent, rabies-based vector such that there is not enough time for an adaptive immune response to be developed. While the high titers garnered by rVSV-GPC do not support this hypothesis, this discrepancy might be due to differences in replication kinetics between the viruses. Since VSV is a relatively fast-growing virus, it likely reaches higher titers and spreads further in the tissues before being eliminated, thus increasing antigen availability. This also corresponds to a linearly higher inflammatory response, which might yield a better GPC "prime". Inactivated LASSARAB achieved appreciable GPC titers (with an EC50 averaging 1:5000 at day 42 post prime), though only if a boost was given 28 days later. When LASSARAB

was adjuvanted with GLA-SE total IgG against LASV GPC titers of total IgG against LASV GPC averaged 1:12500

More than just total IgG titer, a correlate of protection often important for a vaccine is the virus neutralizing titer (VNA)[34]. While the role of VNA is debated in LF, we still wanted to measure them as VNAs are useful as a correlate of protection in several diseases (e.g. rabies). However, the standard plaque reduction assay necessary to measure VNAs against WT LASV requires the use of BSL-4 facilities. To avoid the use of BSL-4, we developed an assay based on VSV pseudotyped with LASV GPC which are competent for only a single round of replication. The pseudotypes express nanolucifarese (NanoLuc) for quantifying levels of neutralization and infection and EGFP (ppVSV-NL-EGFP) for easy titration of the pseudoviruses. For a graphical illustration and more details, see figure 1.

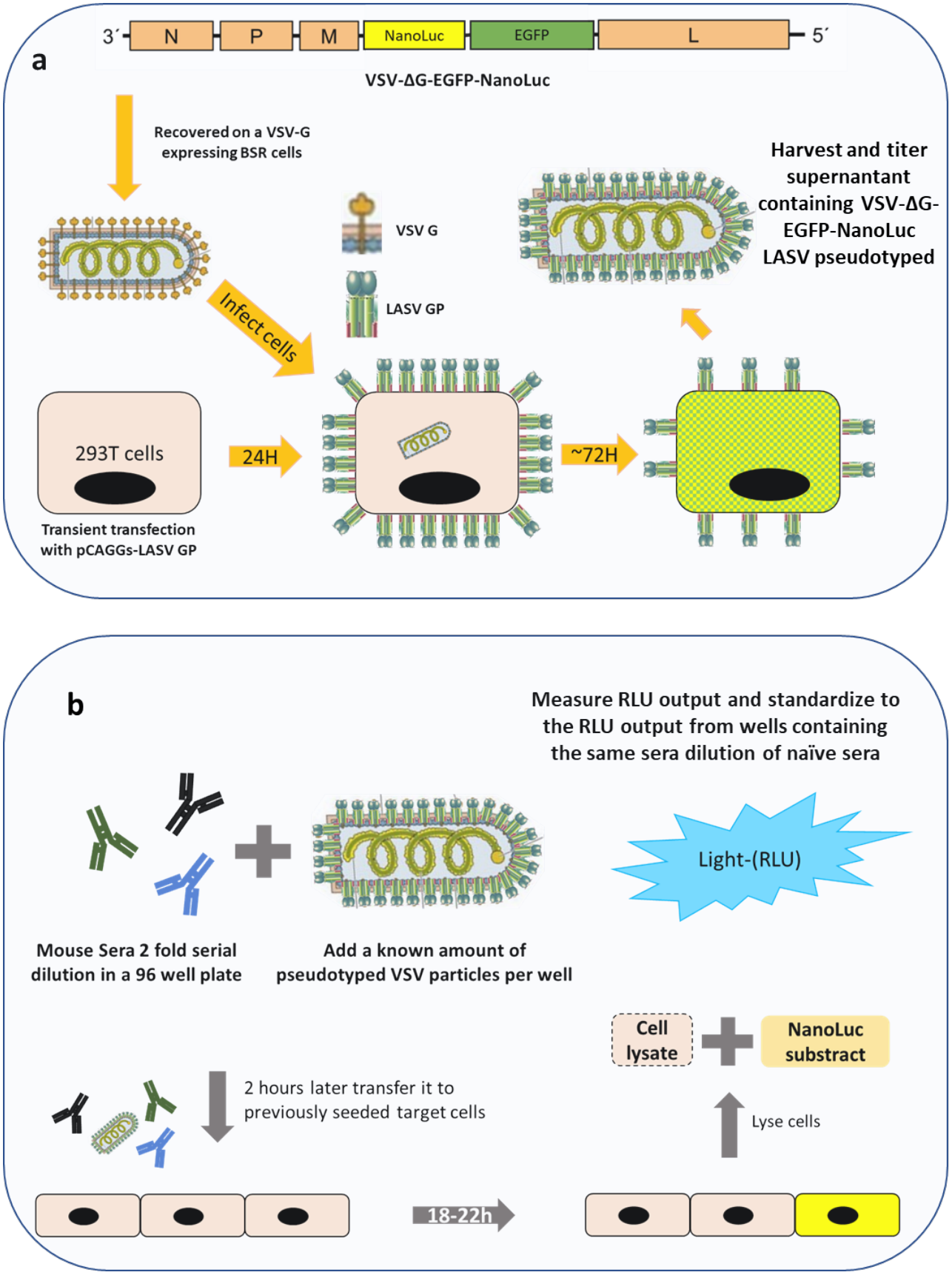


Figure 1. Graphical depiction of the virus neutralizing antibody assay based on VSV pseudotypes expressing NanoLuc.

A) Recovering VSV-ΔG-NL-EGFP, pseudotyping, with LASV GPC, and measuring titer. After virus recovery from BSR cells expressing VSV-G (confirmed by GFP expression), the viral supernatant is used to infect previously transfected 293Ts expressing the viral glycoprotein desired to be pseudotyped (in this case, LASV GPC). After 72 hours post-infection, cells are confirmed to be infected both by cytopathic effect and GFP expression under a fluorescence microscope. At this point, supernatant containing virus is harvested. Virus is titered in VERO cells by overlying cells with serial dilutions of virus. 18-22 hours later, cells are lysed, NanoLuc substrate added, and luciferase signal is measured by a luminometer. The viral titer that generates a relative light unit (RLU) of $5x10^4$ to $1x10^5$ stable for 20 minutes between 2 measures after substrate is added will then be used.

B) Virus neutralization assay using pseudotypes. Sera is diluted 2-fold in serum-free Opti-MEM media from a 1:10 starting dilution (for LASV pseudotypes) or 1:500 (for RABV G pseudotypes) in a 96-well plate. The previously determined amount of pseudovirus is then added to each well of the serial diluted sera and incubated for 2 hours at room temperature. The pseudovirus/sera mix is then overlaid over a confluent monolayer of previously seeded VERO cells and incubated for 18-22 hours. The following day, cells are lysed and the NanoLuc substrate is added. Neutralization is then presented as the percentage of reduction of infection based on the RLU measured in sera dilution normalized to the RLU measured from the same dilution using naïve sera.

To validate this new assay, RABV G pseudotyped pVSV were first tested as the RABV G included in LASSARAB is expected to generate high titers of RABV-specific VNAs. High RABV VNA titers (>1:10000) were detected in the sera of mice immunized with either live or inactivated LASSARAB (see figure 5, chapter 2.1). Equivalent results were obtained with FILORAB1, our internal control which has been previously shown to induce high RABV nAb titers. Because VNA titers obtained can be highly variable depending on the technique or reagents used, the WHO has established international reference standards. These standards, when used in parallel with the sample to be tested, can normalize the nAb titer to IU per volume (usually per ml) and thus results obtained across different assays or reagents can be cross validated. In the case of RABV, a titer >0.5 IU/ml is considered adequate for protection against rabies disease[32,51]. Both live and inactivated LASSARAB achieved VNA titers well above 0.5 IU/ml thus assuring its use as a rabies vaccine and validating the assay.

When sera were assayed with LASV GPC pseudotyped VSV, no reduction in signal was detected at even the lower antibody dilutions, thus indicating that a LASV VNA response was not present. Since previous reports have shown that the supplementation of complement can improve antibody binding (and potentially neutralizing) sensitivity, this was also tested, using

guinea sera as a source of complement (figure S4, chapter 2.2). No major difference was detected between the results from complement or non-complement assays. This LASV pseudotyped assay was also validated using previously published LASV neutralizing mAbs (37.7H, 12.1F, and 25.10C). These antibodies neutralized our pseudotyped VSV at similar concentrations as were previously reported in plaque reduction assays using LASV in BSL-4 conditions.

The lack of LASV VNA induction by LASSARAB is in accordance with the literature as LASV nAbs are rarely detected in humans and animals convalescing from LF infection. The Pinschewer group showed that this was due to the extensive glycan shield present in LASV GPC that promotes nAb evasion[52]. Moreover, in 2017 the Sapphire group accomplished co-crystallization of LASV GPC with a potently neutralizing antibody, 37.7H[31]. 37.7H's binding formed a bridge between GP units and locked GPC in a prefusion stable conformation, a maneuver ostensibly necessary for neutralization[53]. These results suggested that if a stabilized prefusion version of LASV GPC was used as a vaccine antigen, it would more likely induce LASV nAbs, a strategy that has been used in HIV gp120 stabilization studies[54]. However, LASSARAB expressing a prefusion stabilized GPC did not induce nAbs either[55]. Quite the contrary, a lower overall LASV GPC IgG response was detected, conferring no advantage over wildtype GPC present in LASSARAB. Thus, induction of LASV nAbs by vaccination alone appears to be a challenging and ultimately questionable task. Therefore, alternative correlates of protection should be considered for LF.

3.3 LASSARAB's efficacy in a guinea pig challenge model

In parallel to our study of correlates of protection against LF, we sought to test LASSARAB's efficacy in a LASV challenge model. We chose the Hartley outbred guinea pig model and the guinea pig-adapted LASV Josiah strain. This model closely mimics the immune response expected in a genetically diverse population and has a higher probability of reflecting the diverse LF pathology observed[56–58]. Administration of LASSARAB+GLA-SE, reinforced with two boosts (days 7 and 28), conferred asymptomatic protection against LF in 80% of the guinea pigs. This was comparable to the 90% protection afforded by live rVSV-GPC, although all rVSV

immunized guinea pigs displayed symptomatic infection. The results exhibited by our rVSV-GPC group was equivalent to a recent study by Derek Stein et al using the same model[58]. When live LASSARAB or single dosage/rapid boost (day 7 only) inactivated LASSARAB+GLA-SE were administered, LF protection was marginally higher (but not significant) than control. When the humoral response was analyzed, a significantly higher titer of GPC specific IgG was observed in groups that were protected against LF (late boost LASSSARAB+GLA-SE and live rVSV-GPC) in both pre- and post-challenge titers. Interestingly, pre-challenge titers had no nAbs, while post-exposure nAb titers were detected in both survivors and animals that were euthanized after clinical signs, with no difference in titers found between either outcome. Since live LASSARAB was a poor performant in this challenge study, subsequent mentioning of LASSARAB is referring to inactivated LASSARAB+GLA-SE.

3.4 The importance of glycoprotein directed non-neutralizing antibodies in Lassa recall protection

The correlation between GPC-specific IgGs titers and protection against LF despite the absence of neutralizing antibodies led us to investigate whether other immune functions, such as Fc-dependent antibody functions, played a significant role in vaccine-mediated protection. Indeed, in viral diseases such as HIV and influenza, Fc-dependent antibody functions have been shown to be critical for protection, both in context of neutralizing and non-neutralizing antibodies against surface glycoproteins[59–61]. For example, although traditionally thought to depend mainly on their *in vitro* neutralizing potential which simply blocks viral entry or fusion, neutralizing antibodies have also been shown to be dependent upon Fc functions for protection *in vivo*[59]*.* Additionally, Richter and Oxenius showed that the prior existence of LCMV-specific non-nAbs protect against chronic infection, and effectively control LCMV in mice[62]. This was further corroborated by Yamada et al, which reported that chronic infection disrupts Fc receptor function in mice, thus hinting at their potential importance for an effective clearance of an *Arenaviridae* infection[63].

Of the known Fc-mediated effector functions, the most complex and intricate are antibody dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP)[61]. Although classically

described as separate functions, these functions can be better classified as a spectrum of coordinated actions with seemingly different phenotypes but ultimately culminating in the target cell's death. Both ADCC and ADCP start with the formation of an immunological synapse triggered by the crosslinking of Fc receptors present on immune cells with the Fc portions of antibodies bound to cellular antigens[64–68]. Thereafter, depending on the balance of Fc receptors activated, the immune cell either undertakes cellular killing, phagocytosis or both. Phagocytosis of larger cellular targets is usually preceded by cellular death. When NK cells engage in ADCC, several mechanisms are at play, including: the release of cytotoxic granules (perforins and granzyme B) into the immunological synapse; activation of the TNF death receptor family; release of INF-γ and other pro-inflammatory cytokines that promote recruitment and priming of other immune cells while upregulating antiviral signaling in bystander cells[69,70]. Macrophages and other phagocytic immune cells have been shown to predominantly mediate ADCP, however they have also been shown to mediate ADCC[71]. The pleomorphic nature of macrophages combined with the myriad of functions that they perform make macrophage dependent ADCC/ADCP assays harder to evaluate than other immune effector cells.

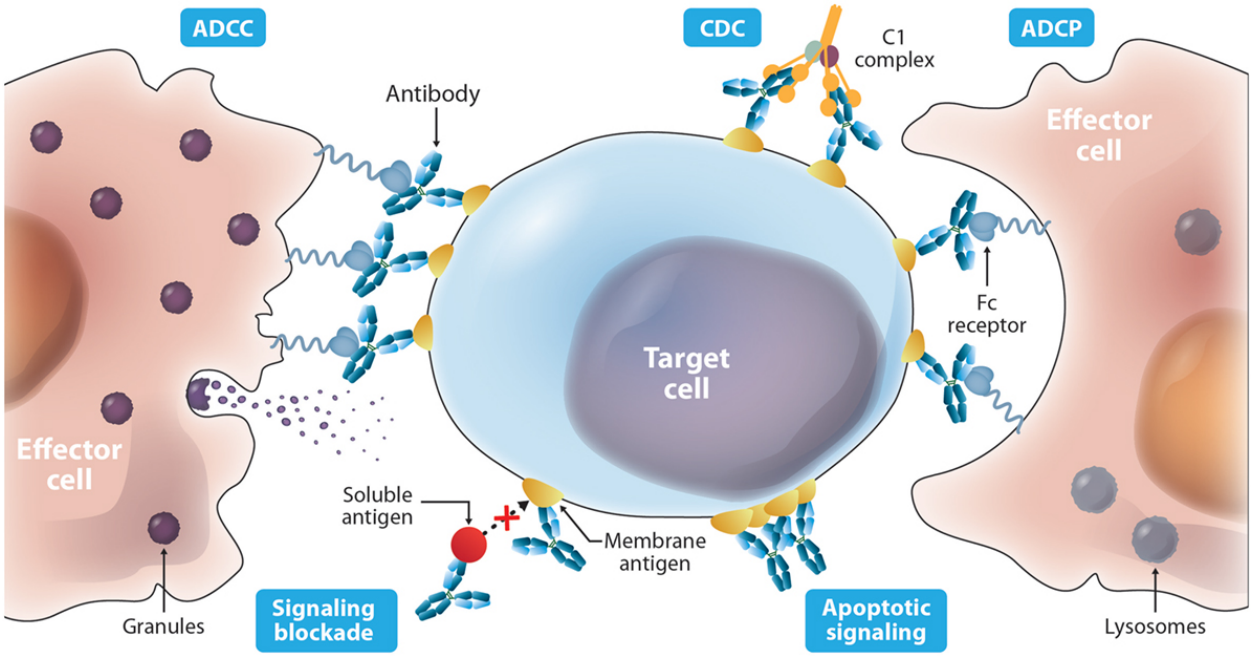


Figure 2. Schematic overview of antibody functions on a target cell. ADCC (left side) and ADCP (right side) are prominently featured with the immunological synapse also represented. Besides ADCC and ADCP, this figure also represents other antibody functions on target cells such as complement dependent cytotoxicity (CDC) and apoptotic signaling by receptor crosslinking and dysfunction (not relevant for viral directed antibodies). Figure

adapted from: *Almagro, J. C., Daniels-Wells, T. R., Perez-Tapia, S. M. & Penichet, M. L. Progress and Challenges in the Design and Clinical Development of Antibodies for Cancer Therapy. Front. Immunol.* ***8****, 1751 (2018)*[72]

To test ADCC and ADCP functions mediated by LASSARAB-induced GPC IgGs, we returned to the mouse model. Although the study of such functions in guinea pig serum would be more appropriate, the lack of availability in guinea pig reagents hindered that approach. A mouse-based approach had added novelty in that little work has been published in mouse-based ADCC/ADCP assays, despite the abundance of literature existing using human immune cells[68].

Classic ADCC assays rely on target cell labeling with membrane impermeable molecules such as chromium 51 or a non-radioactive equivalent reagent (e.g. europium)[73,74]. As cellular cytotoxicity develops, the integrity of the cellular membrane is compromised, and release of these compounds can be detected in the supernatant, either through radioactivity or luminescence. Although they are sensitive, these assays, can have high background signals and signal to noise (S/N) ratios. Moreover, such assays do not allow phenotypic or morphologic characterization of neither the target or effector cells. To circumvent these issues, we developed a flow cytometry-based ADCC assay adapted from the RFAADCC first described by Gomez-Roman but with significant modifications (see figure 3)[75]. As originally described, the assay was performed by labeling both the cellular membrane and cytoplasm of target cells with distinct fluorophores (PKH26 and CFSE respectively). Cellular killing was then measured as the percentage of PKH26 positive/CFSE negative events, since the CFSE signal should be lost when membrane integrity is lost. However, preliminary results showed that this strategy did not work with murine 3T3 target cells since both dyes were still detected in dead cells (as measured by a viability dye). This prompted the switch to the exclusive use of a viability dye (propidium iodine) coupled with expression of GFP in target cells. Higher specificity and sensitivity were accomplished with this approach.

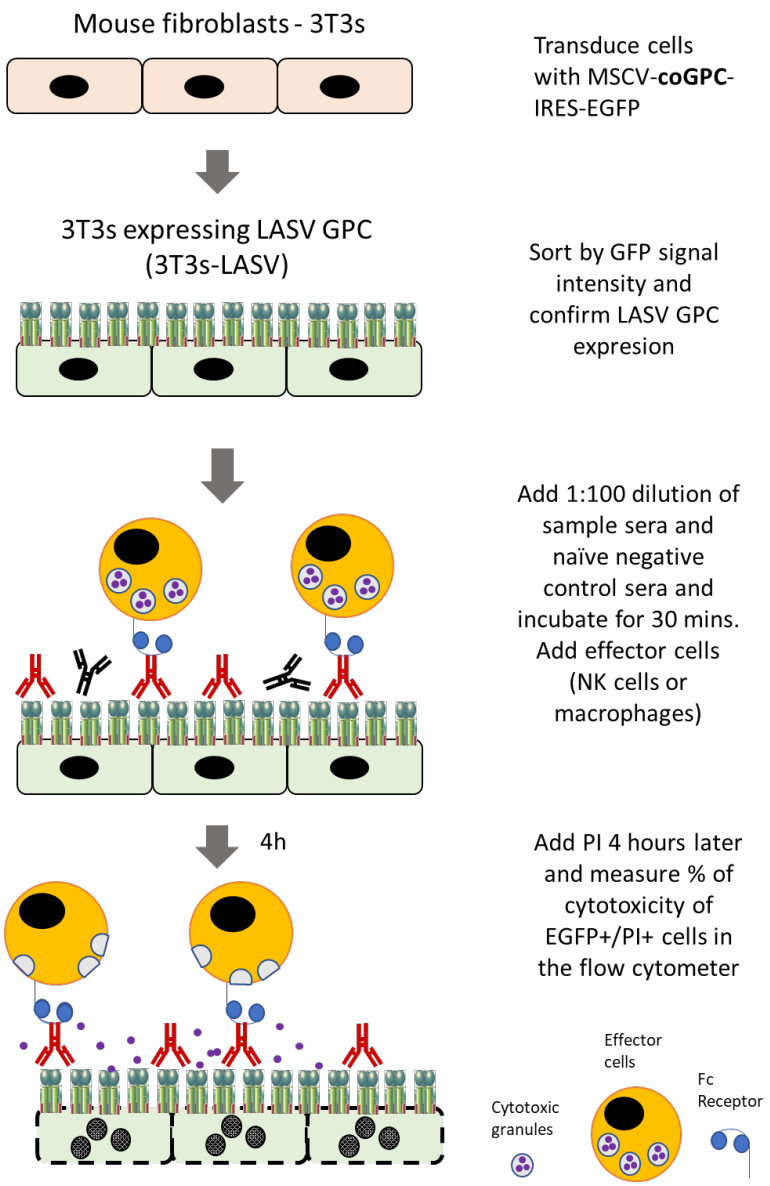


Figure 3. Schematic representation of ADCC/ADCP assay developed for this work. See more details in chapter 2, methods section.

As shown in figure 7 of chapter 2.1.1, sera from mice immunized with inactivated LASSARAB+GLA-SE induce NK cell mediating killing of 3T3 target cells expressing GPC. This was further validated by the concomitant use of a mouse anti-CD32/16, which should fully block ADCC mediated by murine NK cells. To reliably achieve these results, two determining factors were crucial: **1)** Surface expression of GPC, with higher levels of GPC being required for an efficient response to be detected; and **2)** sera concentration, which followed an incomplete S/N ratio bell response with low sera dilutions (1:20) having high background levels and high sera dilutions (1:1000) having a small increment above background only (not shown). We also showed that NK cell mediated ADCC was dependent on IgG, since IgG depleted sera was not able to induce killing above background in contrast to purified IgG. It remains to be explored which IgG sub-isotype was important for ADCC and whether this was epitope dependent. However, given that IgG1 response was barely detected in ELISA while IgG2c accounted for approximately 50-60% of the GPC specific response, one can infer that this sub isotype plays a key role. This is further supported by the finding that Fc activation is strongest with mouse IgG2a/c and the “human

equivalent" IgG1[76]. It's also very likely that ADCC induction depends on the specific GPC epitope that the antibody targets, as is the case with influenza[77]. Indeed, in a study by Fatima et al, GP2 directed mAbs (IgG2a) were found to weakly induce ADCC[78]. Interestingly, we observed strong ADCC induction by mouse macrophages when 12.1F, a human IgG1 LASV nAb was used, although further studies were not pursued. The 12.1F mAb is GP1 directed antibody thought to bind to a more distal section of GPC, which blocks the receptor binding site[30,31]. Thus, in contrast with Influenza where "stalk" IgGs are important for ADCC, "head" directed antibodies in LASV might be optimal for Fc effector functions engagement[60].

Macrophages were also shown to mediate ADCP of 3T3-LASV cells in the presence of LASSARAB sera. This assay is based on the findings of earlier experiments that CFSE remained in the cytosol of dead 3T3 cells and thus, a double positive event of CFSE and previously labeled macrophages could be considered as a phagocytosis event. Viability dye was also used to exclude cellular debris since clumping could lead to false positives. A caveat to this approach is that singlet gating is not possible given the extensive morphological alterations that "full" (post phagocytosis) macrophages display. Furthermore, in contrast to mouse NK cells which only express the activating FcγRIII (CD16), macrophages display the full repertoire of FcγRs. However, as our FcγR blocking experiments show, FcγRIII appears to be the most important receptor for ADCP mediated by J774A.1 macrophages. This was surprising, since FcγRIV (CD16.2) had been shown to have a crucial role in IgG2c activity, while FcγRIII is most important for IgG1[79]. The alleged importance of FcγRIII also contrasts with our ELISA data, which showed high IgG2c titers but neglectable IgG1 titers. Given that the same concentration of Fc blocking mAbs was used, it might be possible that a higher concentration of the 9E9 (anti-CD16.2) mAb was required for blocking ADCP, however this was not further explored.

3.5 Development of a surrogate Lassa Fever mouse model

Our *in vitro* data strongly suggested that Fcγ-dependent functions might play a key role in protection against Lassa. To test its importance *in vivo,* a mouse model is desirable given the ample availability of genetically modified mouse strains and mouse-specific reagents. However, a standard mouse model of LASV did not exist, as introduced in chapter 1.4. This

promped us to generate an alternative model that could evaluate the importance of FcγR functions in a BSL-2 setting. The system we designed employs rVSV-GPC exposure in temporally immunosuppressed mouse[78,80]. We chose the γ chain knockout mouse model (generously provided by Dr. Ravetch) since it has been described to be devoid of most Fc dependent antibody effector functions[81]. This model, combined with α-IFNAR administration, allowed the use rVSV-GPC as a surrogate LASV exposure in a model (mostly) devoid of Fc function. "Mostly", since studies by Barnes et al showed that FcγRI is still partly functional in Fcγ$^{-/-}$ mice, however it was never assessed whether this partial functionality had any *in vivo* relevance[82].

Before discussing the results, it is important to present several caveats of this relatively contrived system. First, although rVSV-GPC should have a similar tropism as LASV given that they have the same glycoprotein on the surface, it does not necessarily cause the same pathology since the backbone is different. Next, the need for immunosuppression (through IFNAR knockdown) to cause pathology by VSV can skew the results towards a higher dependence of the adaptive arm of immune response. Nevertheless, given the lack of pathology that LASV causes in "wild-type" mice through physiological routes, it should be noted that other mouse models of LF have equally marked caveats.

Wild type BALB/c mice and Fcγ-/- BALB/c mice were immunized with inactivated LASSARAB+GLA-SE on day 0 and boosted on day 28. The rapid, day 7, boost was abandoned since it appeared to confer no added efficacy in the guinea pig challenge experiment as well as previous IFNAR-/- based mouse experiments with rVSV-GPC[55]. After rVSV-GPC exposure, a remarkably strong phenotypic difference was observed between the immunized WT and Fcγ$^{-/-}$ mice. While 80% of the WT mice immunized with LASSARAB survived to rVSV-GPC exposure, the Fcγ$^{-/-}$ mice that were immunized with LASSARAB all succumbed to infection (see figure 8, chapter 2). In this experiment we also showed that mock immunized wild type mice and Fcγ$^{-/-}$ mice are equally susceptible to rVSV-GPC since both groups had similar mortality rate (40%). Such results conclusively indicated that Fc functions were of utmost importance for LASSARAB's efficacy, at least in this mouse model.

Another interesting finding in this experiment was that LASSARAB-immunized Fcγ$^{-/-}$ mice succumbed to a further extent (100% lethality) to rVSV-GPC than the Fcγ$^{-/-}$ control mice did

(60% lethality). This also concurred with a visibly more pathogenic display in Fcγ$^{-/-}$ mice than WT (e.g. mucosal hemorrhage). Although this difference in lethality was not found to be significant, it does suggest that, in the absence of Fcγ functions, a higher degree of pathogenicity appears to be at work in GPC-immune mice. This points toward an immunopathological component present in LF disease, a finding that has been previously reported in the literature[83,84]. Although these results were not further investigated in this particular experiment, subsequent data from a newly developed LASV GPC specific T cell assay showed that LASSARAB can induce a strong T cell recall response in WT B6 mice (see figure S8 in chapter 2.2). If an immunopathological component is indeed present, it would attribute an immunoprotective role to GPC-specific IgGs, possibly by preventing excessive T cell cytotoxicity[65].

3.6 Concluding remarks, ongoing work, and future directions

My thesis started, with quite a distinct set of project goals, reflecting my naivety in science. Most prior work on Lassa fever was based on vaccine studies and focused on how cellular immunity was the most important correlate of protection. I thought I should be focusing on that too, at least in the beginning[85]. As time went on, I understood that many questions remained to be answered while, in my mind at least, solidified concepts in the field were based on unsteady grounds. Cellular immunity in LF had been proven to be an important correlate of protection, but as our results hopefully show, humoral response is equally important. Moreover, it can potentially be used in the future as a correlate of protection in LF vaccine development. Approved vaccines for which an absolute correlate of protection has been determined (e.g. rabies neutralizing titer above 0.5 IU/ml or Hepatitis A IgG titer above 10 mIU/ml) could be thought as analogous to an all risks insurance policy[34]. An immune response above that previously determined threshold value protects most individuals against disease. While developing LASSARAB, the results obtained in this work suggest that determining a correlate of protection for LF is possible through GPC specific IgG titers. While this correlate of protection might not be absolute, it appears to be at least a relative (surrogate) correlate of protection measuring a global immune response (humoral and

cellular) that is protective against LF. Indeed, guinea pigs that had the highest GPC specific IgG titers were globally protected against LF disease with no symptom display. The LF correlate of protection appears independent of neutralizing antibody titer (which was rarely detected). Therefore, full LF disease protection might be possible when either GPC-specific IgG (total or sub-isotype) titer, or a normalized ADCC titer are above a certain value. Unfortunately, time constrains did not allow to fully study such parameters. Furthermore, such parameters would need to be reproduced by other groups to gain full acceptance.

LASV humoral response appears to be important for protection—not through viral neutralizing antibodies—but rather through antibody effector functions. Such a finding only opens more questions: 1) How are GPC specific antibodies important for protection independent of neutralizing activity? 2) Are they sufficient by themselves or do they coordinate with a T cellular response in either an immunostimulant or modulatory way? 3) Which antibody sub-isotypes are the most important? 4) Which cellular effector functions (and effectors) are more important, ADCC or ADCP? 5) And finally, how to induce an optimal GPC humoral response? Although these questions were not answered, one can speculate based on results in the guinea pig and murine LASV challenge experiments, as well as previous studies[86,87]. After LASV exposure, most of the control guinea pigs died of acute LF (see figure 6). However, in vaccinated guinea pigs, animals that died from LF disease did so at later timepoints than the control guinea pigs. Moreover, a rather insidious display of LF was observed in such animals until their demise. Such observation suggests a more protracted infection in the vaccinated guinea pigs that were not protected in comparison to control guinea pigs, given the long-drawn display of symptoms. Indeed, LASV RNA was still detected by qPCR in some animals and other non-symptomatic guinea pigs by the end of the study (day 47). Other LASV studies have shown a similar outcome, although these studies ended at earlier timepoints[57,88]. Therefore, besides causing its more acute disease, LASV appears to have the potential to cause late onset disease, or at very least, protracted infection in the tissues without symptom display. Whether this chronic asymptomatic infection is productive or contagious to other individuals remains to be assessed.

Arenaviruses are thought to cause protracted infection through the inhibition of the innate immune response and disruption of APC antigen presentation[63,86,89]. It has been shown that LASV infected APCs are unable to activate T cells and produce inflammatory cytokines, leading

to protracted infection. Moreover, the NP protein of pathogenic viruses has been shown to interfere with antigen processing and presentation by MHC as well as IFN-γ[90]. Such immune disruption is accompanied by defective NK cell responses[91]. In ADCC/ADCP, activation of FcγRs in effector cells promotes the production of inflammatory cytokines (including INF-γ) and drives a controlled inflammation in bystander cells[92]. Thus, it is possible that, through Fc-FcγR activation, GPC directed antibodies can bypass this immune suppression in their respective effector cells and restore IFN-γ production in both APCs and effector cells (e.g. NK, macrophages). This inflammation could then promote APC activation and subsequent T cell activation and recruitment thus leading to viral clearance and prevent chronic infection. Indeed, it has been shown that for LCMV chronic infection to be established, B cells bearing LCMV GPC epitopes are first eliminated, thus hinting at the importance of the humoral response in this arenavirus[89,93,94]. Moreover, throughout protracted LCMV infection, antibody dependent effector functions are disrupted[63]. It remains to be explored whether an equivalent phenomenon is present in LASV chronic infection.

LASSARAB appears therefore as a potential dual inactivated vaccine for both Lassa Fever and Rabies. Currently pre-clinical non-human primate (NHP) studies are underway to test LASSARAB's immunogenicity in this model, and an NHP challenge study is being planned to determine LASSARAB's eligibility for clinical phase 1 trials. These works are currently part of a NIAD/NIH contract to develop a tetravalent Lassa/Marburg/Sudan/Ebola vaccine. However, there is still a long road ahead before (and if) LASSARAB gets approval for human use. Even if the NHP studies are successful, achieving viable and cost-effective production of GMP certified LASSARAB will be another monumental task. Nonetheless, besides an experimental LF vaccine, this work also provided insight for a novel mechanism of protection in the context of LF through Fc-receptor functions. Further studies will be required to dissect the exact molecular mechanisms and, as everything in science, an answer only leads to many more questions.

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Chapter 4

Annexes

Peer Review File of: Non-neutralizing antibodies elicited by recombinant Lassa-Rabies vaccine are critical for protection against Lassa Fever

Tiago Abreu-Mota, Katie R. Hagen, Kurt Cooper, Peter B. Jahrling, Gene Tan, Christoph Wirblich, Reed F. Johnson, and Matthias J. Schnell

*Nature Communications **9**:4223 (2018)*

DOI: 10.1038/s41467-018-06741-w

We want to thank the reviewer for their thoughtful evaluation of our manuscript and address their comments below highlighted in bold.

Reviewer #1 (Remarks to the Author):

The authors present the development and characterization of an inactivated recombinant LASV and rabies vaccine candidate (LASSARAB) expressing a codon-optimized LASV glycoprotein (coGPC). When adjuvanted with a TLR-4 agonist LASSARAB elicited a humoral response against LASV and RABV in both mice and guinea pigs, and protected against LASV challenge. Important evidence for non-neutralizing LASV GPC specific antibodies as a mechanism of protection against Lassa fever is presented for the first time. This is an extremely well written presentation of a complex series of studies. The recent increase in Lassa fever vaccine initiatives by CEPI and others make these results extremely timely and important.

The fact that the immunization induced high titers against Rabies virus glycoprotein, but not LASV GPC confirms that native GPC is not a good inducer of neutralizing antibodies. Hastie et al. Science 02 Jun 2017: Vol. 356, Issue 6341, pp. 923-928 suggest that this is because the most potent neutralizing antibodies recognize quarternary epitopes. This paper should be referenced and discussed in the context of the authors' current results.

The paper has been added and we discuss the related findings in the discussion section.

Minor comments:
1. ABSTRACT
"Overall, these findings are the first to demonstrate an effective inactivated LF vaccine and elucidate novel humoral correlate of protection for LF." Should be: "Overall, these findings demonstrate an effective inactivated LF vaccine and elucidate novel humoral correlate of protection for LF."

While one dose of inactivated LASV did not protect NHPs [Mccormick1993 Journal of Medical Virology 37(1):1-7, in another study 3 doses did protect [Krasnianskii 1993 Vopr. Virusol. 38(6), 276–279].
We agree that with this suggestion and changed the abstract accordingly.

2. “Indeed, proving the urgency and importance of preventive measures, an unprecedented major LF outbreak, with 25.4% high case fatality rate, is currently unfolding in the major city of Lagos and other parts of Nigeria”

Revise: this was a surge not an outbreak as cases occur in Nigeria yearly. While cases were present in Lagos state, the vast majority were not.

We agree with this assessment and corrected this within the text

3. P3 The genus mastomys should be capitalized Mastomys

We did correct this as suggested.

4. P8 reffered to referred.
Has been corrected accordingly.

5. P17 non-humane to non-human
Has been corrected accordingly.

6. P17 “As a major LF outbreak unfolds in Nigeria at the time of manuscript preparation…” Again, a surge not an outbreak.
Has been corrected accordingly.

7. Good job overall with color selection, but may want to revise Figures 4- 6 to be more color-blind friendly.
We adjusted all figures so they are color-blind friendly.

Reviewer #2 (Remarks to the Author):

General remarks

This in an interesting study investigating novel vaccine candidates against the highly pathogenic Lassa virus (LASV). The Old-World arenavirus LASV is the causative agent of a severe hemorrhagic fever with high mortality in humans and represents one of the most important emerging pathogens according to the World Health Organization. Despite significant efforts over the past decades, the development of a safe and efficacious vaccine against LASV remains an unmet challenge. A particular problem in LASV vaccine design is the notoriously low immunogenicity of LASV envelope glycoprotein (GP)-1 implicated in receptor binding and target for neutralizing antibodies. During natural infection, anti-viral CD8T cells represent a major correlate of protection, whereas neutralizing antibodies (nAb) appear late in convalescence and tend to be of low titer. An elegant recent study by Pinschewer and colleagues (reference 45) provided evidence for N-glycan shielding as a likely mechanism behind low

immunogenicity of LASV GP1, similar to envelope GPs of other viruses, such as HIV-1. Recent proof-of-concept studies using recombinant nAb against LASV provided evidence for protection, but the extent of protection did not always correlate with nAb titers.

In the present study, Abreu-Mota and colleagues designed bivalent LASV rabies virus (RABV) vaccines based expressing LASV GPC within a well-characterized RABV vaccine backbones with favorable safety profile (LAASARAB). Using an existing recombinant LASV candidate vaccine based on recombinant vesicular stomatitis virus (rVSV-LASVGP) as benchmark, the authors first studied the humoral immune response to LASSARAB in a murine model. Interestingly, immunization with live LASSARAB and a variant lacking the RABV G protein failed to induce potent antibody responses. However, inactivated vaccine formulations of LASSARAB, in particular when combined with a TLR4 agonist adjuvant and applied in a prime-boost regimen induced robust titers anti-LASVGP IgG. The newly developed ELISA involved purified rVSV-LASCGP as antigen, allowing the detection of antibodies capable of binding the LASV GP in its native pre-fusion conformation, as displayed on virions and at the surface of infected cells, which is a decisive advantage. Using a state-of-the art neutralization assay based on VSV pseudotypes, only negligible nAb titers were detected, suggesting that virus-binding, non-nAb may contribute to protection, which is a reasonable assumption. As a LASV challenge paradigm the guinea pig model was used, which is appropriate and widely applied for evaluation of drugs and vaccines against LASV prior to NHP studies. Inactivated LASSARAB combined with adjuvant in a prime-boost application conferred a level of protection comparable to rVSV-LASVGP, which is one of the most promising live vaccine candidates. Consistent with the evaluation of the vaccine in mice, survival did not correlate with nAb titers, which were negligible. Using a combination of elegant assays, the authors provide evidence for enhanced effector cell activation and clearance mediated by the non-nAb in survivors implicating antibody-dependent cellular cytotoxicity (ADCC) and cellular phagocytosis (ADCP).

The study addresses in important and timely issue in vaccine design against a major emerging pathogen. Although not conceptually novel, the design of a bivalent RABV/LASV vaccine for application in Western Africa is of interest. The study is well-conceived, developed in a logical manner, and is overall well performed. A particular strength is the application of a novel ELISA allowing the detection and quantification of virus-binding non-nAb. A decisive advantage of the inactivated LASSARAB vaccine formulation developed here is its capacity to elicit protective immunity without biosafety concerns in immunocompromised individuals. This is of utmost importance given the population composition in affected regions. The results will contribute to improve rationale LASV vaccine designs. However, some points should be addressed prior to publication.

We want to thank reviewer two for her/his vigorous evaluation and supporting the importance of the study. We agree with several of the concerns raised and addressed them below.

Specific comments:

Major points:

1. In Fig. 2a, the authors employ immunofluorescence to assess the expression of LASVGP by the different vaccine platforms. It would be better to use flow cytometry to quantify expression of the

GPs and to show co-expression of LASV GP and RABV G.

That is a reasonable request and the flow cytometry data has been added to Figure 2.

2. A hallmark of LASV GP1 is its dense glycan shield that correlates with its poor immunogenicity (reference 45) and fully glycosylated LASV GP shows an apparent molecular mass of 40-45 kDa in SDS-PAGE. In Fig. 2c the apparent mass of LASV GP1/GP2 seems < 40kDa. Please comment. Also, previous studies revealed the presence of mainly high-mannose sugars on LASV GP1 (Illick, M.M., Branco, L.M., Fair, J.N., Illick, K.A., Matschiner, A., Schoepp, R., Garry, R.F., Guttieri, M.C., 2008. Uncoupling GP1 and GP2 expression in the Lassa virus glycoprotein complex: implications for GP1 ectodomain shedding. Virol J 5, 161). Have the authors looked at the type of N-glycans present on LASV GP expressed by their LASSARAB vaccine?

Regarding the first question in point 2, indeed we misidentified LASV GP1 as GP2 in our Western Blots while only LASV GP2 was being detected (as later confirmed by GP2 specific human 22.5D mAb). Unfortunately, as we did not possess a GP1 mAb that detects monomeric GP1 on western blot at the time of writing, we focused on proving the presence of both GP1 and GP2 by ELISA along with the presence of a fully conformational LASV GPC on the virion surface 37.7H mAb (confirmed in ELISA – figure S1g) with previously described mAbs in reference 43.

As such, to provide the information requested we now include a Western blot probed with guinea pig survivor sera that detects monomeric LASV GP1 alongside GP2 and has been included in both figure 2 and figure S1. Indeed, as stated, we can confirm that LASV GP1 is running with molecular size, as previously described (from 48 kDa to 42 kDa), thus suggesting that correct GP1 glycosylation is occurring. To confirm such findings and to corroborate with the results of Ilick and Branco&Garry (Characterization of the Lassa virus GP1 ectodomain shedding: implications for improved diagnostic platforms, 2009 Virology journal) and more recent works, we treated LASSARAB-inactivated particles with the endoglycosidases Endo H and PNGase F and have now included the results as part of Supplemental figure 1. (S1f) Our results showed a mobility shift for GP1 when treated with Endo H (from 45 kDa to a gradient between 45 kDa to 35 kDa) and when treated with PNGase (further shift to 20-23 kDa), thus indicating the presence of N-Glycans on LASV GPC. (S1e) Similar results are observed with GP2, which results in a mobility shift from 42-38 kDa to 34-30 kDa when treated with Endo H and in a further reduction to 20-23 kDa when treated with PNGase F.

Since RABV P protein runs in a similar size and fuzzy pattern as LASV GP1 it is hard to discriminate GP1 in LASSARAB particles resolved in a SDS-PAGE page although a faint enhancement of signal can be observed (now indicated with an arrow on figure 2d). GP1 becomes notoriously apparent in SDS-PAGE when purified LASV GPC antigen is resolved in SDS-PAGE (now included as Supplemental figure 1b).

The presence of GP1 monomer with a molecular size of 42-48 kDa is also observed in the ELISA antigen together with GP2 (S1 a, b and c) in both western blot and SDS-PAGE, thus indicating that a similar glycosylation pattern is present in LASV GPC antigen used for the detection of GPC binding IgGs.

It should be noted that depending on the cell line used to grow the virus we did observe some slight variation in size and pattern on GPC/GP2 in western blot (data not included but can be provided if requested).

3. While strong evidence is provided for a role of non-nAb, and likely ADCC and ADCP in protection, it would be important to better define the actual immunological correlate of protection. It would be very interesting to perform serum transfer and ideally transfer of purified IgG, in the guinea pig challenge model, allowing an assessment of protective non-nAb titers.

We agree this would be an interesting addition to the paper, it is nevertheless, by itself, a time consuming and expensive experiment (due to the requirement of both a BSL-4 facility and guinea pig/NHP model) that would only complement the results we obtained *in vivo,* in mice, with our surrogate LASV challenge mouse model. Applying our ADCC/ADCP assays for guinea pig sera would also not be possible to be conducted with accuracy since Guinea pig specific reagents (NK/macrophage cell isolation kits) are not available as well as guinea pig IgG sub isotype profiling. Moreover, while this experiment could confirm that passive sera transfer of antibody from immunized guinea pigs is sufficient for protection in the guinea pig model, it can also introduce a confounding factor. Since LASSARAB immunization also induces CD8+ and CD4+ T cellular responses those controls would also be required if a definitive protective non-NAb titer induced by LASSARAB is to be established.

These concerns formed the rationale behind the experiment with Fcγ-R KO mice (Figure 8). With this experiment, we sought to emulate more closely what would happen if, after a LASSARAB immunization, mice were exposed to a virus with a similar tropism as LASV (since it expresses the same glycoprotein) but in the absence of Fc receptor functions.

4. Has complement-dependence of antibody neutralization been checked?

That is a very good suggestion. Indeed, we had checked the complement-dependence of antibody neutralization while developing the VNA assay and found that there seemed to be no function for complement-mediated neutralization of LASV pseudotypes by antibodies elicited by our vaccine.

We had not included these results since they were negative and served as a basis for developing the VNA assay, but we have now included them as part of supplemental figure S3 since other

researchers might have the same question.

5. The potent anti-LASV GP antibody response upon vaccination with inactivated LAASARAB (Fig. 4 and S2) is in stark contrast to the low antibody titers in mice immunized with live, replicating virus. It would be interesting to look at the frequency of antigen-specific CD4T cells and B cells in the two situations.

The potent response is indeed interesting, and we see have seen a similar response for some other viral antigens (e.g., MERS-CoV) but not for all (e.g., Nipah). To a certain extent, the response seems to go against the dogma that live vaccines are more potent than killed. The overall hypothesis is that the immune response against RABV G might quickly block the spread of the vector (at least in intramuscular immunization) and therefore prevent a potent IgG immune response against LASV GPC. However, RABV G immune response was not compromised, as shown in Figure S2b, to a certain extent corroborating previous findings that LASV GPC is a very poor immunogen by itself (Ref: 43, 81 and 82).

In the case of the RABV G deleted vector, we saw an increase in GPC response compared to the RABV G-containing vector, but adjuvanted killed vaccine was still more potent thereby indicating that RABV vector is being cleared before inducing a significant immunological response to LASV GPC.

Because of the poor response of the replication competent rabies vectors and higher advantages of inactivated vaccine, this study was then largely directed toward the development of a killed vaccine and no further experiments were conducted with live (replication competent) rabies vectors.

6. In the LASV challenge model, inactivated LASSARAB is compared to live rVSV-LASVGP used as benchmark. Have the authors compared inactivated LASSARAB and inactivated rVSV-LASVGP in combination with GLA-SE?

We included the VSV vaccine in the form it is currently utilized because it is a leading vaccine candidate. To study VSV in its inactivated form would certainly be very interesting, but nevertheless it was outside the scope of this work. Of note, a VSV-based inactivated vaccine would not confer protection to RABV which would be surely a major disadvantage for the intended region.

7. The detection of viral RNA 50 days post challenge (Fig. 6d) is intriguing. Have the authors tried to recover infectious virus from serum or organs of these animals? Do immune-privileged sites (testis, kidney etc.) harbor infectious virus and serve as reservoirs?

We were equally surprised by the detection of viral RNA 50 days post challenge. A decisive factor for doing such a prolonged monitoring post challenge was that we observed late clinical symptoms (day 20 post challenge), and there is very little information in the literature regarding persistent infection of LASV. As such, the persistence of the LASV infection was an unforeseen result that will be addressed in a following work since recently both (40) and (42) had similar findings albeit in an

NHP model and in a shorter timeframe. Our collaborators at the IRF are currently studying the persistence of the virus at different sites.

Minor points:
All of reviewer two's minor points listed below have merit, and therefore we did change the text of the manuscript accordingly.

1. Introduction, like 19: in the clinic, the SOC for LASV is the off-label use of ribavirin, which shows some efficacy when used early in disease, but can be associated with unwanted side effects, e.g. hemolytic anemia.

2. Line 122: one reason for the more robust growth of LAASARAB lacking the RABV G may be that the GPs interfere during entry due to different fusion pHs and the dependence of LASV GP on LAMP1 for fusion.

3. Please complete the legend for Fig. S2 labelling the curves and be consistent in the nomenclature of live vs. inactivated virus between main text and figures.

4. The robust levels of virus-binding non-nAb elicited by the LASSARAB + GLA-SE formulation reported here are in line with a recent study that reported a similar increase in virus-binding non-nAb to LASV GP1 using a polymersome nanocarrier in combination with the TLR4 agonist MPLA as adjuvant (Galan-Navarro C, Rincon-Restrepo M, Zimmer G, Ollmann Saphire E, Hubbell JA, Hirosue S, Swartz MA, Kunz S. 2017. Oxidation-sensitive polymersomes as vaccine nanocarriers enhance humoral responses against Lassa virus envelope glycoprotein. Virology 512:161-171). Please discuss.

Reviewer #3 (Remarks to the Author):

Abreu-Mota present a nice study of a novel Lassa fever vaccine and provide interesting mechanistic studies. The studies are generally well done and should be of interest to Nature Comms readers.

Comments
Fig 1 - rVSV-GPC is a little confusing – the N, P, M, L genes are portrayed as being identical to the Rabies vector but I gather they are VSV genes? - they could be portrayed as a different color (or size?). Or is the whole rabies gene block inserted? – in which case flanking VSV or rabies genes could be illustrated to make this clear.

Yes, they are VSV genes. We will change the figure accordingly to avoid such confusion

I gather the BNSP333 vector is similar to a vector used for animal vaccination against rabies (line 93) – has BNSP333 been used either as a widespread animal vaccine or used in human trials? – if not are there issues of concern that would make this pathway difficult? – this would lessen the enthusiasm for the work as it could make it another interesting but non-translatable vector. I note the related Ebola studies were published many years ago.

We give some background regarding the vector below but the most important fact here is that we do develop a deactivated (killed) not a live rabies virus-based vaccine against LASV. This is very important because pregnant women and children are a major target of the vaccine. For certain other approaches we consider immunization of animal hosts with the live virus, but not humans. This is now clearly stated in the publication.

The vector:

The BNSP333 vector is based on the SAD-B19 vaccine strain of rabies virus. This vaccine strain has been widely used for live oral immunization since the 70s, as can be seen in here: http://www.who.int/rabies/vaccines/oral_immunization/en/.

BNSP333 was further attenuated by a 333 mutation in its native glycoprotein that completely abrogates neurovirulence even in SCID (severely immunocompromised mice), as can be observed in our works as well. Furthermore, this vector is permissible to recovery in GLP/GMP conditions.

Currently, 3 RABV-based vaccines based on BNSP333 are being manufactured and formulated using good manufacturing practices (GMP). There are all utilized as deactivated vaccines, so the attenuation of the vectors is mostly an advantage for production. Together with LASSARAB (also based on BNSP333 platform), they are the basis for the tetravalent vaccine development NIH contract (HHSN272201700082C) against EBOV, SUDAV, MARV and LASV and are on target to reach human clinical trials.

Several studies using BNSP333 for several different diseases (Hendra, Ebola and MERS) have been published within the last few years (as recent as 2017) and can be verified here in the paper's literature: (54-61)

Fig 3a - Was the rVSV-GPC group worse than the control rVSV-EGFP group? - why would this be so?

Yes this is true - rVSV-EGFP has an extra gene (GFP) and contains the original VSV glycoprotein, hence has likely a different tropism. Therefore VSV-GPC seems to be neurotropic. It is however

safe if used in peripheral inoculations for the purpose of immunization in immunocompetent mice/guinea pigs.

Fig 3c - The high pathogenicity of BNSP333 in suckling mice – is this a problem?

Our work presents an intracranial exposure of virus in highly susceptible mice that do not possess a fully developed immune system. The high pathogenicity was expected, and the main objective was to confirm that the addition of LASV GPC to BNSP333 did not augment killing.

We also conducted the same experiment in the highly susceptible adult SCID mice, and as it can be confirmed, a mature innate immune response in the absence of adaptive immune response is sufficient to clear the virus even after an intracranial exposure. As such, the virus should not be of concern to even severely immunocompromised people (if used as replication competent).

Furthermore, and as described above, the objective is to use the vaccine as an inactivated vector and, as such, no live virus will actually be exposed to humans.

Fig 4 and text – the difference between the replication competent vectors and "inactivated" vectors is not clear. The terminology "reffered from now on" in the text is confusingly different to the Figures.

This has been corrected.

It is not clear why the unadjuvanted inactivated vector would be better that the live vector, assuming the "dose" is equivalent – was 10 µg of the live vector given? Why was only the un-activated vector given 3 times? (this is also a concern for the later protection experiments)

The dose equivalent of live/dead vaccine is not a straightforward concept since live vaccines replicate in the tissues and killed do not, and as such antigenic exposure is different depending on tissue/virus/immunogen. As such, it is more appropriate to measure a live vaccine dosage as a measure of live infectious units. Of note, the dosage equivalent of live infectious units (based on total particles) used as a deactivated vaccine, would likely be far too low to be effective (since it won't replicate). Thus inactivated vaccines to be effective need be used at higher particle concentrations than replication competent equivalent.

The inactivated vaccine was given 3 times (and, later on, switched to 2 times [on Day 0 and Day 28] since the Day 7 boost does not seem to make a difference) to account for the lack of replication of an inactivated vector, and also to follow the typical rabies virus immunization

schedule. The live vectors were not boosted because we previously showed that this approach does not work for replication competent RABV. Also, by using inactivated vaccine, safety concerns are greatly reduced and boosts can be employed more easily.

Line 277 the statement "Furthermore, we hypothesize that ADCC might be epitope-dependent given that 3 different mouse LASV GPC-specific mAbs did not induce ADCC killing above background in contrast with the sera or purified IgG (not shown)." – some more context for this statement is needed – to which regions did they bind and did the Mabs have the same Fc isotype? Have Fc-defective (GLGR) mutations been studied for these mabs – that would provide more definitive evidence in my view.

Our main objective with this experiment was to prove that ADCC was dependent on IgG, and, to a further extent, verify if any GPC specific mAbs (that are IgG2a/b subclass) can induce ADCC, which was not the case. We will retract that statement, since the mAbs used in that experiment are currently being characterized, the epitope dependence of ADCC in LASV will be published as a separate work.

The numbers of animals in Fig 8b are small for unclear reasons. The vaccinated Fcγ knock out animals look almost worse than controls – if confirmed this might suggest a role for Fc-mediated function in partial post infection control in this admittedly contrived model, as noted in the discussion.

We have now edited the text and the figure to better reflect the numbers of animals per group since the animal numbers are not small (10 per group in the LASSARAB vaccinated animals so 20 in total which was the main experiment). For controls we had 5 per group (so 10 in total). Moreover, these numbers were large enough to be statistically significant

Mouse and human FcgR are different and there is some scepticism about the Fcg knock out model used – alternate models, including knock in models, have been studied. This could be noted in the discussion.

That is a very good point and shall be fully noted in the discussion.

Minor comment
Line 37. You could note this is also known as the common African rat.

True and addressed.

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to author)

The authors have in my opinion addressed the major points of criticism in a satisfactory manner and I have no further comments.

Reviewer #3 (Remarks to author)

I am satisfied with the response.