DEVELOPMENT AND CHARACTERIZATION OF LASSA VIRUS VACCINES AND MOUSE MODEL

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
ALLV	Allpaahuayo virus
ALT	alanine transaminase
AMAV	Amapari virus
APC	antigen presenting cell
APS	ammonium persulfate
AST	aspartate transaminase
ATCC	American Type Culture Collection
βME	β-mercaptoethanol
BCNV	Bear Canyon virus
BSA	bovine serum albumin
BSL	biosafety level
CDC	Centers for Disease Control and Prevention
ConA	concanavalin A
CPE	cytopathic effect
CPXV	Cupixi virus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DIC	disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline

DPT	diphtheria-pertussis-tetanus
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FBS	fetal bovine serum
FLEV	Flexal virus
GTOV	Guanarito virus
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
i.c.	intracranial
ICTV	International Committee on Taxonomy of Viruses
IFA	immunofluorescence assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
IPPYV	Ippy virus
IU/L	international units per liter
i.v.	intravenous
JUNV	Junín virus
kb	kilobases
kDa	kiloDaltons (10^3 Daltons)
KO	knock-out

LB	Luria broth
LASV	Lassa virus
LATV	Latino virus
LCMV	Lymphocytic choriomeningitis virus
LD_{50}	median lethal dose
LP2000	Lipofectamine 2000
MACV	Machupo virus
MDDC	monocyte-derived dendritic cells
MDM	monocyte-derived macrophages
MHC	major histocompatibility complex
MOBV	Mobala virus
MOPV	Mopeia virus
MP	macrophage
mRNA	messenger ribonucleic acid
N/A	not available
N/D	not determined
NHP	non-human primate
NK	natural killer cell
NML	National microbiology laboratory
NOD	non-obese diabetic
nt	nucleotide
OD	optical density
OLVV	Oliveros virus

O/N	overnight
PAMP	pathogen-associated molecular patterns
PARV	Paraná virus
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS/T	PBS with 0.1% TWEEN 20
PCR	polymerase chain reaction
PFU	plaque-forming unit
p.i.	post-infection
PICV	Pichinde virus
PIRV	Pirital virus
PRNT	plaque reduction neutralization assay
PVDF	polyvinylidene fluoride
RAG	recombination-activating gene
RIP	radioimmunoprecipitation
rVSV	recombinant Vesicular stomatitis virus
RT	room temperature
RT-PCR	reverse transcription-PCR
SABV	Sabiá virus
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SEM	standard error of mean
sfc	spot-forming unit

sGP1	soluble glycoprotein 1
S.O.C.	Super Optimal Culture
SP	signal peptide
STAT1	signal transducer and activator of transcription 1
TAMV	Tamiami virus
TCRV	Tacaribe virus
TDM	transmembrane domain
TEMED	tetramethylethylenediamine
TNF	tumor necrosis factor
USAMRIID	United States Army Medical Research Institute of Infectious Diseases
VEE	Venezuelan equine encephalitis
VHF	viral hemorrhagic fever
VLP	virus-like particle
VSV	Vesicular stomatitis virus
WB	Western blotting
WBC	white blood cell
WWAV	Whitewater Arroyo virus

ABSTRACT

Lassa virus (LASV) is the causative agent of a hemorrhagic fever endemic in West Africa, which results in over 200,000 infections and 3000 to 5000 deaths annually. It has previously been demonstrated that a live attenuated recombinant Vesicular Stomatitis Virus expressing the LASV glycoproteins (VSVΔG/LVGPC) is protective in a lethal non-human primate model of Lassa fever. The objective of the present study was to compare the immune response to the VSVΔG/LVGPC vaccine to a novel non-replicating vaccine candidate based on Lassa virus-like particles (VLPs) in the mouse. Additionally, there is a lack of an adequate small animal model for Lassa fever, and LASV is not lethal in immunocompetent mice. We thus investigated the role of the immune system during LASV infection using various immunodeficient mice. In the present study, the VSVΔG/LVGPC vaccine was shown to induce an adaptive immune response, as determined by ELISA and a cytokine ELISPOT assay in immunocompetent mice. The response was stronger with increasing vaccine doses and the number of booster injections. Additionally, the cytokine response preferentially shifted towards epitopes from the N-terminal part of the LASV glycoprotein following the booster injections. A novel vaccine based on Lassa VLPs was also generated, but the immune response to this vaccine candidate was much lower than for the VSVΔG/LVGPC vaccine, even when the VLPs were combined with an adjuvant. Furthermore, we found that mice deficient in the interferon system are susceptible to lethal LASV infection. Complete lethality was observed in STAT1 deficient mice, which are unresponsive to Type I and Type II interferons. Moreover, partial lethality was observed in IFN-γ deficient mice. In contrast,

mice lacking major components of the adaptive immune system, as well as wild-type mice, did not show any apparent signs of disease. In conclusion, these results suggest that both the VSVΔG/LVGPC and Lassa VLP vaccine candidates can stimulate an adaptive immune response in the mouse, although the immune response is stronger for the VSVΔG/LVGPC vaccine than for Lassa VLPs. Furthermore, we have established a novel mouse model of LASV infection and lethality, which could potentially be used for vaccine and drug testing against LASV.

1. Introduction

1.1. Arenaviruses

1.1.1. Taxonomy and classification

Lassa virus (LASV) is a member of the *Arenaviridae* family, which contains a single *Arenavirus* genus, comprised of 23 virus species (Table 1) (International Committee on Taxonomy of Viruses (ICTV) 2010a). However, several putative new arenaviruses from Africa and North America have been discovered in recent years and are not yet included in the ICTV list, such as Lujo virus, Catarina virus, Kodoko virus and Merino Walk virus (Briese et al. 2009; Cajimat et al. 2007; Cajimat et al. 2008; Charrel, de, X, and Emonet 2008; Coulibaly-N'golo et al. 2011; Gunther et al. 2009; Lecompte et al. 2007; Milazzo et al. 2008; Palacios et al. 2008; Palacios et al. 2010).

Lymphocytic choriomeningitis virus (LCMV), the prototypical arenavirus, was the first arenavirus to be discovered in 1933 during a St. Louis encephalitis epidemic (Armstrong and Lillie 1934), and thereafter became a popular tool in the field of viral immunology, particularly for studying the mechanisms of persisting viral infection and immunopathology (Oldstone 2002; Oldstone 2007; Zinkernagel R.M. 2002). Arenaviruses are zoonotic, and in nature most cause chronic infection in rodents from Africa, the Americas and Europe, with each arenavirus infecting a single species of rodent or in some cases a number of very closely related species. Tacaribe virus (TACV) is an exception to this general rule since it has been isolated from bats. Most arenaviruses do not cause human disease; however, some are known to infect humans, and can even cause severe disease, such as a viral hemorrhagic fever (VHF) that can be fatal (Table 1).

	Virus	Natural host	Geographic distribution	Disease in humans
Old World Arenaviruses	Ippy virus (IPPYV)	*Arvicanthis sp.*	Central African Republic	–
	Lassa virus (LASV)	*Mastomys natalensis*	West Africa	Lassa fever
	Lymphocytic choriomeningtis virus (LCMV)	*Mus musculus*	Europe, Americas	Lymphocytic choriomeningitis
	Mobala virus (MOBV)	*Praomys sp.*	Central African Republic	–
	Mopeia virus (MOPV)	*Mastomys natalensis*	Mozambique, Zimbabwe	–
New World Arenaviruses	Allpaahuayo virus (ALLV)	*Oecomys bicolour, Oe. paricola*		–
	Amapari virus (AMAV)	*Oryzomys capito, Neacomys guianae*	Brazil	–
	Bear Canyon Virus (BCNV)	*Peromyscus californicus*		–
	Chapare virus	Unknown	Bolivia	Hemorrhagic fever
	Cupixi virus (CPXV)	*Oryzomys sp.*		–
	Flexal virus (FLEV)	*Oryzomys spp.*	Brazil	–
	Guanarito virus (GTOV)	*Zygodontomys brevicauda*	Venezuela	Venezuelan hemorrhagic fever
	Junín virus (JUNV)	*Calomys musculinus*	Argentina	Argentine hemorrhagic fever
	Latino virus (LATV)	*Calomys callosus*	Bolivia	–
	Machupo virus (MACV)	*Calomys callosus*	Bolivia	Bolivian hemorrhagic fever
	Oliveros virus (OLVV)	*Bolomys obscurus*	Argentina	–
	Paraná virus (PARV)	*Oryzomys buccinatus*	Paraguay	–
	Pichinde virus (PICV)	*Oryzomys albigularis*	Colombia	–
	Pirital virus (PIRV)	*Sigmodon alstoni*	Venezuela	–
	Sabiá virus (SABV)	Unknown	Brazil	Brazilian hemorrhagic fever
	Tacaribe virus (TCRV)	*Artibeus spp.*	Trinidad	–
	Tamiami virus (TAMV)	*Sigmodon hispidus*	Florida, U.S.A.	–
	Whitewater Arroyo virus (WWAV)	*Neotoma albigula*	New Mexico, U.S.A.	Hemorrhagic fever with liver failure

References: (Buchmeier, Bowen, and Peters 2001; Delgado et al. 2008; International Committee on Taxonomy of Viruses (ICTV) 2010b).

Table 1.1. The *Arenaviridae* family.

No arenavirus vaccines are currently available for human use, except for Junin virus (JUNV), and therapeutic agents are limited. In addition, Lassa virus (LASV) and Machupo virus (MACV) can be transmitted from human-to-human, especially during nosocomial outbreaks. Consequently, arenaviruses causing VHFs (Sabiá virus (SABV), Guanarito virus (GTOV), JUNV, MACV and LASV) are classified as biosafety level 4 (BSL-4) agents and are listed in the Category A Pathogen List as defined by the Centers for Disease Control and Prevention (CDC).

The family *Arenaviridae* is divided into two major groups, the Old World (Africa, Europe and Asia) and New World (North and South Americas) groups (Figure 1.1.). The New World complex is further divided into three clades, A (ALLV, BCNV, FLEV, PARV, PICV, PIRV, TAMV and WWAV), B (AMAV, CPXV, GTOV, JUNV, MACV, SABV and TCRV) and C (LATV and OLVV) (Figure 1.1.). However, each of the five Old World species is monophyletic (Emonet et al. 2006). Regarding the genetic diversity among LASV strains, phylogenetic analyses have demonstrated that Lassa viruses are part of one large clade, which is composed of four geographic lineages (Bowen et al. 2000). Lineages I through III include LASV strains from Nigeria whereas lineage IV contains strains from Guinea, Liberia and Sierra Leone. Sequence diversity among LASV strains was established to be at a maximum of 26.8% at the nucleotide (nt) level and 14.8% at the amino acid (aa) level based on sequence comparison of the NP gene (Bowen et al. 2000). The GP1 region of the GPC gene was found to be more variable than GP2 (21.6% vs. 20.2% at the nt level and 7.5% vs. 5.8% nt at the aa level, respectively) (Bowen et al. 2000).

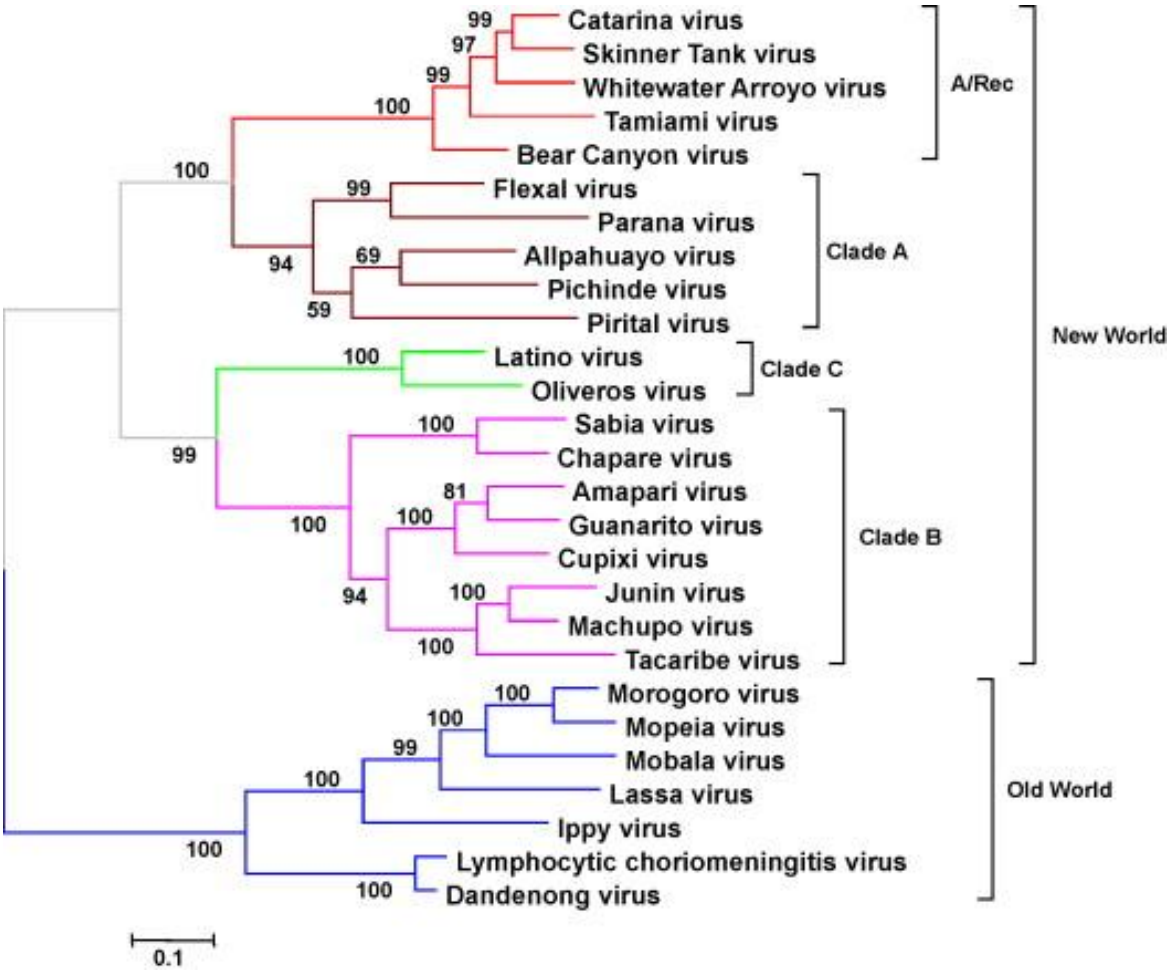


Figure 1.1. Phylogeny of the arenaviruses.

This phylogenic tree is based on the amino acid sequence of the nucleoprotein.

Source: Emonet et al., 2009.

1.1.2. Genome and virion structure

Arenaviruses have a genome consisting of two single-stranded RNA segments with negative polarity and an ambisense organization (Figure 1.2.). The large segment, L (~7.2 kb), encodes the viral RNA polymerase, L (~200 kDa) and the matrix protein, Z (~11 kDa), whereas the small segment, S (~3.5 kb), encodes the nucleoprotein, NP (~ 63 kDa) and the envelope glycoproteins, GP1 (~44 kDa) and GP2 (~38 kDa). The glycoproteins are produced by posttranslational cleavage of a polyprotein precursor, GPC by the cellular enzyme subtilase SKI-1/S1P (Lenz et al. 2001). The 5' and 3' untranslated terminal sequences of the genomic segments are composed of a 19 nucleotide sequence, which has reverse complementarity and is relatively conserved among the different arenaviruses. Both genomic segments have an intergenic, noncoding region located between the two open reading frames, which has been hypothesized to fold into a stable secondary structure and is most likely involved in regulating transcription termination. The morphology of the virions ranges from spherical to pleomorphic with a diameter of 50-300 nm (Figure 1.3.B) (Arenaviridae 2005). Ribosomes can be present in the viral particles, giving the viral particles a sand-like appearance under electron microscopy (EM), hence the Latin name *arena* meaning "sand". GP1 is linked to GP2 by disulfide bonds; the latter is embedded in the viral envelope. The matrix protein, Z, contains a RING-finger domain and binds zinc. Z is a structural protein forming a layer underneath the viral envelope, and thus protects the ribonucleoprotein complex. The latter is formed of the most abundant viral protein, NP, and the viral genomic RNA segments (Figure 1.3.A). It has been speculated that a soluble GP1 (sGP1) protein exists; however, only

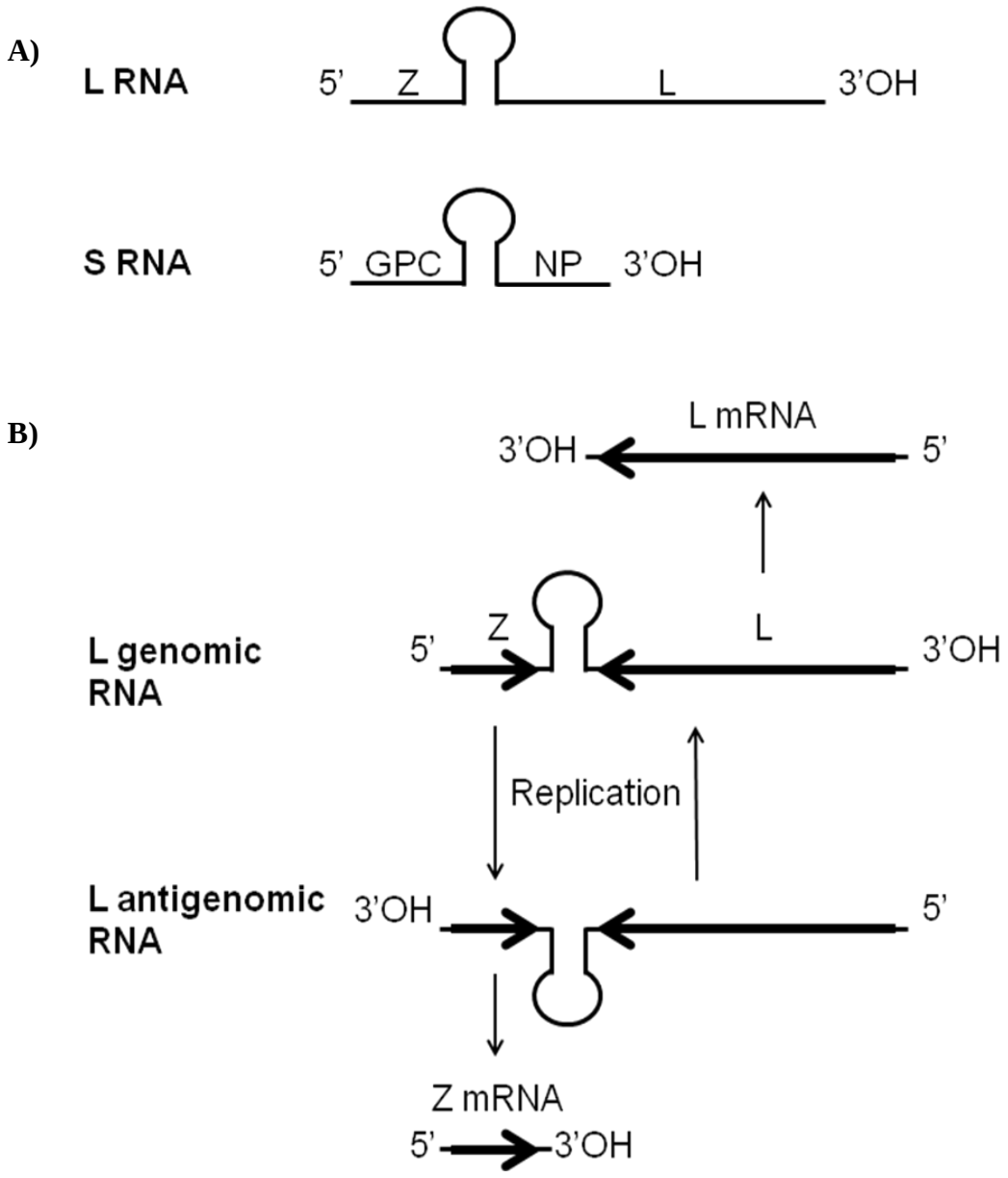


Figure 1.2. Ambisense genomic organization and replication of arenaviruses.

A) Ambisense gene organization. The intergenic region is shown schematically as a stem-loop secondary structure. B) Replication and transcription of the arenavirus genome.

Adapted from: Emonet et al., 2009.

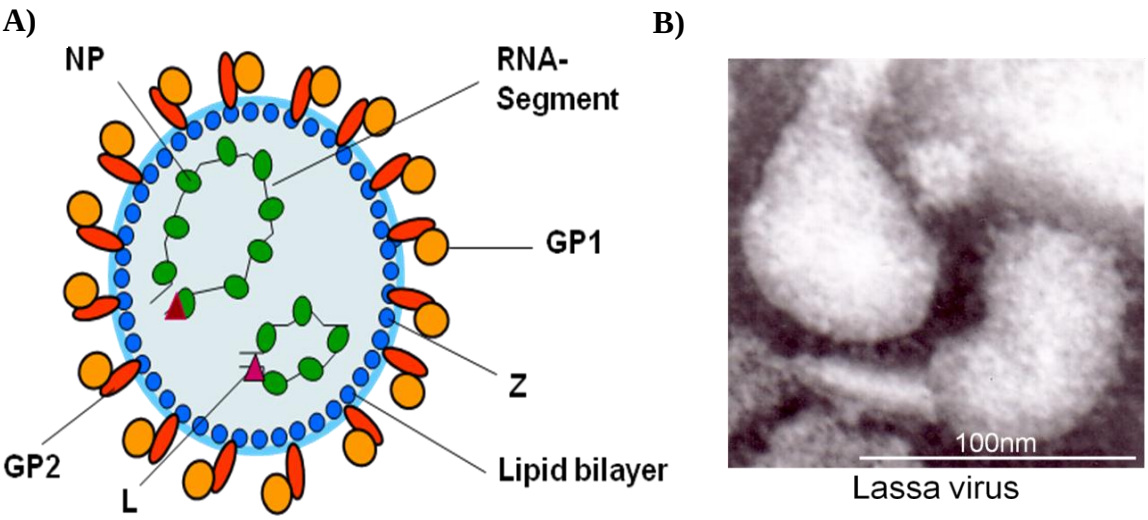


Figure 1.3. Lassa virus structure and morphology.

A) Schematic representation of a LASV virion structure. The LASV genome encodes five proteins: an RNA polymerase, L; a matrix protein, Z; the nucleoprotein, NP; and two envelope glycoproteins, GP1 and GP2. B) Electron microscopic image of LASV. Bar indicates 100 nm.

preliminary data are available as of now (Branco et al. 2010b; Branco and Garry 2009; Illick et al. 2008).

1.1.3. Lassa virus life cycle

Initial binding to a host cell is mediated by GP1 which binds to the cellular receptor α-dystroglycan in the case of LASV (Cao et al. 1998; Kunz et al. 2005) (Figure 1.4.). Alpha-dystroglycan is a ubiquitous cell surface protein and consequently LASV can infect a broad range of cell types. Following virus attachment to a host cell, virions are internalized in endocytic vesicles and nucleocapsids are released into the cytoplasm by pH-dependent fusion of the viral envelope and endosomal membranes (Borrow and Oldstone 1994; Glushakova et al. 1992). The ambisense coding strategy of arenaviruses means that only the NP and L mRNAs, which are in the 3' to 5' direction, can initially be transcribed by the viral polymerase (L protein) (Buchmeier, Bowen, and Peters 2001). As for the GPC and Z proteins, a round of replication is required before they can be synthesized since full-length antigenomic RNAs must be produced before their mRNAs can be transcribed. Full-length genomic RNAs are also produced using the antigenomic RNAs as templates. Newly synthesized viral proteins and genomic RNA segments assemble into virions near the host cell plasma membrane where the glycoprotein spikes are embedded prior to budding. The budding process will be discussed in further detail in Chapter 4.

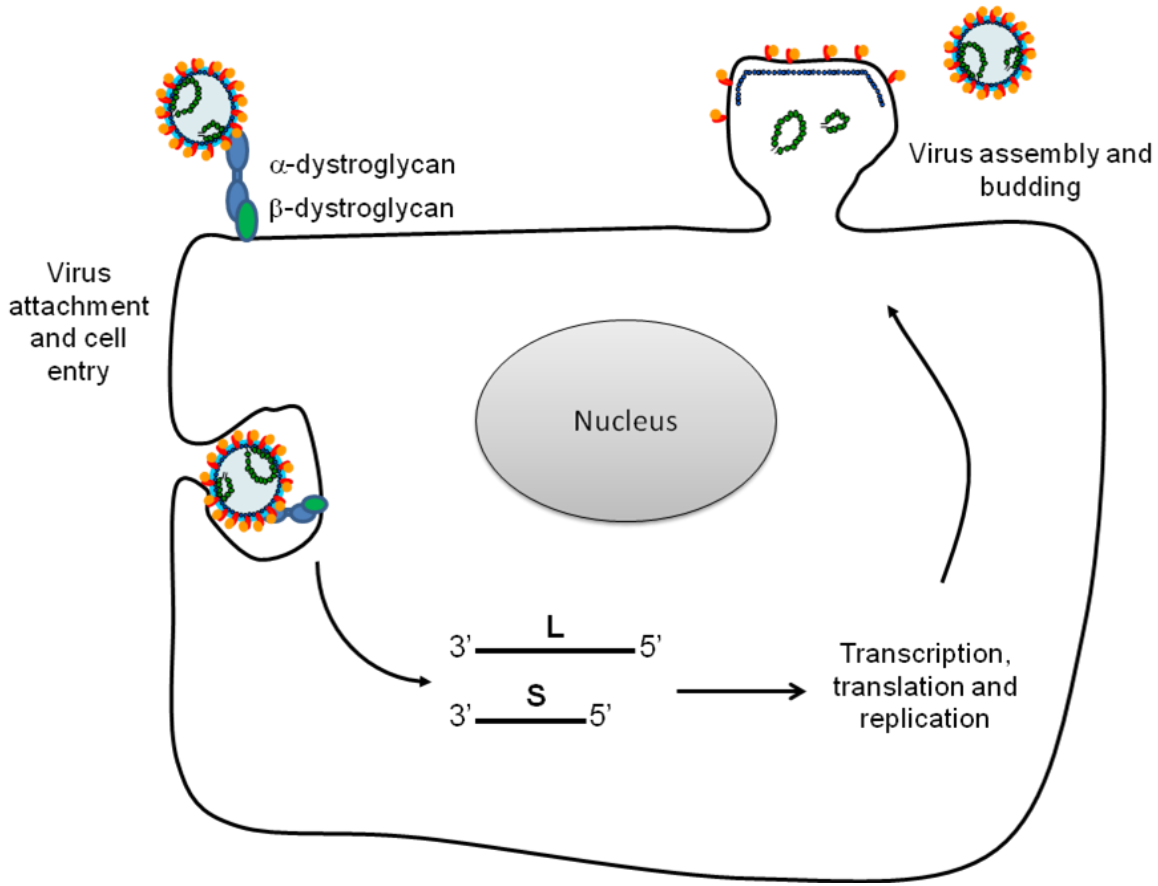


Figure 1.4. Lassa virus life cycle.

Virus attachment is mediated through GP1, which interacts with α-dystroglycan on the host cell surface. The virion is then endocytosed and the nucleocapsid is released into the cytoplasm where viral transcription, translation and replication occur. Virus budding takes place at the host cell plasma membrane following virus assembly.

1.2. Lassa fever

1.2.1. Discovery of Lassa fever

On January 12 1969, L.W., a 69-year-old missionary nurse working in Lassa, Nigeria, complained of a backache. The following week she developed a sore throat, had difficulty swallowing and her oral temperature was 100°F. Over the next few days her condition deteriorated: she appeared dehydrated, her neck and face began to swell and hemorrhagic signs were present on her skin. On January 25, she was transferred to the Bingham Memorial Hospital in Jos, Nigeria. At her arrival, L.W. was in shock with low blood pressure, irregular pulse and signs of cardiac failure. She died the following day (Frame et al. 1970). This new disease was termed Lassa fever after the town where L.W., the first reported confirmed case, worked (Frame et al. 1970). The causal agent, named Lassa virus, was then isolated and based on its characteristics was classified as a new member of the *Arenaviridae* family (Buckley and Casals 1970; Buckley, Casals, and Downs 1970; Murphy 1975; Rowe et al. 1970; Speir et al. 1970).

1.2.2. Clinical presentation of Lassa fever

The incubation period for Lassa fever lasts on average from 7 to 18 days, after which non-specific symptoms develop, such as fever, weakness, general malaise and headache (McCormick et al. 1987a; McCormick and Fisher-Hoch 2002; Mertens et al. 1973; Monath et al. 1974a; Monath and Casals 1975). Chest pain, vomiting, diarrhea, abdominal pain, a painful sore throat, pharyngitis and a dry, nonproductive cough are other symptoms that are present in more than 50% of patients (McCormick et al. 1987a; Mertens et al. 1973). Physical examination reveals an increased respiratory rate, pulse rate, temperature and a normal or low blood pressure. No characteristic skin rash is

present, and neither are petechiae or ecchymoses observed. Hence, a broad spectrum of clinical manifestations and severity of disease have been observed for Lassa fever.

1.2.2.1. Clinical and virological laboratory parameters

Clinical laboratory values in Lassa fever patients were established based on a prospective case-control study performed in Sierra Leone involving 441 human Lassa fever cases (McCormick et al. 1987a). On admission, the average hemoglobin was 10.7 g/dl, whereas the hematocrit was often elevated (mean, 50.1), most likely as a result of dehydration. The mean white blood cell (WBC) counts were normal on admission, as were the differential counts. However, in another study, it was observed that early in the disease a moderate leukopenia occurs and a marked leukocytosis can appear later in the disease (Monath and Casals 1975). Additionally, lymphopenia and neutrophilia have been observed in severe Lassa fever cases (McCormick and Fisher-Hoch 2002).

Numerous studies have been performed to determine what clinical and virological parameters correlate with survival or death during LASV infection (Johnson et al. 1987; McCormick et al. 1986; McCormick and Fisher-Hoch 2002). In humans, a correlation was found between higher AST (aspartate transaminase) levels and death. When admitted to hospital, the mean AST levels of LASV-infected patients was 171 UI/L for survivors (n=89, 90% CI 114-228) versus 923 UI/L for patients who died (n=41, 90% CI 685-1180) (McCormick and Fisher-Hoch 2002). On the other hand, ALT (alanine transaminase) was only slightly elevated. A viremia greater than 10^3 $TCID_{50}$/mL was found to be associated with a higher mortality rate with an odds ratio of death of 3.7 (90% CI 1.9-7.2) (Johnson et al. 1987). It was also observed in the same study that viremia decreased progressively to undetectable levels from the sixth day of illness in

non-fatal cases of Lassa fever whereas fatal cases remained viremic until death. Overall, a case-fatality of 78% occurred in patients with concomitant elevated viremia and AST (McCormick and Fisher-Hoch 2002). These data suggest that those patients that die have difficulty in clearing the virus and also have elevated AST enzyme levels indicating tissue damage.

1.2.2.2. Severe cases and complications

Severe cases are associated with clinical symptoms of shock (low blood pressure, increased heart rate and respiratory rate), neck and facial edema and respiratory distress. Bleeding is sometimes present (gums, nose, conjunctives, venipuncture sites) with conjunctival hemorrhages being associated with a poor prognosis (McCormick et al. 1987a). Neurological manifestations are infrequent but confusion, severe encephalopathy with or without seizures have been noted in some cases (Cummins et al. 1992; Gunther et al. 2001b; Ikerionwu et al. 1978; McCormick et al. 1987a; Monath and Casals 1975). Hemorrhage, abortion, pleural and pericardial effusion and deafness have all been reported as complications of Lassa fever (McCormick et al. 1987a; Monson et al. 1987; Price et al. 1988).

Several studies have described the hemorrhagic manifestations of Lassa fever (McCormick et al. 1987a; McCormick and Fisher-Hoch 2002; Monath and Casals 1975). McCormick et al. reported that 53 out of 306 (17%) confirmed cases of Lassa fever, versus 19 out of 339 (6%) in the febrile control group, presented with hemorrhage, in a prospective case-control study performed in Sierra Leone (McCormick et al. 1987a). Of the 53 Lassa cases with hemorrhagic signs, 19 had hemetemesis, 3 had blood in the stools, 18 had vaginal bleeding (of which 11 were pregnant women who aborted), 2 had

epistaxis, 4 had hematuria and 5 had several sites of bleeding. Twenty of them succumbed to the disease. The mechanism underlying the hemorrhagic manifestations of Lassa fever is not currently well understood but some studies have nevertheless explored this issue. The mean platelet counts appear to be lower in severe Lassa fever cases (Cummins et al. 1989; Fisher-Hoch et al. 1988), but were only found to be statistically significant in the study by Fisher-Hoch et al. On the other hand, platelet dysfunction, as assessed by aggregation studies, was observed in severely ill Lassa fever patients in both studies, but coagulation abnormalities and disseminated intravascular coagulation (DIC) were absent (Fisher-Hoch et al. 1988; McCormick et al. 1987a). Involvement of platelet dysfunction in the hemorrhagic manifestations of Lassa fever, rather than coagulation abnormalities or DIC, is supported by animal studies (Cummins et al. 1989; Fisher-Hoch et al. 1987; Lange et al. 1985). The mechanism has been suggested to involve the presence of a platelet inhibitor in the plasma of infected individuals, interfering with normal hemostasis (Cummins et al. 1989). Furthermore, this inhibitory factor appears to have global effects on cellular function since plasma from LASV-infected individuals (13 out of 15 samples) prevented neutrophil superoxide generation upon stimulation with a chemotactic peptide in comparison to control plasma samples (1 out of 9 samples) (Roberts et al. 1989). In contrast, Hensley et al. have shown in a recent study in cynomolgus monkeys (n=6) infected with LASV that levels of fibrin degradation products (D-dimers) markedly increased during the course of infection and polymerized fibrin was present in the spleen, liver and kidney of infected animals in histopathological studies (Hensley et al. 2011). Altogether, these data demonstrate discrepancy in the hematological features observed during LASV infection and further data is required to

clarify whether the hemorrhagic manifestations of Lassa fever arise as a consequence of platelet dysfunction, coagulation abnormalities or DIC.

The major sequelae associated with Lassa fever infection is acute sensorineural deafness occurring in the convalescent phase of the disease (Cummins et al. 1990; Frame 1989; McCormick et al. 1987a; Rybak 1990). A prospective audiometric evaluation of 49 confirmed cases of Lassa fever revealed that 14 (29%) of these patients, and none of the controls, had a sensorineural hearing deficit, which can be unilateral or bilateral (Cummins et al. 1990; Rybak 1990). Importantly, the hearing impairment was permanent in two thirds of the affected patients (9 out of 14). In addition, all of the deaf patients developed anti-Lassa antibodies prior to the clinical onset of their hearing loss, which developed 5 to 12 days following the disappearance of their fever. In contrast, the development of hearing impairment was not associated with the peak AST levels or severity of the disease, and ribavirin antiviral therapy did not appear to reduce the risk or severity of this complication. The authors thus suggested that the immune response and not the virus itself would be responsible for causing the hearing loss associated with Lassa fever infection (Cummins et al. 1990; Liao, Byl, and Adour 1992). Recent data suggest that hearing loss can also occur during the acute phase of the disease (Ibekwe et al. 2011; Okokhere, Ibekwe, and Akpede 2009). Interestingly, although not statistically significant, a higher case fatality rate was observed in acutely infected patients with sensorineural hearing loss (3 out of 5, 60%) than those without (7 out of 32, 21.9%) (Ibekwe et al. 2011).

1.2.2.3. Pathology of Lassa fever

Post-mortem examinations of a small sample of human Lassa fever cases (10 cases total) have revealed that the gross pathology cannot account for the death of the individuals. Based on histological data and organ viral titers, the liver appears to be the main target organ as it is the major site of viral replication and tissue damage, although LASV can be recovered from several organs, such as the lymphoreticular system, kidneys, adrenal glands, lungs and heart (Walker et al. 1982b; Winn, Jr. and Walker 1975). In addition, the severity of hepatic necrosis observed in several cases is sufficient to suggest it plays a major role in the fatal outcome but it does not correlate with the severity or duration of the disease prior to death (Winn, Jr. and Walker 1975).

1.2.3. Epidemiology

Conservatively, over 200,000 infections are estimated to occur each year in West Africa, resulting in 3000 to 5000 deaths (Fisher-Hoch and McCormick 2004). Lassa fever is endemic in Guinea, Sierra Leone, Liberia and Nigeria (Gunther and Lenz 2004). Seroepidemiological surveys have also demonstrated the presence of antibody-positive individuals in Ivory Coast, Ghana and Benin (Akoua-Koffi et al. 2006; Emmerich, Gunther, and Schmitz 2008; Frame 1975), but only one clinical case of Lassa fever has been reported in these countries (Gunther et al. 2000). In addition, LASV-infected *M. natalensis* (Safronetz et al. 2010) and antibody-positive individuals were identified in Mali (Atkin et al. 2009; Frame 1975; Richmond and Baglole 2003). There have also been 28 reported cases of travelers importing Lassa fever to Europe and North America since 1969 (E-alert 24 July: Case of Lassa fever imported into Germany from Sierra Leone 2006; Amorosa et al. 2010; Atkin et al. 2009; Gunther et al. 2000; Kitching et al. 2009;

Macher and Wolfe 2006), of which 36% died, with the last case imported in the USA in 2010 (Amorosa et al. 2010).

1.2.4. Transmission

The virus is primarily transmitted to humans by contact with the urine and feces of the reservoir host *Mastomys natalensis*, also known as the multi-mammate rat (Fichet-Calvet et al. 2007; Keenlyside et al. 1983; Lecompte et al. 2006; McCormick et al. 1987b; Monath et al. 1974b; Wulff, Fabiyi, and Monath 1975). Another possible route of transmission from rodent-to-human is through the ingestion of infected multi-mammate rats (ter Meulen et al. 1996). In addition, person-to-person spread can also occur, particularly in hospitals, often resulting in significant outbreaks of disease and breakdowns in the local health care system. In common with many other VHF outbreaks, nosocomial transmission is believed to occur through close contact with blood and secretions from infected patients (Frame et al. 1970; Fraser et al. 1974; Monath et al. 1974b).

1.2.5. Laboratory diagnosis

LASV infection can be diagnosed through various molecular and immunological methods. Conventional virus culture and subsequent immune detection by immunofluorescence assay (IFA) are the gold standard assays for the diagnosis of Lassa fever but can take days to weeks to perform and require a BSL-4 laboratory. Diagnosis of infected patients is thus performed primarily by genomic detection through reverse transcription-polymerase chain reaction (RT-PCR), which is currently the method of choice, or antigen capture (sandwich enzyme-linked immunosorbent assays, ELISA). Virus is ideally detected in a serum sample, but can also be detected in urine, throat

swabs, breast milk, cerebrospinal fluid, pleural and pericardial transudates (McCormick and Fisher-Hoch 2002). Serological assays (indirect ELISA, indirect IFA, immunoblot) are usually reserved for epidemiological studies. A recent study demonstrated that LASV-specific IgM can be detected for as long as months or years after LASV infection and therefore should not be used as a diagnostic marker for acute LASV infection (Branco et al. 2011). No commercial reagents are currently available for the diagnosis of LASV infection and therefore each laboratory currently designs their own diagnostic tools.

1.2.6. Animal models of LASV infection

LASV inoculation of non-human primates (NHP), guinea pigs and mice has been found to result in disease and lethality to variable levels (Table 2). In addition, several animal models have been developed based on the inoculation of various animal species with other arenaviruses, which results in clinical symptoms similar to Lassa infection in humans such as LCMV-WE (Lukashevich et al. 2002), Pichinde virus (Aronson, Herzog, and Jerrells 1994; Buchmeier and Rawls 1977; Jahrling et al. 1981) and Pirital virus (Sbrana et al. 2006; Xiao et al. 2001).

1.2.6.1. Non-human primates

Several NHP species have been evaluated as potential models of Lassa fever and interestingly, variability was observed in the susceptibility of different macaque species to become infected as well as succumb to the disease (Peters et al. 1987). LASV infection of hamadryas baboons (*Papio hamadryas*), of Old World origin, has been reported to result in a disease resembling human Lassa fever (Evseev et al. 1991). Squirrel (*Saimiri scirreus*) and capuchin monkeys (*Cebus capucinos*), both of New World origin, were

found to be resistant to LASV lethality and only a mild infection was observed. In capuchin monkeys receiving a high virus dose (6.1 $\log_{10}$ PFU), a low viremia (ranging from 1.2 to 2.6 $\log_{10}$ PFU/mL) was detected up to day 16-17 post-infection (p.i.), whereas no viremia could be detected in monkeys which received a low virus dose (1.1 $\log_{10}$ PFU). In contrast, lethality occurred following LASV inoculation in rhesus macaques

Animal model	**Lethality**	**Selected references**
Rhesus, Cynomolgus, African green monkey	100%*	(Callis, Jahrling, and DePaoli 1982; Fisher-Hoch and McCormick 1987; Hensley et al. 2011; Jahrling et al. 1980; Peters et al. 1987; Stephen and Jahrling 1979; Walker et al. 1982a)
Hamadryas baboons	N/A	(Evseev et al. 1991)
Marmoset monkey	100%	(Carrion, Jr. et al. 2007a)
Capuchin, squirrel monkey	0% (mild infection)	(Peters et al. 1987)
Guinea pig	100% in strain 13 30% in outbred Hartley	(Jahrling et al. 1982; Jahrling 1983)
Mouse**	0-100%***	(Lukashevich 1985; Lukashevich 1992)

* Depending on the virus dose
** Intracranial challenge only
*** Depending on the mouse genotype and LASV strain
N/A: not available

Table 1.2. Animal models for LASV infection.

(*Macaca mulatta*), African green monkeys (*Chlorocebus sabaeus*) and cynomolgus macaques (*Macaca fascicularis*), which are Old World species.

A low virus dose (1.1 $\log_{10}$ PFU) resulted in 100% lethality in rhesus and African green monkeys (6/6 and 3/3, respectively and data not available for cynomolgus macaques) (Peters et al. 1987). In contrast, a high virus dose (6.1 $\log_{10}$ PFU) resulted in 100% lethality in cynomolgus macaques (12/12), but only partial lethality in rhesus and African green monkeys (6/10 and 2/3, respectively) (Peters et al. 1987). These data suggest that increasing the virus challenge dose can result in an "interference" phenomenon in certain monkey species, as defined by the authors (Peters et al. 1987). Furthermore, a critical viremia value of 4 $\log_{10}$ PFU/mL was identified as the cut-off to determine whether a macaque would succumb to LASV infection, similarly to the observations made in humans (Johnson et al. 1987). In addition, seroconversion was observed in all five macaque species, regardless of the viremia levels and survival outcome observed. Intermittent virus shedding from the nasopharynx and urine was also detected in all groups until the fourth week p.i.

The rhesus macaque model of LASV infection and lethality is the most extensively studied for the pathogenesis of Lassa fever and the first one to be reported (Jahrling et al. 1980; Stephen and Jahrling 1979). Rhesus monkeys inoculated subcutaneously (s.c.) with 10^4 PFU of LASV strain Josiah developed viremia by day 5, peaking on days 10 to 14, at which time 6 out of 10 animals died (60% lethality rate). The infected animals developed a fever, demonstrated hemorrhagic signs (petechial rash, bleeding from the gums and nares) and all had detectable antibodies by day 10 post-challenge as detected by indirect IFA. It was noted that the animals in which viremia exceeded 10^4 PFU/mL died whereas

the survivors' viremia remained below this value. An extensive study was later performed to investigate the physiopathology of LASV infection in rhesus macaques as well (Walker et al. 1982a). The findings in this study were in agreement with previous data which indicate that rhesus macaques can serve as a suitable model for Lassa fever in humans, except for meningoencephalomyelitis, pulmonary vasculitis, systemic arteritis and skeletal myositis which appear more important in rhesus monkeys than humans (Walker et al. 1982a).

Data from cynomolgus macaques are similar to the observations made in rhesus macaques (Baize et al. 2009; Hensley et al. 2011; Peters et al. 1987), although the "interference" phenomenon is absent in the cynomolgus macaques (Peters et al. 1987). Clinical parameters, such as pericardial effusion, bleeding at venipuncture site, epistaxis, petechial hemorrhage, facial edema and convulsions, are comparable to data from rhesus monkeys and human Lassa fever cases (Hensley et al. 2011). Similarities to rhesus macaques and humans were also found in laboratory parameters and histopathologic observations (Hensley et al. 2011). In addition, neuropathology at terminal stages of the disease was observed in cynomolgus macaques, consistent with the neurological symptoms observed in NHPs and human Lassa fever cases (Hensley et al. 2011).

The pathogenesis of Lassa fever in squirrel monkeys, which become sick but do not die from LASV infection, was also investigated in a short study in which four animals were inoculated intramuscularly (i.m.) with LASV and sacrificed at days 7, 12, 14 and 28 post-infection (Walker et al. 1975). The animals demonstrated clinical signs of disease and virus was recovered from all organs tested, with lymphoreticulotropism, hepatotropism and nephrotropism appearing to be more important than infection of other

organs. Viremia was also detected in all animals, including the one sacrificed on day 28, in which infectious virus was recovered from several organs. Hence, the squirrel monkey, although not a lethal model of Lassa fever, was considered a potential animal model to study Lassa fever pathogenesis as it reflected the disease observed in humans.

A recent study demonstrated that the common marmoset (*Callithrix jacchus*) is susceptible to LASV infection (Carrion, Jr. et al. 2007a). Subcutaneous inoculation of 10^3 or 10^6 PFU of LASV strain Josiah (n=2 in each group) resulted in a systemic infection with weight loss and viremia noticeable by day 8 p.i. and severe morbidity 15 to 20 days following infection. However, lethality has not been reported *per se* in the literature, as the animals were sacrificed once euthanasia criteria were met in the two studies reporting the use of marmosets as a model for LASV infection (Carrion, Jr. et al. 2007a; Lukashevich et al. 2008). Nevertheless, Carrion et al. concluded that LASV infection of marmosets reflects the disease observed in human Lassa fever patients. This animal model thus offers a potentially interesting intermediate alternative to larger macaques and smaller animal models such as the mouse and guinea pig to study Lassa fever, especially due to its size of 320 to 450 g being advantageous in the BSL-4 setting (Carrion, Jr. et al. 2007a).

1.2.6.2. Guinea pigs

Guinea pigs were also found to be susceptible to LASV infection; however, the degree of lethality depended on the guinea pig strain. Studies of outbred Hartley guinea pigs resulted in partial lethality and poor correlation of the symptoms with the description of human Lassa fever (Jahrling et al. 1982; Walker et al. 1975). NHP models were thus concluded to be superior to the guinea pig as a model for Lassa fever (Walker et al.

1975). Following a study which demonstrated that an adapted strain of another arenavirus, Pichinde virus, induced a lethal infection in strain 13 but not outbred Hartley guinea pigs (Jahrling et al. 1981), the susceptibility of strain 13 guinea pigs to LASV was evaluated (Jahrling et al. 1982). It was determined that 100% lethality was obtained in strain 13 guinea pigs infected with a virus dose of 2 PFU or more s.c. (inoculum doses were: 0.02; 0.2; 2; 24; 2,400; 240,000 PFU of LASV strain Josiah). Strain 2 guinea pigs also uniformly succumbed to LASV infection at a dose of 2,400 PFU, whereas no more than 32% lethality was observed in outbred Hartley guinea pigs when testing the same virus doses as for the strain 13 guinea pigs (Jahrling et al. 1982). In addition, it was found that viremia titers were higher in strain 13 than in outbred Hartley guinea pigs, and lethally-infected animals of this latter group had higher viremia levels than the survivors. This suggests that viral replication is more efficient in strain 13 guinea pigs (Jahrling et al. 1982). In contrast, no significant differences were seen in the humoral immune response among the different guinea pig strains and neutralizing antibodies were only detected at day 45 p.i. in the surviving outbred guinea pigs.

1.2.6.3. Mice

Mice have also been reported to be susceptible to LASV infection, resulting in lethality (Lukashevich 1985; Lukashevich 1992; Lukashevich et al. 2005). However, this was only achievable through intracranial (i.c.) inoculation and the degree of susceptibility depended on the mouse genotype and age, in addition to the virus dose (Lukashevich 1985). Lukashevich' group found that mice could be divided into three different groups based on their susceptibility to LASV infection following i.c. inoculation. The first group was highly susceptible to LASV disease, with almost 100% lethality achieved at day 6-8

p.i. and included newborn and young adult C3H/Sn mice. The second group was resistant to LASV lethality (100% survival observed) and included adult C3H/Sn mice (aged 64 or 105 days) and BALB/c mice (aged 1, 15 or 50 days). Lethality ranged from 30 to 60% in the third group, which consisted of C57BL/6 mice (aged 2 or 50 days) and AKR mice (aged 50 days). CBA mice, which had not been evaluated in this study, were later reported to be susceptible to LASV infection and lethality (Lukashevich 1992; Lukashevich et al. 2005).

In conclusion, LASV infection of non-human primates more closely resembles the clinical, laboratory and histopathological features of human cases than guinea pigs and mice. The mouse would be an interesting model due to its smaller size and vast availability of immunological reagents. Unfortunately, the only route of infection resulting in disease and lethality is intracranial inoculation, which results in a disease distinct from human Lassa fever. Among the various NHP species tested, the available data on capuchin monkeys and hamadryas baboons is not sufficient to offer an interesting alternative at the moment. Furthermore, housing baboons within BSL-4 containment is very challenging and will be unlikely to improve our understanding of LASV pathology forward very quickly. Regarding squirrel monkeys, one interesting feature is that the low pathogenicity rate more closely reflects human Lassa fever and this model appears to be suitable for studying the pathogenesis of human Lassa fever. However, the fact that lethality was not observed in this animal species makes it unsuitable for vaccine and drug efficacy testing. On the other hand, several studies on rhesus and cynomolgus monkeys suggest that these two models are very similar, and both have been used for vaccine and drug efficacy testing for Lassa fever in the past. In addition, the common marmoset

(*Callithrix jacchus*) might also become a very useful alternative in the near future due to its advantage of being much smaller than the other monkey species.

1.2.7. Pathogenesis and immunology of LASV infection

1.2.7.1. Infection

As mentioned previously, humans are believed to become infected with LASV through skin abrasions or inhalation of particulates from rodent's urine. The virus probably then spreads in the human body via the reticuloendothelial system (Baize et al. 2004; Lukashevich et al. 1999; Mahanty et al. 2003; Walker et al. 1982b; Winn, Jr. and Walker 1975), resulting in a multisystemic disease. Recent data from experimental infection of cynomolgus monkeys provides evidence that dendritic cells (DCs) are the primary target cells *in vivo,* as in many other VHF infections, and that the virus is present initially in lymphoid tissues. It later migrates to the liver and adrenal glands to finally reach endothelial cells of various tissues, including the central nervous system and epithelial cells throughout the body, resulting in a multisystemic infection (Hensley et al. 2011).

1.2.7.2. Host immune response

Immunity to Lassa fever

LASV infection in humans is generally believed to provide life-long immunity, although mild secondary infections might occasionally occur, as noted by an increase in antibody titers in some individuals (McCormick et al. 1987b). An antibody response has been demonstrated to occur during Lassa infection against the glycoproteins, NP and the Z protein but the importance of mounting an immune response targeted against one Lassa antigen versus another one has not been investigated (Barber, Clegg, and Lloyd 1990;

Gunther et al. 2001a; Hummel, Martin, and Auperin 1992; Lukashevich, Clegg, and Sidibe 1993; ter Meulen et al. 1998). Most evidence suggests so far that the humoral immune response to LASV does not play a role in clearance of the virus during natural infection. Viremia is present throughout the course of the disease in humans, during which non-neutralizing antibodies are detected (Johnson et al. 1987). In addition, no correlation has been observed between clinical outcome and appearance of antibodies to LASV (Johnson et al. 1987). Measurable amounts of neutralizing antibodies only appear later, during the convalescence phase of the infection (Tomori et al. 1987). Nevertheless, LASV-immune plasma studies in animals have demonstrated that antibodies from convalescent animals can be protective (Jahrling 1983; Jahrling and Peters 1984). Recent data from cynomolgus monkeys also showed that antibodies appear earlier and to higher titers in survivors than in non-survivors (Baize et al. 2009). Although only six animals were included in this study, these findings stress the importance of differentiating the immune response of survivors versus fatalities, which is not always done. Furthermore, detailed studies of the humoral immune response, such as quantitative analysis of the different immunoglobulin isotypes and IgG subclasses present in fatal vs. non-fatal Lassa fever cases, are lacking.

Regarding the T cell response, the first demonstration of cytotoxic cells playing a role in LASV infection was described in 1986 by showing that splenocytes from guinea pigs inoculated with LASV produced target cell lysis in a cytotoxic T lymphocyte (CTL) assay (Jahrling and Peters 1986; Peters et al. 1987). In addition, the same study demonstrated that immune splenocytes obtained early (7 to 21 days) after infection with LASV protected recipient guinea pigs against LASV in an adoptive transfer experiment.

However, spleen cells harvested 47 to 57 days after infection did not provide protection. Further experiments to characterize the effector cells mediating short-term protection are thus necessary to fully understand the involved mechanism. In a more recent study, Ter Meulen et al. demonstrated that LASV seropositive individuals can have a very strong $CD4^+$ T cell response to LASV NP, strain Josiah (8/13 individuals) as assessed by a proliferation assay (ter Meulen et al. 2000). Furthermore, La Posta et al. showed that cross-protection against LCMV challenge of mice primed with a vaccinia recombinant virus expressing the full-length LASV glycoprotein precursor can be mediated by cytotoxic $CD4^+$ T cells specific for a particular LASV glycoprotein epitope (La Posta et al. 1993).

Evidence emerging from vaccine studies currently suggests that cell-mediated immunity is responsible for vaccine-induced protective efficacy rather than the B cell immune response (Vaccine Section 1.2.9.), which is concordant with data from natural infection with Lassa fever. For example, T cell depletion, but not B cell depletion, resulted in loss of survival in a protection efficacy study of the Mopeia/Lassa reassortant vaccine (Lukashevich et al. 2005).

Altogether, these data suggest that the antibody response raised against LASV is not efficient in viral clearance and provides evidence that it is rather the cell-mediated immune response which plays a role during natural LASV infection. However, this conclusion mostly comes from indirect evidence and further studies are thus required to confirm the importance of the cell-mediated response over humoral immunity.

Antigenicity of Lassa viral proteins

Several laboratories have attempted to identify immunodominant T cell epitopes which could serve in vaccine design. $CD4^+$ T cell epitopes of LASV NP have been identified using T cell clones generated from a LASV seropositive individual (ter Meulen et al. 2000). In addition, a $CD4^+$ T cell epitope in the fusion domain of the Lassa GP2 (amino acids 289-301 according to the GPC sequence), which is highly conserved among Old and New World arenaviruses, has been identified (Meulen et al. 2004). As well, thirty-seven $CD4^+$ T cell epitopes from seven pathogenic arenaviruses, covering the 4 arenaviral proteins, were also identified using HLA-DRB1*0101 transgenic mice since HLA-DRB1*0101 restricted $CD4^+$ T cell epitopes display broad reactivity with other HLA-DR molecules (Kotturi et al. 2010).

Concerning the identification of CTL epitopes, Boesen et al. have investigated the presence of HLA-A2.1-restricted CTL epitopes within the LASV GPC and NP using computer-assisted algorithms (Boesen, Sundar, and Coico 2005) since HLA-A2 is represented in ~50% of the population worldwide (Botten et al. 2006). Three out of five selected GPC-derived peptides (GPC_{60-68},SLYKGVYEL; $GPC_{258-266,}$ LLGTFTWTL; and $GPC_{441-449}$, YLISIFLHL) but none of the NP-derived peptides stabilized the expression of HLA-A2 molecules. The three selected GP-derived peptides were also shown to induce T cell responses *in vivo* in HLA-A2.1 transgenic mice, as observed by IFN-γ secretion and target cell lysis in a CTL assay. In a similar approach, Botten et al. have demonstrated that immunization of HLA-A*0201 transgenic mice with either GPC_{42-50}, GLVGLVTFL; GPC_{60-68}, SLYKGVYEL; and $GPC_{441-449}$, YLISIFLHL, protected the mice against

challenge with a recombinant vaccinia virus expressing LASV GPC, as seen by a significant reduction in mean viral titers (Botten et al. 2006).

A common CTL NP epitope has also been identified among Old World arenaviruses (Oldstone et al. 2001). In this study, Oldstone et al. evaluated the possibility that the known LCMV immunodominant CTL epitope consisting of amino acids 118 to 126 from NP could cross-react with other arenaviruses. Indeed, the authors observed CTL cross-reactivity among three Old World arenaviruses (LCMV, LASV and MOPV), but not the New World arenavirus SABV. The possibility of generating a multivalent vaccine against multiple Old and New World arenaviruses was further demonstrated by immunizing HLA transgenic mice with an epitope cocktail covering GPC and NP proteins from 7 arenaviruses (Kotturi et al. 2009). $CD8^+$ T cell responses were induced against the 7 arenaviruses and immunized mice were also protected against challenge with a recombinant vaccinia virus expressing LCMV, LASV or SABV GPC. Highly conserved, cross-reactive $CD8^+$ T cell epitopes (LCMV $GPC_{447-455}$ and LASV $GPC_{441-449}$) were also demonstrated to exist and provide cross-protection against heterologous arenavirus challenge in the mouse (Botten et al. 2010).

Overall, these studies present a proof of concept regarding the possibility of targeting specific T cell epitopes in designing a vaccine against LASV. In addition, these data suggest that the generation of a cross-protective vaccine among various arenaviruses might be possible. On the other hand, all of these data were obtained in artificial models such as humanized transgenic mice bearing only one HLA type or by the lack of protection studies or use of an inadequate challenge model. The practicality of identified immunodominant T cell epitopes thus remains inconclusive.

Immunopathogenesis

The currently available data on the immunology of LASV infection suggests that the immune response to the virus is deficient in fatal cases. A study of certain cytokines (IFN-γ, IL-1β, IL-6, IL-10, IL-12, TNF-α) and chemokines (IL-8 [CXCL8], IP-10 [CXCL10], RANTES [CCL5]) in human patients infected with LASV in West Africa has revealed that the levels of these immune modulators were all elevated in non-fatal cases of Lassa fever (Mahanty et al. 2001). In contrast, their levels in blood samples from fatal cases were all low or undetectable, with the exception of one patient who had high levels of TNF-α, IL-8 (CXCL8) and IL-1β. Although broad variability in the cytokine/chemokine levels within a given study group was present and the total number of patients was low (n=59), this data is supported by *in vitro* studies published by others. For instance, Lukashevich *et al.* have found that monocyte-derived macrophages (MDM), but not monocytes (MO) from human peripheral blood mononuclear cells (PBMC) origin, were permissive to infection with LASV and MOPV, another Old World arenavirus believed to be non-pathogenic (Lukashevich et al. 1999). Both TNF and IL-8 (CXCL8) secretion were suppressed in LASV-infected MDM and human umbilical vein endothelial cells (HUVEC). In contrast, IL-8 (CXCL8) secretion was not suppressed in MOPV-infected HUVEC, which may be related to differences in the pathogenecity of these two viruses. It was also found in a different study that immature monocyte-derived dendritic cells (MDDC) were permissive to LASV infection and secreted IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4) and MCP-1 (CCL2) chemokines (Mahanty et al. 2003). However, these cells were not activated, as observed by the absence of secretion of various cytokines (TNF, IL-1β, IL-6, IL-10, IL-2, IFN-γ and IL-12), lack of a significant

expression of co-stimulatory molecules (CD86, CD80, CD40) and inability to elicit T cell proliferation. Lack of T cell activation or proliferation by LASV-infected DCs, as well as induction of weak memory responses, has also been demonstrated in another *in vitro* study (Pannetier et al. 2011).

These data are supported by a subsequent study which also demonstrated immune suppression of human MDDC and macrophages (MP) infected with LASV (Baize et al. 2004). However, their data was in contrast to the findings from Mahanty et al. regarding the presence of chemokines in culture supernatants (Mahanty et al. 2003). Mahanty et al. showed that IL-8 (CXCL8), MIP-1α (CCL3) and MIP-1β (CCL4) chemokines were present in the supernatants of LASV-infected DCs, whereas no difference was observed between mock and LASV-infected DCs by Baize et al. In addition, Baize et al. supported their findings by demonstrating that immature DCs did not migrate toward LASV-infected DCs in a chemotaxis assay. It was suggested by Baize et al. that the observed discrepancy was due to a difference in the experimental conditions, such as different virus strains. Baize et al. also observed that immature DC produced higher viral titers than MP and mature DC (Baize et al. 2004). The authors further suggested that the lower virus yields observed in MP in comparison to immature DC could be explained by the ability of infected MP to produce Type I IFNs, since virus production increases following blockage of the Type I IFNs in these cells but not in DC with a neutralizing antibody (Baize et al. 2006).

The role of interferons in Lassa infection remains unclear. It was demonstrated in a study by Asper et al. that LASV is susceptible to the antiviral activity of IFN-α and IFN-γ, but not TNF, as seen by a decrease of viral titers following incubation of Huh7 cells

(human origin) or Vero cells (nonhuman primate origin) treated with the above mentioned cytokines (Asper et al. 2004). Interestingly, the authors demonstrated that the susceptibility to IFN activity was slightly higher with the highly pathogenic AV strain than the milder NL and CSF strains of LASV. In contrast to this study in which Huh7 and Vero cells were used, Baize et al. were unable to demonstrate a direct role for IFN-γ in controlling viral replication in human-derived antigen-presenting cells (APCs) (Baize et al. 2006). Another contrasting result was found in an earlier study by Peters et al. in which LASV strain Josiah was resistant to the antiviral effects of IFN-α as assessed by pre-treatment of Vero cells followed by virus infection and plaque assay to determine viral titers (Peters et al. 1989).

The NP of various arenaviruses, including LASV, has been shown to have an antagonistic effect on the Type I IFN response (Martinez-Sobrido et al. 2006; Martinez-Sobrido et al. 2007). This appears to be mediated through exonuclease activity preventing IRF-3 translocation into the nucleus (Hastie et al. 2011) or removing the viral pathogen-associated molecular pattern (PAMP) RNA ligands (Qi et al. 2010). Additionally, LASV NP mutants were shown to induce a strong Type I IFN response in human MO and MP *in vitro* (Carnec et al. 2011). However, given the fact that the NP of both pathogenic and non-pathogenic arenaviruses were found to interfere with Type I IFN production (Martinez-Sobrido et al. 2007) and that Baize et al. have observed a difference in the ability to secrete Type I IFN in MP and DC (Baize et al. 2006), the role of interferons in Lassa fever pathogenesis remains elusive.

Experimental evidence obtained in the common marmoset demonstrated that immunophenotypic alterations are present in the spleen of LASV-infected animals

(Carrion, Jr. et al. 2007a). More specifically, overall numbers of $CD3^+$ and $CD20^+$ lymphocytes were decreased and the architecture of the lymphoid follicles was disrupted. In addition, the architecture of lymph nodes was also impaired and a marked reduction in the intensity of MHC-II antigen staining was noted in the spleen and liver of the infected animals. These findings support previous data suggesting an impairment of the immune response during severe Lassa fever. Moreover, a recent study performed in cynomolgus macaques suggests that the activation of the immune response in fatalities (n=2) is weaker and delayed in comparison to the survivor group (n=4), especially regarding the T cell response (Baize et al. 2009). In addition, IL-6 was present in the fatalities only. Unfortunately, the sample size was limited and further studies are thus required to confirm these results. However, the cytokine and chemokine data are supported by a study by Hensley et al., which has shown that certain pro-inflammatory cytokines and chemokines (IL-1β, IL-6, MCP-1 [CCL2] and eotaxin [CCL11]) were elevated in LASV-infected cynomolgus macaques (Hensley et al. 2011).

Although the general consensus is that fatal LASV infection is associated with an immunosuppressive state, Branco et al. have recently suggested the opposite (Branco et al. 2011). The authors demonstrated that there was a strong correlation between survival and low levels of IL-6, IL-8 [CXCL8], IL-10 and CD40L molecules in human cases of Lassa fever. Their results contrast with the findings of Mahanty et al. (Mahanty et al. 2001) and this discrepancy could be explained by the distinct parameters used to classify subjects as fatal or non-fatal acute LASV cases. For example, Branco et al. only included samples from individuals for which antigen detection was positive, whereas Mahanty et al. used LASV-specific IgM seropositivity as a criteria for including a case in the acute

LASV-infected group. The latter is less specific and has been shown to potentially also include patients not currently infected with LASV since detectable LASV-specific IgM levels can remain for long periods of time following acute LASV infection (Branco et al. 2011).

In conclusion, based on cytokine and immunomodulatory molecule profiles observed *in vitro* and in various animal models and human Lassa fever cases, it is generally believed that LASV infection is associated with an immunosuppressive state in severe cases (Baize et al. 2009; Carrion, Jr. et al. 2007a; Fisher-Hoch et al. 1987; Mahanty et al. 2001). Immune suppression of the primary innate immune cells would result in the inability to induce an antiviral state and to elicit an adaptive immune response, which would be essential to clear the infection. However, recent data suggest the opposite. Further research is thus necessary to clarify this issue, as well as to determine whether the humoral or cell-mediated immune response is responsible for the final virus clearance and immunity.

1.2.8. Therapy

The protective efficacy of immune plasma as a treatment against LASV has been a matter of debate since the early seventies. Preliminary studies in human patients infected with LASV were only short reports of individual cases (Clayton 1977; Keane and Gilles 1977; Leifer, Gocke, and Bourne 1970; Monath and Casals 1975; White 1972) and were not performed as properly organized clinical studies with appropriate controls. Thus unambiguous conclusions regarding the efficacy of LASV immune plasma therapy could not be drawn from these reports. Studies in guinea pigs (Jahrling 1983) have demonstrated that treatment of lethally challenged animals was possible with LASV-

immune plasma if given at days 0, 3 and 6 p.i. Importantly, the efficacy of plasma therapy was found to correlate with the presence of neutralizing antibodies (from the convalescence phase) as assessed by a plaque reduction neutralization test (PRNT), but not with the total antibody response as determined by an IFA test. In addition, a higher dose of plasma was required to protect guinea pigs challenged with a heterologous virus strain. Similar results and conclusions were reached in a later study with cynomolgus monkeys (Jahrling and Peters 1984). The importance of geographical matching of the immune plasma with the virus strain infecting the patient to be treated is also supported by the observation that differences in neutralization efficacy are present among different LASV strains *in vitro* when not matched (Jahrling and Peters 1986). The relationship between the timing to initiation of immune plasma therapy and survival was investigated by Jahrling et al., who concluded that the efficiency decreased as the treatment was delayed (1/8, 1/3 and 5/6 dead/total cynomolgus monkeys with initiation of treatment on day 0, 4 and 7 p.i., respectively) (Jahrling, Peters, and Stephen 1984). One concern which has been raised regarding plasma therapy against Lassa fever infection is the fact that immunologic enhancement of disease has been reported for other arenaviral diseases (Eddy et al. 1975; Maiztegui, Fernandez, and de Damilano 1979). However, this phenomenon was not observed in a Lassa fever cynomolgus macaque study, in which there was no demonstration of increased viremia or accelerated death (Jahrling and Peters 1984). It should be noted though that neurological sequelae, which are thought to result from an immunopathologic process, have not been directly evaluated in this study and thus only obvious neurological damage would have been noticed. Human immune plasma from convalescent patients is limited due to the difficulty in accessing these samples and

the low neutralizing antibody titer measured in these samples in comparison to immune plasma from laboratory animals (Jahrling and Peters 1984). This theory has been used to explain the failure of immune plasma therapy in human cases (McCormick et al. 1986) in comparison to the promising results that had been previously obtained in laboratory animals.

Ribavirin (Sidwell et al. 1972), a nucleoside with broad-spectrum antiviral activity, is the only commercially available antiviral drug that has been shown to be successful as a treatment for LASV infection (Huggins 1989). Both subcutaneous and intramuscular injection of ribavirin on days 0 or 5 p.i. in rhesus macaques, with continued treatment at 8-hr intervals until day 18, resulted in a low viremia and complete survival of the animals in comparison to the untreated animals (Jahrling et al. 1980; Stephen and Jahrling 1979). The rhesus macaques that received the early ribavirin treatment on day 0 developed a mild clinical illness and two of the four animals had no signs of clinical illness at all. Conversely, all animals that received ribavirin from day 5 to 18 p.i. developed a moderately severe disease but survived with no apparent long-term sequelae. In terms of the toxicity of ribavirin use, a preliminary evaluation of a proposed intravenous (i.v.) regimen for treatment of human Lassa fever patients was performed in rhesus macaques and demonstrated that ribavirin treatment does affect hematological parameters, but only transiently, with hemograms reversing to normal values upon cessation of ribavirin treatment (Wannarka, Stephen, and Canonico 1982).

Since both ribavirin and passive immunization require an early initiation of treatment to be effective, an alternate approach based on the combination of both therapies was evaluated in cynomolgus macaques (Jahrling, Peters, and Stephen 1984). It

was shown in this study that this treatment regimen resulted in a significantly enhanced efficacy as compared to each treatment used individually. In brief, 100% survival was achieved in monkeys with a combination therapy, even if treatment was initiated as late as day 10 p.i. Unfortunately, this success was not paralleled in a study involving human Lassa fever patients, in which a 22% case-fatality rate (7/32) was observed in the combination group in comparison to a 20% case-fatality rate (6/30) in the ribavirin alone group and 32% (17/53) in the immune plasma alone group (McCormick et al. 1986). This study nevertheless demonstrated that ribavirin is effective in human cases of Lassa fever, especially when administered within the first six days after the onset of fever (case-fatality rate of 9%, n=11). It was also shown that both i.v. and oral administration of the drug are effective (McCormick et al. 1986).

Overall, the above data suggest that ribavirin treatment is safe, and will increase survival, most likely as a consequence of the decreased viremia. However, its efficacy, both in terms of increasing the survival rate and ameliorating the clinical disease course, depends upon an early start of the treatment. However, contrasting data emerged from human and animal studies, suggesting that further evaluation of this approach is required to determine its efficacy as a therapy against Lassa fever. Recent guidelines do not recommend a liberal use of ribavirin for post-exposure prophylaxis, but does propose its use following high-risk exposure (Bausch et al. 2010). Regarding the immune therapy, the available data suggest that although the immunological studies of natural LASV infection and vaccines strongly imply that cell-mediated immunity plays a major role in controlling and clearing Lassa infection, the development of monoclonal antibodies as a novel therapeutic approach against Lassa fever could be worth investigating in the future.

1.2.9. Vaccine design strategies

Several approaches can be undertaken to develop novel vaccine candidates. Live attenuated, recombinant, subunit, whole inactivated virus and DNA vaccines are only some of the vaccine design strategies that have been developed so far in order to combat viral infections.

Many of the commercially available vaccines are live attenuated, including the Yellow fever vaccine and Varicella-zoster virus vaccine (Ulmer, Valley, and Rappuoli 2006). Live attenuated vaccines can be generated in several ways (Graham and Crowe Jr. 2006). Phenotype attenuation can be achieved by inducing genomic mutations through multiple passages of the virus. Reverse genetics can also allow the introduction of mutations or deletions altering the virulence of the virus. A foreign microbial gene can also be inserted into the recombinant virus, in order to protect against a different infectious agent. Several variants of this approach have been developed, including recombinant vaccinia (Walsh and Dolin 2011) or Vesicular Stomatitis Virus (VSV) (Lawson et al. 1995; McKenna et al. 2003; Roberts et al. 1999) vaccine vectors harboring genes from other pathogenic viruses. Another strategy is to use a different route of inoculation. For example, adenovirus inoculated through the respiratory route causes disease whereas oral administration results in protective immunity without disease manifestations (Edmondson et al. 1966). Live attenuated vaccines are believed to be more efficacious than non-replicating or subunit vaccine candidates and to be better at generating life-long immunity. This is mostly due to the fact that they can activate both arms of the adaptive immune system and can activate the response locally and systemically (Graham and Crowe Jr. 2006). They also usually include multiple antigens,

ensuring that protective immunity is induced. One of the main concerns with the use of live attenuated vaccines is reversion of the virus into a virulent strain through the acquisition of novel mutations, or reassortment in the case of segmented RNA viruses. In addition, live vaccines are thought to be less stable than nonliving vaccines during storage and transport, which can be an important factor in tropical climates. There is also a risk of transmission of the live vaccine to close contacts of vaccinees, which could be problematic in immunocompromised individuals and pregnant women. Vaccination of immunocompromised individuals can also be a problem itself, especially in regions where HIV/AIDS is prevalent.

Inactivated whole virus is another approach that has been successful for many viruses, such as Influenza, Hepatitis A virus, Rabies, Japanese encephalitis and Poliovirus. Inactivation is most often achieved through the use of chemical agents such as formalin or detergents (Ulmer, Valley, and Rappuoli 2006). Advantages include a very low risk of infection and the inclusion of multiple antigens. On the other hand, one limitation is that no mucosal immunity is induced when the parenteral route of immunization is used (Graham and Crowe Jr. 2006). Additionally, an exaggerated immune response has been reported for at least two formalin-inactivated vaccines, against measles and respiratory syncytial virus, with deleterious outcomes occurring in humans (Fulginiti et al. 1967; Kim et al. 1969; van Drunen Littel-van den Hurk et al. 2007).

Recombinant proteins and virus-like particle (VLP) vaccines are classified as subunit vaccines and are replication-incompetent because they lack any genomic nucleic acid (Noad and Roy 2003; Roy and Noad 2009). Acellular vaccines appear safer than whole vaccine candidates because there is no risk of reactivation causing disease and of

severe side effects. On the other hand, they are usually less efficient than live vaccines at stimulating a strong and protective immune response and often require to be combined with an adjuvant to enhance the immune response towards the viral antigens. Nevertheless, there are some successful vaccines which are based on this vaccine strategy. The human papillomavirus vaccine is based on VLPs and has been shown to be very effective so far for the prevention of cervical cancer in vaccinated women (Ghim, Jenson, and Schlegel 1992; Kirnbauer et al. 1992; Rose et al. 1993; Zhou et al. 1991). The Hepatitis B virus vaccine is also based on VLPs.

DNA vaccines are also replication-incompetent. However, intramuscular or intradermal injection results in the DNA uptake by host cells followed by antigen protein expression, thus having the potential to stimulate cell-mediated immunity more efficiently than subunit vaccines. Moreover, there is no induction of antivector immunity. The safety of DNA vaccines has been shown to be excellent so far in clinical trials, and no plasmid DNA integration has been observed. Unfortunately, initial studies have shown that DNA vaccines are less protective and less immunogenic than other vaccine candidates, most likely due to low transfection efficiency (Graham and Crowe Jr. 2006; Ulmer, Valley, and Rappuoli 2006). However, there have been recent developments in the optimization of gene delivery and protein expression and DNA technology continues to be an active area of research in vaccine design and development.

Synthetic peptides have been used experimentally, but a malaria clinical trial with a peptide-based vaccine has been deceptive (Ada 2001). This vaccination strategy is safe but the immune response is usually weaker than for any other vaccine strategy, which is most likely due to the lack of conformational epitopes in this vaccine formulation

(Graham and Crowe Jr. 2006). They are thus useful to identify immunodominant T cell epitopes but do not represent a promising approach as a novel vaccine design strategy.

1.2.9.1. Vaccines against Lassa fever virus

Numerous vaccines have been developed throughout the years with the hope of decreasing the burden of Lassa fever in West Africa where it is endemic, and to protect laboratory workers from being infected while manipulating the virus itself or infected animals. Several vaccine strategies have been developed, ranging from inactivated to live recombinant and DNA vaccine candidates (Table 1.3.).

1.2.9.2. Mopeia virus

The first description of a potential vaccine candidate against LASV occurred in 1979 when Mozambique virus (now named Mopeia virus)-inoculated monkeys were found to be protected against a lethal dose of LASV (Kiley, Lange, and Johnson 1979). However, the use of a live virus such as MOPV is not a currently acceptable vaccine for human use since clear evidence demonstrating that it is not pathogenic in humans does not exist. MOPV infection in humans has not been investigated and NHP studies were only superficial. In a study by Walker et al. (Walker et al. 1982a), histopathologic data from rhesus macaques immunized with MOPV followed by LASV challenge demonstrated the presence of lesions in several tissues, including the central nervous system, but it was not possible in this study to determine whether these lesions were due to MOPV or LASV infection. Liver and kidney cellular damage of MOPV-infected rhesus monkeys was also reported by Lange et al. (Lange et al. 1985). On the other hand, rhesus macaques infected with MOPV were described to produce no viremia, nor fever nor any significant histological, biochemical or hematological abnormalities in

comparison to rhesus monkeys infected with LASV or Mobala virus, another arenavirus believed to be non-pathogenic in humans (Peters et al. 1987). Additionally, studies in strain 13 guinea pigs suggest that MOPV is non-pathogenic in this animal species (Peters et al. 1987). Further studies would thus be required to provide evidence that MOPV is not pathogenic in humans before it can be considered a potential vaccine for human use.

Vaccine platform	**LASV protein(s)**	**Animal model**	**% Survival (survivors/ challenged)**	**References**
Live replication-competent vaccine candidates				
Mozambique virus (Mopeia virus)	None	Rhesus macaques	100% (3/3)	Kiley *et al*., 1979
ML29 reassortant	NP and GPC (L and Z from MOPV)	CBA mice	100% (10/10)	Lukashevich, 1992; Lukashevich *et al.*, 2005; Moshkoff *et al.*, 2007; Carrion Jr. *et al.*, 2007; Lukashevich *et al.*, 2008
		Guinea pigs	100% (10/10)	
		Marmosets	100% (6/6)	
Vaccinia recombinant	NP	Outbred guinea pigs	100% (6/6)	Clegg and Lloyd, 1987 Auperin et al., 1988 Morrison et al., 1989
	GPC	Outbred guinea pigs	100% (11/11)	
	NP GPC NP + GPC	Outbred guinea pigs	94% 79% 58%	
	GPC	Rhesus macaques	100% (4/4)	Fisher-Hoch et al., 1989
	GP1	Cynomolgus and/or rhesus macaques	0% (0/2)	Fisher-Hoch et al., 2000

	GP2 GP1 + GP2 GPC NP GPC+NP		0% (0/2) 100% (2/2) 86% (6/7) 27% (3/11) 88% (7/8)	
Salmonella recombinant	NP	Mice*	36% (5/14)	Djavani *et al.*, 2000; 2001
VSVΔG/LASVGPC	GPC	Cynomolgus macaques	100% (4/4)	Geisbert, Jones *et al.*, 2005
YFV17D/LASV-GPC	GPC GP1+GP2	Strain 13 guinea pigs	80% (4/5) 83% (5/6)	Bredenbeek *et al.*, 2006 Jiang et al., 2010
Replicon vaccine candidates				
Venezuelan equine encephalitis virus replicon	LASV GPC LASV NP LASV GPC + LASV NP LASV GPC /EBOV GP LASV GPC + EBOV GP	Strain 13 guinea pigs	100% (5/5) 100% (5/5) 100% (5/5) 100% (5/5) 80% (4/5)	Pushko et al., 2001
Inactivated vaccine candidates				
Gamma-irradiated purified LASV	Whole virus	Rhesus macaques	0% (0/3)	McCormick *et al.*, 1992
DNA vaccine candidates				
DNA vaccine encoding LASV NP	LASV NP	Mice (LCMV and Pichinde virus only)	N/D**	Rodriguez-Carreno *et al.*, 2005

* intracranial challenge with LCMV
** N/D, not determined.

Table 1.3. Vaccine candidates against LASV infection.

1.2.9.3. Vaccinia recombinant constructs

The first genetically engineered vaccine against LASV was reported in 1987 with the generation of a recombinant vaccinia virus containing the LASV NP gene (Clegg and Lloyd 1987). This preliminary experiment demonstrated that expression of the NP protein only was sufficient to protect outbred guinea pigs against a lethal dose of LASV (n=6). A recombinant vaccinia virus expressing the LASV GPC (named V-LSGPC) was later found to also confer protection to outbred Hartley guinea pigs against LASV: 100% survival was achieved in animals vaccinated with V-LSGPC versus 20% survival in the group vaccinated with wild-type vaccinia versus 33.3% survival in the unvaccinated group (Auperin et al. 1988). However, no anti-LASV antibodies were detected prior to LASV challenge at 21 days post-immunization. Vaccine efficacy to prevent LASV illness was not sterilizing as the V-LSGPC-vaccinated animals developed a transient low-grade fever and viremia was present, though at a greater than 10-fold reduction in comparison to the unvaccinated animals (Auperin et al. 1988). Vaccinia vaccine candidates expressing LASV GPC (79% survival), NP (94% survival) or both together (58% survival) were also found to offer partial protection against a lethal LASV challenge in outbread guinea pigs (Morrison et al. 1989). Fourteen percent survival was observed in unvaccinated animals and 39% survival in animals vaccinated with wild-type vaccinia as controls.

The efficacy of the V-LSGPC vaccine candidate in rhesus monkeys was also evaluated in a follow-up study (Fisher-Hoch et al. 1989). The rhesus macaques did not show any clinical signs of disease or laboratory abnormalities following the immunization, and they were later challenged with LASV. Two animals were challenged

with LASV at day 37 and another two primates at day 284 post-vaccination. All four animals had a brief viremia following LASV challenge and one, challenged at day 37 post-vaccination, remained intermittently febrile for 3 months despite the inability to recover virus after day 7 post-challenge, in either the blood or tissue samples at necropsy. In addition, the four vaccinated animals presented slight laboratory abnormalities representative of a mild LASV illness such as increased serum glutamic-oxaloacetic transaminase (i.e. AST), decreased platelet function, lymphopenia and neutrophilia. Regarding the immune response to the vaccine, the animals presented a LASV-specific humoral immune response to LASV GPC following immunization, which increased post-challenge. However, no neutralizing antibodies were detected before challenge as the result of the vaccine.

An extended study of the vaccinia vaccine platform was later performed to determine whether the expression of GPC, the individual GP subunits, GP1 and GP2, or NP alone or in combination (Auperin et al. 1988; Morrison et al. 1991) would protect NHPs against LASV challenge (Fisher-Hoch et al. 2000). The efficacy of the vaccine decreased while the viremia levels increased as the vaccine-to-challenge time interval was prolonged: the two animals with the longest vaccine-to-challenge intervals, 488 and 700 days, died. One of these animals had received a vaccinia recombinant with LASV GPC and NP and the second animal had reveived one with LASV GPC. Similarly to other studies, no correlation was observed between the antibody levels and survival/death of the NHPs and once again the animals demonstrated some laboratory abnormalities suggesting a mild LASV disease in the vaccinated animals. Regarding the efficacy of incorporating the various Lassa viral proteins into the vaccinia vector, it was concluded

from this study that expression of GP1 or GP2 individually was unsuccessful (none of the animals in these two groups survived) and thus expression of both together was required to achieve 100% survival of the animals. Only three of the eleven macaques immunized with the recombinant vaccinia expressing the LASV NP survived following LASV challenge (a 27% survival rate) (Fisher-Hoch et al. 2000), in contrast to a previous study in guinea pigs in which 100% survival was achieved (Clegg and Lloyd 1987). Whether this discrepancy is due to a difference in the animal species, vaccinia constructs, route and dose of injection or other experimental parameters remains unknown. Interestingly, it was noted that all animals vaccinated with the construct expressing the LASV NP and receiving the highest LASV challenge dose (10^4 PFU) died, and on average 2 days earlier than the controls, with higher viremia levels (Fisher-Hoch et al. 2000).

1.2.9.4. Alphavirus replicons

Individual and bivalent vaccines against LASV and EBOV have been generated using an RNA replicon system developed based on the attenuated strain of Venezuelan equine encephalitis (VEE) virus (Pushko et al. 1997; Pushko et al. 2001). In this system, gene expression is limited to the cells initially infected with the replicon particles and this vaccine platform is thus replication-incompetent. Strain 13 guinea pigs (n=5) immunized with monovalent or bivalent vaccines expressing LASV NP, LASV GPC and EBOV GP were challenged 28 days following the second booster injection with a lethal dose of homologous virus (Pushko et al. 2001). All animals survived with the exception of one animal in the group which was immunized with a combination of a VEE vector expressing LASV GPC and one vector expressing EBOV GP (LASVGPC+EBOVGP group). Two animals also succumbed to EBOV challenge in the group immunized with a

single VEE vector expressing both EBOV GP and LASV GPC (LASVGPC/EBOVGP group).

1.2.9.5. Recombinant *Salmonella*

In order to evaluate the feasibility of developing a mucosal vaccine against Lassa fever, Djavani et al. generated an attenuated form of *Salmonella typhimurium* expressing the LASV NP and investigated the immune response in mice following intragastric delivery of the vaccine construct (Djavani et al. 2000). BALB/c mice received the vaccine on day 0 and a boost on day 21. Local mucosal IgA and serum IgG were both present, in addition to an NP-specific CTL response as determined by a ^{51}Cr release assay. Similar results were obtained with a control vaccinia construct expressing the LASV NP which was also administered intragastrically. Furthermore, the recombinant *Salmonella* vaccine partially cross-protected mice from a lethal i.c. challenge with LCMV (Djavani et al. 2001). Five out of 14 mice (36%) which received the *Salmonella* expressing the LASV NP survived, whereas 4 out of 12 animals (33%) vaccinated with the vaccinia construct survived. All mice that received a vaccinia vector expressing LCMV NP survived (n=20). Whether mucosal administration of a Lassa fever vaccine offers protection upon homologous virus challenge remains to be evaluated.

1.2.9.6. Inactivated Lassa virus

Inactivated LASV was found to be ineffective in a NHP model of LASV (McCormick et al. 1992). In this study, three rhesus macaques received six doses of gamma-irradiated LASV in combination with the diphtheria-tetanus-pertussis (DPT) vaccine as an adjuvant. Two control macaques were not vaccinated. All five NHPs were then challenged with a lethal dose of LASV. Interestingly, a difference was observed in

the humoral immune response between the two groups. Although no IgM antibodies were detected, IgG antibody levels were detected in the vaccinated monkeys by IFA and radioimmunoprecipitation (RIP) prior to LASV challenge. In contrast, only one of the three vaccinated macaques developed neutralizing antibody in a PRNT assay. The IgG antibody levels increased much faster in the vaccinated animals post-LASV challenge as compared to the unvaccinated ones. IgM antibodies developed in all five animals by day 12 following LASV infection but no neutralizing antibodies were detected post-challenge. Although a difference was observed in the humoral immune response between the two groups, viremia, leukocyte counts, platelet function and AST levels were comparable among all five monkeys. In addition, while the unvaccinated macaques died on days 12 and 13 post-challenge, the time to death in the vaccinated animals was delayed (days 21, 15 and 21). This data suggests that NHPs vaccinated with a gamma-irradiated LASV vaccine are able to mount a humoral immune response against LASV. However, although death was delayed in these animals, all eventually succumbed, suggesting that this inactivated vaccine adjuvanted with DPT is ineffective in protecting against a lethal dose of LASV. It should be noted that irradiation may have been a suboptimal inactivation method resulting in the destruction of key structural epitopes and DPT, whilst potent, is not a clinically relevant adjuvant system.

1.2.9.7. Mopeia/Lassa reassortant virus

In 1992, Lukashevich described the generation of a Mopeia/Lassa reassortant virus named ML29. Co-infection of Vero cells with the two wild-type viruses resulted in the isolation and purification of a reassortant virus containing the MOPV L RNA segment, encoding the L polymerase and Z, and the LASV S RNA segment, encoding NP

and GPC (Lukashevich 1992). CBA mice inoculated i.c. with LASV were protected when adoptive transfer of immune spleen cells from ML29-immunized animals was performed 2 to 3 hours p.i. Interestingly, the opposite results were observed when the same experiment was performed with MOPV instead of the ML29 reassortant virus: CBA mice succumbed to LASV i.c. challenge following adoptive transfer of splenocytes from MOPV-immunized animals. This was unexpected since a NHP study had previously shown that MOPV infection results in protection of macaques subsequently challenged with LASV (Kiley, Lange, and Johnson 1979), as described above (Section 1.2.9.1.). However, the route of inoculation and the animal model differed between the two studies and this could explain the observed discrepancy regarding the cross-protective effect of MOPV.

In a second publication, the authors demonstrated that depletion of T cells, but not B cells, resulted in the loss of the therapeutic effect of ML29-immune splenocytes adoptively transferred to CBA mice i.c. challenged with LASV (Lukashevich et al. 2005). Furthermore, the ML29 reassortant, as well as MOPV, protected strain 13 guinea pigs against a lethal dose of LASV. Safety of this potential vaccine was also studied in two rhesus macaques which received 10^3 PFU of the ML29 reassortant s.c. No clinical, hematological or chemical abnormalities were observed but viremia was detected up to day 21 post-vaccination by RT-PCR but not by plaque assay. ML29 antigen-specific IgG antibodies were detected by day 14 post-immunization in both animals but neutralizing antibodies were undetectable. IFN-γ positive cells were present by day 7 following *in vitro* stimulation with ML29 virus, as assessed by ELISPOT. The animals were not challenged with LASV.

The ML29 gene expression profile in infected cells revealed that it resembles the profile of mock-infected cells rather than the one of MOPV-infected cells (Lukashevich et al. 2008). The authors suggest that the attenuated phenotype of the ML29 reassortant in cell culture is due to the presence of the L RNA segment of MOPV origin and to mutations introduced during selection of this virus in comparison to the parental strains (Lukashevich et al. 2005; Moshkoff, Salvato, and Lukashevich 2007). Protective efficacy of this vaccine against heterologous challenge with a distantly related LASV strain and LCMV was also evaluated (Carrion, Jr. et al. 2007b). Strain 13 guinea pigs were vaccinated with the ML29 reassortant, generated from the LASV strain Josiah and later challenged with LASV strain Josiah/SL, LASV strain 803213/NIG or LCMV strain WE. The vaccine fully protected the animals from the two LASV strains and although the animals challenged with LCMV WE did not survive, their death was delayed in comparison to the unvaccinated animals which received LCMV WE (16-21 days versus 13-14 days post-challenge, respectively). These experiments suggest that the ML29 reassortant can protect strain 13 guinea pigs against homologous and heterologous LASV strains, and can also induce some level of cross-protection against LCMV, a different Old World arenavirus.

The safety, immunogenicity and efficacy of the ML29 vaccine candidate was also assessed in the common marmoset, *Callithrix jacchus* (Lukashevich et al. 2008), a newly developed animal model for LASV infection (Carrion, Jr. et al. 2007a) (Section 1.2.6.1.). No clinical symptoms or significant hematological and biochemical changes were observed in the ML29-immunized marmosets. In addition, six vaccinated marmosets were challenged with LASV-Josiah and survived without showing any clinical,

hematological and biochemical manifestations of illness whereas the four unvaccinated animals that were challenged met humane end-point criteria by day 17 to 21 following LASV challenge. Viremia was detected in all unvaccinated control animals but only in three of the vaccinated ones and was undetectable by day 15 post-challenge, as assessed by both plaque assay and RT-PCR.

1.2.9.8. DNA vaccine

Another vaccine platform has been a DNA vaccine encoding the full-length LASV NP (Rodriguez-Carreno et al. 2005). Unfortunately, homologous challenge with LASV was not performed in this study. However, this vaccine platform was shown to reduce viral titers following heterologous i.p. challenge in the mouse and to contain protective epitopes against both Old World and New World arenaviruses (LCMV and Pichinde virus, respectively). The authors thus suggested that the development of a "pan-arenavirus" vaccine might be possible in the future. However, the fact that these results were only observed in BALB/c mice and not C57BL/6 mice indicates that the host genetic background influences greatly the effectiveness of a vaccine and weakens the probability that protection against multiple and genetically distinct arenaviruses could be achieved in humans due to the great genetic diversity of this host species.

1.2.9.9. Yellow Fever recombinant vaccine

One of the newest vaccine candidates is the recombinant Yellow Fever vaccine (YFV17D) expressing the full-length LASV GPC protein (YFV17D/LASV-GPC) (Bredenbeek et al. 2006). The chimeric YFV17D/LASV-GPC vaccine elicited an antibody response in outbred Hartley guinea pigs following a boost immunization but only partially protected strain 13 guinea pigs against a lethal LASV challenge following a

single immunization dose (4/5 of the animals survived). The partial protection in this case, in contrast to the complete protection of many other live-attenuated vaccine candidates, could be explained by the fact that the YFV17D/LASV-GPC vaccine contains the GPC gene of LASV strain AV and that the challenge was performed using a heterologous virus strain, Josiah. In addition, combining GPC with other viral antigens, such as NP, and increasing the efficiency of GPC cleavage into the GP1 and GP2 subunits might also help to increase the protective efficacy of this vaccine in the future. Despite the lack of complete protection against LASV challenge, this particular vaccine was considered to be promising for several reasons, including the fact that the YFV17D vaccine is well-known for eliciting long-lasting immunity against Yellow fever and has been used safely and efficaciously in humans for over 70 years (Monath, Cetron, and Teuwen 2008). In addition, several chimeric YFV17D vaccines have been evaluated in the recent past and are currently in clinical trials (Monath, Cetron, and Teuwen 2008). Finally, the overlap in the regions of endemicity for Yellow Fever and LASV makes this vaccine platform suitable for preventing human infection by both viruses simultaneously (Jiang et al. 2010). Unfortunately, further studies of this vaccine platform have been impaired by the fact that the YFV17D/LASV-GPC construct was found to be unstable in the long term (Jiang et al. 2010). The authors thus generated new vaccine constructs expressing the LASV GP1 and GP2 separately and immunized strain 13 guinea pigs with a mixture of these two YF17D-based viruses (Jiang et al. 2010). Five out of six of the animals survived to a lethal dose of LASV but disappointingly presented signs of disease such as weight loss and elevated body temperature. LASV was also detected in the various sampled tissues at necropsy on day 25 post-LASV challenge. This vaccine thus

potentially offers several advantages over the other proposed vaccines but requires further optimization before it can be considered a realistic approach.

1.2.9.10. Recombinant VSV vaccine

Our laboratory, in collaboration with Geisbert et al., has developed live attenuated recombinant vaccines against LASV, Ebola virus (EBOV) and Marburg virus (MARV) that are based on the VSV vector with the native VSV glycoprotein removed and functionally replaced by the full-length glycoprotein from either LASV (VSVΔG/LVGPC), EBOV (VSVΔG/ZEBOVGP) and MARV (VSVΔG/MARVGP) (Garbutt et al. 2004). These vaccines have been demonstrated, in preliminary studies, to protect NHPs against homologous lethal EBOV, MARV (Jones et al. 2005) and LASV infections (Geisbert et al. 2005). In the latter study, four cynomolgus macaques were immunized i.m. with VSVΔG/LVGPC (LASV GPC, strain Josiah) and two macaques with VSVΔG/ZEBOVGP. The vaccines did not cause any apparent signs of illness and the six NHPs were thus challenged 28 days later with a lethal dose of LASV strain Josiah. The two macaques immunized with the irrelevant vaccine, the VSVΔG/ZEBOVGP construct, showed signs of illness by day 3 following LASV infection and were euthanized on days 11 and 13 because of the severity of their symptoms. On the other hand, the four macaques immunized with the VSVΔG/LVGPC vaccine were completely protected from LASV infection. The biochemical and hematological parameters did not change in comparison to pre-challenge values, except for a slight increase in ALT levels in two of the animals on day 7 and a third one on day 10 post-challenge. Additionally, there was a slight decrease in the platelet counts in three of the four animals on day 7 post-challenge in the VSVΔG/LVGPC-immunized

macaques. LASV RNA was detected by RT-PCR in the blood of one of the two control animals by day 3 post-challenge, but by day 7 all animals from both groups were viremic to a similar extent. The VSVΔG/LVGPC-immunized macaques had cleared the viremia by day 10 post-challenge whereas LASV RNA levels continued to increase in the control monkeys until they reached the humane end-point and were euthanized on day 11 and 13 post-challenge. The immune response was also studied and it was found that the VSVΔG/LVGPC vaccine elicited IgG antibodies detectable by day 14 post-vaccination in two of the four monkeys and by day 28 post-vaccination in the other two macaques. However, the presence of neutralizing antibodies was only detected 14 days post-challenge in the four VSVΔG/LVGPC-immunized macaques. The cellular immune response was assessed by intracellular cytokine staining of IFN-γ and TNF-α in $CD4^+$ and $CD8^+$ T lymphocytes. Only three of the four VSVΔG/LVGPC-immunized monkeys had a detectable level of IFN-γ and TNF-α positive T cells post-challenge. No correlation between humoral or cellular immunity and protection can thus be drawn from this study.

Overall, various vaccine strategies have been developed against Lassa fever, but some of them appear more promising than others. Live attenuated vaccines appear superior in inducing an adaptive immune response to Lassa viral antigens and providing protection in animal models in comparison to non-replicating vaccine candidates. The inactivated LASV was unsuccessful in providing protection against a lethal challenge in the rhesus macaque model. However, death was delayed in this case, suggesting that the use of different conditions, such as the use of another inactivation procedure or adjuvant, or the development of subunit vaccines might be a possible alternative to live vaccines. One possible limitation of replication-competent vaccines is that they have a limited use

in immunocompromised patients, especially in Sub-Saharan Africa, where HIV rates are the highest. On the other hand, reverse genetics systems for LCMV and LASV are now available, which open the door to the design of novel recombinant vaccines against arenaviruses (Albarino et al. 2011a; Bergthaler et al. 2006). There is also a new study describing the generation of a recombinant chimeric JUNV/LASV vaccine (Albarino et al. 2011b). This chimera was generated from cDNA based on the Candid1 vaccine against JUNV and viral glycoprotein exchange was performed with LASV. However, immunogenicity and protection studies have not yet been performed with this novel potential vaccine candidate (Albarino et al. 2011b). If a vaccine based on an arenavirus genetics systems is eventually shown to confer protection against multiple pathogenic arenaviruses, this could offer an interesting alternative vaccine candidate.

1.3. Global objectives and hypotheses

1.3.1. Global rationale

Lassa virus is an "Old World" arenavirus that causes a hemorrhagic fever and is endemic in West Africa. LASV causes the majority of VHF cases in Africa and LASV infections are among the most imported VHF cases. The virus is transmitted to humans mainly by the urine and feces of the multimamate rat, *Mastomys natalensis*. However, person-to-person spread can also occur, particularly in a hospital setting, often resulting in significant outbreaks of disease and a breakdown in the local health care system. Years of continued effort have shown that rodent control is currently unrealistic and that ribavirin antiviral therapy, although appearing effective, is not presently approved for Lassa fever by the US Food and Drug administration, and is far too expensive for use in

Africa. There is thus a need for the understanding of the immune response to LASV and for the development of a vaccine.

Development of a successful vaccine is supported by the fact that a natural infection with LASV provides long-term immunity (Fisher-Hoch and McCormick, 2004). In addition, several vaccines against Lassa fever have been made but so far none of them has been successful (Fisher-Hoch and McCormick, 2001; 2004). As there is still a need for an effective vaccine, this project proposes to create two vaccines, using two different vaccine platforms, and test their efficacy and ability to generate an immune response in a mouse model. Our laboratory, in collaboration with Geisbert et al., has developed a live-recombinant vaccine against LASV, the VSVΔG/LVGPC vaccine (Geisbert et al. 2005). In preliminary studies, the vaccine has shown the ability to induce strong humoral and cellular immunity, and protected NHPs against a lethal LASV challenge (Geisbert et al. 2005). In addition, we have developed a recombinant subunit vaccine. Upon expression of GPC, Z and NP in a eukaryotic system, we have produced virus-like particles (VLPs), which are enveloped LASV particles lacking the viral genome. Although they resemble authentic virus particles, they are replication-incompetent. There is currently no available data comparing the immunogenicity of the various Lassa viral antigens. Nevertheless, the glycoproteins have been shown to provide protection in previous vaccine studies. In addition, an issue regarding the inclusion of NP in a vaccine against Lassa fever has been raised following the study in which the recombinant vaccinia vaccine expressing the NP appeared to be detrimental in NHPs (Fisher-Hoch et al. 2000). We thus decided to use the glycoprotein in both vaccine platforms as immunogen. The matrix protein Z was also

expressed in the VLPs as it is known to be the driving force for LASV VLP budding (Perez, Craven, and de la Torre 2003; Strecker et al. 2003).

In addition, there is a lack of a small animal model to study vaccines and immunity to LASV. Three different animal species can be used as animal models for LASV: the mouse, the guinea pig and the non-human primate models. In the mouse model, only intracranial injection has been shown to result in death of the animal and can thus be used as a lethal challenge model (Lukashevich, 1985). The mouse model is advantageous in comparison to the other two animal models, because there are significantly more immunological reagents available, and many knock-out mouse strains have been developed, allowing us to carefully dissect the immune response to the vaccine. This mouse model could permit the study of the immunology and pathogenesis of Lassa fever, and the immune response to vaccines against LASV.

1.3.2. Hypotheses

This PhD project can be divided into two complementary sections. The first concerns the development and characterization of two vaccine platforms against Lassa fever. The second one is the establishment of a novel mouse model of Lassa fever.

1.3.2.1. Development and characterization of vaccines against Lassa fever

- The VSVΔG/LVGPC vaccine, which is a live attenuated vaccine, induces a strong humoral and cellular immune response in the mouse.
- Lassa virus-like-particles will be generated and used as a subunit vaccine, which will induce a weaker immune response in comparison to the

VSVΔG/LVGPC vaccine. However, this could be overcome by the use of an adjuvant to boost the immune response to the VLP vaccine.

1.3.2.2. Establishment of a novel mouse model of Lassa fever

- Susceptibility to LASV infection and lethality is achievable in the mouse using immunodeficient mice.

1.3.3. Objectives

1) To characterize the humoral and cellular immune response to a live-attenuated vaccine, the VSVΔG/LVGPC vaccine, and to compare this vaccine candidate to a new subunit vaccine based on LASV virus-like particles;
2) To establish a novel small animal model of Lassa fever using immunodeficient mice for vaccine testing.

2. Materials and Methods

2.1. Cell lines

Human embryonic kidney 293T cells (Hans Schnittler, TU-Dresden, Germany) and Vero E6 African green monkey kidney cells (American Type Culture Collection, ATCC CRL-1586) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 10% of γ-irradiated fetal bovine serum (FBS) (Wisent, St Bruno, QC) and 2 mM L-glutamine (Invitrogen). The cells were grown in a 37°C humidified 5% CO_2 incubator. For Vero E6 cells, the medium was also supplemented with 100 units/ml penicillin G (Invitrogen) and 100 μg/ml streptomycin sulphate (Invitrogen). The 293T cells were maintained by splitting them 1:6 when 90% confluent, and the Vero E6 cells were split 1:8 when 100% confluent. To split the adherent cells, media was discarded, cells were washed once with phosphate buffered saline (PBS), and 0.25% trypsin (Invitrogen) was added to detach the cells. Plain DMEM was added to inactivate the trypsin at a 1:1 ratio and the cell suspension was cultured with supplemented DMEM as mentioned above.

2.2. Viruses

Recombinant VSV (rVSV) was generated based on the reverse genetics system developed by Dr. John Rose and collaborators (Lawson et al. 1995) and kindly provided by Alex Silaghi (University of Manitoba and NML, Winnipeg, Canada). LASV strain Josiah was kindly provided by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID, Frederick, Maryland, USA). For *in vitro* experiments, the viral stock of LASV strain Josiah prepared by Thomas Strecker in April 2005 ($1.23X10^5$ PFU/mL as determined by plaque assay) was used, whereas the viral stock of LASV

strain Josiah ($6.2X10^6$ PFU/mL as determined by plaque assay) prepared by Friedericke Feldmann in May 2007 was used for the animal experiments.

2.2.1. Recombinant VSV virus stock preparation

Vero E6 cells were split one day prior to infection in T-150 flasks. Virus was diluted in plain DMEM and a 10 mL inoculum was added to each T-150 flask after the medium was removed. Virus adsorption was performed for 1 hr at 37°C, 5% CO_2 and the flasks were rocked every 15 min. The inoculum was removed and 35 mL of DMEM supplemented with 2% FBS and 2 mM L-glutamine was added. The flasks were incubated at 37°C, 5% CO_2 until 3+ cytopathic effect (CPE) was visible (20% of the cell monolayer still intact). The cells were gently detached with a cell scraper and supernatants were harvested and centrifuged at 2,500 rpm for 10 min at 4°C. Supernatant was transferred to a new tube and FBS was added to obtain a final concentration of 10%. The virus stock was aliquoted and stored at -80°C or in liquid nitrogen for long-term storage.

2.2.2. Plaque assay

Vero E6 cells were grown as for cell culture (see above) in 6-well plates. Confluent Vero E6 cells were infected with serially diluted VSVΔG/LVGPC strain Josiah or LASV strain Josiah. The 10-fold serial dilutions were prepared by adding 0.05 ml of the virus stock into 5 ml plain DMEM (10^{-2} dilution) and then transferring 0.5 ml into the next tube containing 4.5 ml plain DMEM (10^{-3} dilution to 10^{-8} dilution). Vortexing was performed before each transfer. Medium was removed from the cells and 0.5 mL of each virus dilution was added to each well of the 6-well plate in duplicate. For the mock, 0.5 mL of plain DMEM was added to the cells rather than virus. Plates were rocked in a

circular motion to cover them entirely every 15 min during adsorption (one hour at 37°C, 5% CO_2). The agar overlay was prepared by mixing 2X MEM (Invitrogen) containing 4% FBS with autoclaved 2% (W/V) low melting point agarose (Invitrogen) in a 1:1 ratio. After adsorption, the virus inoculum was removed and 2 mL of the agar overlay was poured into each well of the 6-well plate and allowed to solidify for 30 min at room temperature (RT), after which the cultures were incubated in a 37°C humidified 5% CO_2 incubator until plaques were visible. The cells were then stained and fixed with 2 mL crystal violet working solution (1 part stock crystal violet, 1 part 37% formaldehyde and 8 parts ddH_2O) per well for 24 hr. The crystal violet stock solution was composed of 2% crystal violet, 2 parts 100% ethanol, 1 part 37% formaldehyde, and 7 parts ddH_2O. The agar overlay was then removed and the number of plaques was counted. The following formula was used to determine the plaque-forming unit (PFU) count using the dilutions that gave between 20 and 200 PFUs in a well:

$$\text{PFU/mL} = \frac{\text{mean PFU count}}{\text{0.5 ml}} \text{ X dilution factor}$$

2.3. SDS-PAGE and Western blotting (WB)

Samples were mixed with 4X gel loading buffer (25 mL 0.5M Tris-HCl pH6.8; 4g SDS; 20 mL glycerol; 0.2 g bromophenol blue in 50 mL H_2O) containing β-mercaptoethanol (βME) (0.8 mL βME in 9.2 mL 4X gel loading buffer). Samples were heated at 100°C for 10 min, once immediately after adding the 4X gel loading buffer and once prior to gel loading. Magic Mark XP Western Blot Standard (Invitrogen) and SeeBlue Plus 2 Prestained Standard (Invitrogen) were used as molecular weight standards.

Samples were loaded on 10% or 12% polyacrylamide Tris-glycine gels. A 10% resolving gel was prepared as follows: 4.8 mL of sterile ddH_2O, 2.5 mL of 40% acrylamide/bis solution (Bio-Rad Laboratories, California, USA), 2.5 mL of 1.5 M Tris-HCl pH 8.8, 100 μL of 10% SDS (Fisher Scientific, Ontario, Canada), 100 μL of 10% ammonium persulfate (APS, Sigma-Aldrich) and 4 μL tetramethylethylenediamine (TEMED, Fisher Scientific). A 12% resolving gel was prepared as follows: 4.3 mL of sterile ddH_2O, 3.0 mL of 40% acrylamide/bis solution, 2.5 mL of 1.5 M Tris-HCl pH 8.8, 100 μL of 10% SDS, 100 μL of 10% ammonium persulfate and 4 μL TEMED. The 4% stacking gel was made up of 3.15 mL of sterile water, 0.5 mL of 40% acrylamide/bis solution, 1.25 mL of 1.0 M Tris-HCl, 50 μL of 10% SDS, 50 μL of 10% APS and 8 μL TEMED.

Electrophoresis was performed at a constant voltage (120 V) in 1X Tris-glycine buffer (10X Tris-glycine buffer: 30.28g Trizma base, 144.13g Glycine F in 1L of ddH_2O, sterile). Electrophoretic transfer of proteins from the gels to polyvinylidene fluoride membranes (PVDF, Hybond-P, GE HealthCare Buckinghamshire, UK) was carried out using semi-dry transfer (Trans-Blot SD semi-dry transfer cell, Bio-Rad) at 65 mA per gel for 2 hr. For the transfer, the gel and membrane were sandwiched between two filter pads. The first filter pad was soaked in anode buffer (75 mL of 0.67 M Boric acid, 200 mL of methanol and 725 mL of ddH_2O). The PVDF membrane was incubated in methanol for 5 min and then in anode buffer. The gel was also soaked in anode buffer briefly then placed on the membrane. The final filter pad was wetted in cathode buffer (75 mL of 0.67 M Boric acid, 50 mL of methanol and 875 mL of ddH_2O) and laid on top of the gel.

Following transfer, the membranes were blocked with blocking buffer (0.1% Tween20 in PBS (PBS/T) and 6% skim milk) overnight (O/N) at 4°C. Membranes were then sequentially incubated with the primary antibody in blocking buffer for 1 hr at RT, washed four times for 10 min in PBS/T, incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) for 1 hr at RT and washed four times 15 min in PBS/T and two times 5 min in PBS. Bound antibodies were visualized using chemiluminescence, according to the manufacturer's directions (ECL^{TM} Plus Western Blotting Detection Reagents Kit, GE HealthCare), and exposed to X-ray film (Hyperfilm, GE HealthCare) with desired film exposures.

Anti-LASV GP2 rabbit polyclonal antibody #3 or #4 was used at 1:2,000 dilution, anti-LASV Z rabbit polyclonal antibody was used at 1:40,000 dilution and anti-LASV NP rabbit polyclonal antibody was used at a 1:6,000 dilution. These anti-LASV antibodies were kindly provided by Thomas Strecker (Institute of Virology, Philipps University Marburg, Marburg, Germany). The secondary antibody was an anti-rabbit immunoglobulin G (IgG)-HRP-conjugated antibody (Sigma-Aldrich, St. Louis, MO) used at a 1:2,000, 1:25,000 or 1:70,000 dilutions, respectively.

2.3.1. Membrane stripping

To remove both primary and secondary antibodies to reprobe with a different antibody, membrane stripping was performed. After chemiluminescence, the membrane was washed 3 times with PBS/T. Membrane stripping was performed at 60°C for 30 min using stripping buffer (62.5 mM Tris-HCl, 2% SDS, 0.1M βME, pH 6.7). The membrane was then rinsed once in ddH_2O and washed 3 times with PBS/T. The WB protocol was then repeated.

2.4. Mouse studies

CBA mice were obtained from Taconic (Germantown, NY, USA) and BALB/c and C57BL/6 mice from Charles River Laboratories (Saint-Constant, Quebec, Canada).

2.4.1. Animal ethics

All experiments involving animal work were conducted following approval by the Canadian Science Centre for Human and Health Animal Care Committee (AUD # H-06-011) based on the guidelines of the Canadian Council on Animal Care.

2.5. Quantitative ELISA

2.5.1. LASV antigen preparation

Vero E6 cells were infected with a 1:100 dilution of LASV strain Josiah (virus stock of $1.23X10^5$ PFU/mL). A 10 mL inoculum was added to each T-150 flask and virus adsorption was performed for 1 hr at 37°C, 5% CO_2 and the flasks were rocked every 15 min. The inoculum was removed; 35 mL of DMEM supplemented with 2% FBS and 2 mM L-glutamine was added, and the cells were incubated at 37°C, 5% CO_2. LASV-infected cells were collected when CPE was observed with cells still attached, and transferred to 50 mL centrifuge tubes for centrifugation at 3,000 rpm for 10 min. The pellets were resuspended in 4 mL borate saline (50 mM H_3BO_3; 120 mM NaCl; 24 mM NaOH, pH 9) per flask and centrifuged for 10 min at 3000 rpm. Supernatants were discarded and pellets were washed with 45 mL borate saline and centrifuged for 10 min at 3,000 rpm. The pellets were resuspended in 2 mL borate saline/1% Triton X-100 per flask, transferred to a 15 mL centrifuge tube and frozen at -20°C until ready for γ-irradiation (5 Mrad). The suspension was then sonicated (Vibra Cell, Sonics and Materials, Danbury, CT) for 10 min at an intensity of 50% and centrifuged for 10 min at

10,000 xg. The supernatant was then collected, aliquoted and stored at -20°C. Prior to use, the protein concentration of the LASV cell lysate antigen was measured using the DC Protein Assay (Bio-Rad) according to the manufacturer's protocol. A serial dilution of bovine serum albumin (BSA) served as the protein standard.

2.5.2. Mouse serum samples

Mouse serum samples were collected from the saphenous vein or by cardiac puncture using Microtainer serum separator tubes (BD, New Jersey, USA) and centrifuged at 12,000 rpm for 2 min before harvesting the serum fraction and storage at -20°C.

2.5.3. ELISA protocol

Coating for the standard curve was performed with goat anti-mouse Fab (Bethyl Laboratories, Texas, USA) in PBS at a dilution of 1:1,000 (1 µg/mL), whereas a LASV-infected cell lysate (5 µg/mL) was used for the mouse serum samples. The plates were incubated for 1 hr at 37°C before the coating solution was discarded and blocking was performed O/N at 4°C with 2% skim milk in PBS. For the standard curve, a two-fold serial dilution of mouse purified IgA, IgM, IgG1, IgG2a, IgG2b or IgG3 (Mouse Reference Serum, Bethyl Laboratories) was performed with concentrations ranging from 0 to 1000 ng/mL in blocking buffer. The mouse serum samples were diluted 1:100 in blocking buffer. The plates were then incubated for 1 hr at 37°C before washing the plates and adding the detection antibody at 1:1,000 for 1 hr at 37°C. The plates were washed again and the substrate was added according to the manufacturer's protocol before reading the optical densities (OD) at a wavelength of 405.

For the data analysis, the OD of the blank was substracted from all the standard and sample OD values. The immunoglobulin (Ig) protein concentration of the mouse serum samples was extrapolated from a standard curve generated using GraphPad InStat V5.04 (GraphPad Software, CA, USA).

2.6. ELISPOT assay

2.6.1. Preparation of mouse splenocytes

Each spleen was individually homogenized with a 5 mL syringe plunger in 5 mL plain RPMI 1640 (Invitrogen) on a cell screen and the homogenized solution was filtered through a 40 µm nylon cell strainer (BD Biosciences, Ontario, Canada). The cell screen was washed once with 7 mL of plain RPMI 1640 and the homogenized spleen was then centrifuged for 6 min at 300 xg. The red blood cells were lysed by resuspending the pellet in 5 mL lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 1 mM EDTA, ph 7.6) for 4 min at RT. Twenty mL of plain RPMI 1640 was added and the sample was centrifuged for 10 min at 200 xg. The pellet was washed twice before being resuspended in 10 mL of supplemented RPMI (10 mM MEM non-essential amino acids; 100 mM sodium pyruvate; 5 mL HEPES buffer; 10% FBS; 100 units/ml penicillin G; 100 µg/ml streptomycin sulphate; $5X10^{-3}$ M 2-mercaptoethanol) and incubated in a 37°C humidified 5% CO_2 incubator during the cell counting in a 50 mL centrifuge tube with the cap loose. Ten µL of the cell suspension was added to 10 µL of 0.4% trypan blue (Invitrogen) and 80 µL of Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium (Invitrogen). Ten µL of this mixture was then added to a hemocytometer and the number of trypan blue-free cells in 4 square millimiters was counted. The cell density was obtained by the following formula:

Number of cells/mm^3 = Number of cells/mm^2 X dilution X 10

2.6.2. ELISPOT protocol

The ELISPOT assay was performed according to the manufacturer's protocol (BD Biosciences) for the following mouse cytokines: IFN-γ, TNF, IL-2, IL-4, IL-10 and IL-12p70. Briefly, 96-well filter plates (Millipore, Massachusetts, USA) were covered with the appropriate capture antibody in coating buffer (PBS) and incubated O/N at 4°C. The unbound antibodies were discarded and the wells were washed once with blocking solution (RPMI 1640 containing 10% FBS, 1,000 units/ml penicillin G, 1,000 μg/ml streptomycin sulphate and 2 mM L-glutamine). The plates were then incubated with blocking solution for 2 hr at RT. Plain RPMI 1640 (negative control), a mitogen (positive control) or a LASV peptide pool (Section 2.6.2.1.) was added to the wells in triplicate for cell stimulation. The positive control Concanavalin A (ConA) (Sigma-Aldrich) was used as a mitogen to stimulate non-specific cytokine production, at a final concentration of 2.5 μg/mL for IFN-γ, TNF, IL-2 and IL-4 and a final concentration of 25 μg/mL for IL-10 and IL-12p70. In addition, the influenza peptide IYSTVASSL (GenScript, New Jersey, USA) was used as a second negative control at the same concentration as the LASV peptides (3 μg/mL per peptide). The wells were then plated with mouse splenocytes at a final density of $4X10^5$ cells per well. The plated cells were incubated for 20 hr in a 37°C humidified 5% CO_2 incubator. Following incubation, plates were washed twice with ddH_2O to lyse and remove the cells and three times with wash buffer I (PBS containing 0.05% Tween-20). The plates were then incubated with the appropriate detection antibody in dilution buffer (PBS containing 10% FBS) for 2 hr at RT. Plates were washed again three times with Wash buffer I before adding the enzyme conjugate, Streptavidin-

HRP (BD Biosciences), in dilution buffer for 1hr at RT. The wells were then washed four times with wash buffer I and twice with wash buffer II (PBS) before adding the final substrate solution (AEC Substrate Reagent Set, BD Biosciences) prepared according to the manufacturer's instructions. Spot development was monitored for a maximum time period of 60 min and the color development was stopped by soaking the plates in ddH_2O for 1 min before letting the plates dry O/N in the dark. The spots were then counted automatically using the AID EliSpot Reader (Autoimmun Diagnostika, Strassberg, Germany) and the data was analyzed using the AID EliSpot Software (Autoimmun Diagnostika) version 3.5.

2.6.2.1. LASV peptides

One hundred and twenty peptides spanning the LASV GPC strain Josiah sequence were synthetically synthesized (Mimotopes, Victoria, Australia). These peptides were 15 amino acids long with an 11 amino acid overlap. They were divided into 3 pools of 40 peptides: Pool A (spanning amino acids 1 to 170 of the GPC protein sequence), Pool B (amino acids 160 to 331) and Pool C (amino acids 321 to 491) (Figure 2.1.). The powdered peptides were resuspended individually in a 20%H_2O/80%DMSO solution at a concentration of 40 mg/mL and vortexing was performed to help dissolve the peptides. The peptide pools were then prepared by adding 25 µL of each peptide, which resulted in a stock concentration of 1000 µg/mL per peptide and stored at -80°C. The peptide pools were diluted to obtain a final concentration of 3 µg/mL per peptide for the ELISPOT assay. Considering the dilutions necessary to obtain a final concentration of 3 µg/mL per peptide for the ELISPOT assay, the final DMSO concentration was 0.24%. The control

samples containing no LASV GPC peptides were adjusted to contain the same concentration of DMSO.

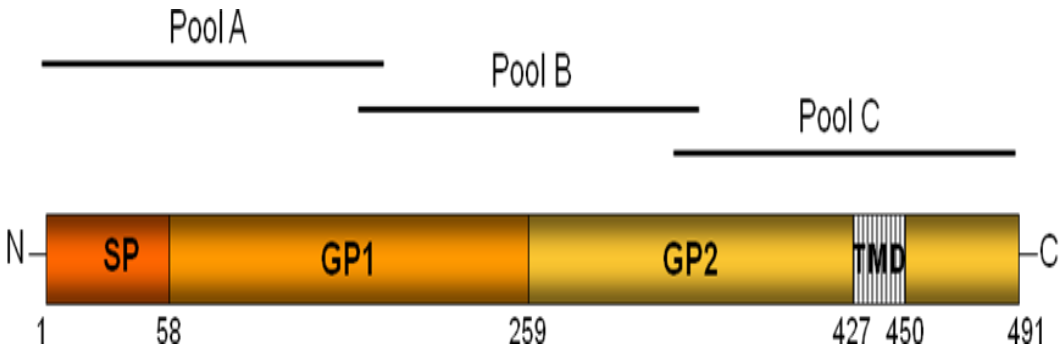


Figure 2.1. The full-length LASV glycoprotein structure.

Schematic representation of the LASV GPC protein showing the sections included in each peptide pool. SP: signal peptide; TMD: transmembrane domain.

2.7. Biosafety

Handling of LASV was performed under BSL-4 conditions. Handling of rVSV viruses was performed under enhanced BSL-2 conditions. Experiments with viruses were conducted in a biosafety cabinet as outlined in the Health Canada Laboratory Biosafety Guidelines (http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html).

All animal procedures were performed in a BSL-2 facility unless otherwise specified according to the Canadian Science Centre for Human and Animal Health Animal Care Committee guidelines, and were in accordance to the principles of the Canadian Council on Animal Care.

2.8. Statistical analysis

Statistical analysis was performed with GraphPad InStat V5.04 (GraphPad Software, CA, USA) using two-way analysis of variance (ANOVA). All data are shown as mean ± standard error of mean (SEM). Statistical analysis to compare the values for each peptide pool versus unstimulated samples was only performed when a statistical difference was found between the control group and treatment groups for a given peptide pool. Differences between groups were considered statistically significant when the p value was less than 0.05 ($p<0.05$).

3. Characterization of the immune response to the VSVΔG/LVGPC vaccine

3.1. Introduction

Replication-competent vaccines are well known for inducing a strong immune response and powerful immunity as a single injection in comparison to sub-unit and non-replicating vaccine vectors. These vaccines can be produced in different ways, including the use of recombinant viral vectors able to express a foreign antigen. One such example is the recombinant VSV (rVSV), which has been recovered from plasmid DNA coding for antigenomic RNA under the control of a T7 promoter using reverse genetics (Lawson et al. 1995), and which has been shown to have the capacity to express foreign genes (McKenna et al. 2003). VSV is a rhabdovirus causing a relatively harmless disease in cattle, swine, horses and rodents. Although VSV can occasionally infect humans, most infections are asymptomatic and symptomatic individuals only report mild flu-like symptoms, with the exception of one reported case of encephalitis (Lichty et al. 2004). The pathogenicity of VSV is believed to be associated with its high replication rate, and in particular the VSV glycoprotein G (VSV G) (Lichty et al. 2004). The early rVSV vaccines added an extra gene expressing the antigenic protein of interest into the VSV genome (Kretzschmar et al. 1997; Schnell et al. 1996a; Schnell et al. 1996b). However, in later studies the VSV G was replaced by a gene expressing the glycoprotein of a foreign virus (Roberts et al. 1999). Given the fact that VSV G is absent in the vaccine vector and that the recombinant viruses have decreased growth kinetics in comparison to wild-type VSV, these vectors can be considered highly attenuated (McKenna et al. 2003; Roberts et al. 1999). Pre-existing immunity is also uncommon since there are relatively few human infections and the immune response predominantly targets the VSV G, which is lacking

in the rVSV of interest to us (Lichty et al. 2004). Additionally, VSV is a non-segmented negative-strand RNA virus replicating in the host cell's cytoplasm, and thus no reassortment, recombination or integration has been reported and is thus unlikely to occur. Another advantage of VSV over other viral vectors is that its genome is relatively simple, easy to manipulate and the virus replicates well in vitro.

Given the promising development of vaccine vectors based on the recombinant VSV, our laboratory, in collaboration with Geisbert et al., has developed live-recombinant vaccines against LASV, EBOV and MARV (Garbutt et al. 2004), which have been shown to protect non-human primates against homologous viral challenge (Geisbert et al. 2005; Jones et al. 2005; Qiu et al. 2009). Additionally, the VSVΔG/LVGPC vaccine showed the ability to induce both humoral and cellular immune responses, as assessed by the presence of LASV GPC-specific total IgG, neutralizing antibodies and IFN-γ and TNF-α intracellular cytokine staining in $CD4^+$ and $CD8^+$ T lymphocytes (Geisbert et al. 2005). However, a detailed understanding of the immune response to the VSVΔG/LVGPC vaccine is a prerequisite before pursuing human clinical trials. We thus studied the development of LASV-specific immunoglobulin isotypes and IgG subclasses by ELISA, as well as the T cell response by a cytokine ELISPOT assay following immunization of mice with the VSVΔG/LVGPC vaccine.

3.2. Materials and methods

3.2.1. Cloning and rescue of the VSVΔG/LVGPC vaccine

3.2.1.1. Reverse transcription-Polymerase chain reaction (RT-PCR)

RT-PCR was performed to obtain a cDNA template of the LASV full-length GPC gene, strain Josiah, in order to insert it into the VSVXN2ΔG vector. RT-PCR was

performed using the Qiagen OneStep RT-PCR Kit (Qiagen, Ontario, Canada) with LASV strain Josiah RNA as the template. The LASV RNA template was obtained by RNA extraction using the QIAamp Viral RNA MiniSpin Kit (Qiagen). The RT-PCR reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler or a Biometra T1 thermocycler. The mastermix contained: 28 µL RNase free water, 10 µL 5X Buffer, 2 µL of 10 mM dNTP, 3 µL of 10 µM forward primer, 3 µL of 10 µM reverse primer, 2 µL enzyme mix, 2 µL RNA template. The cycling conditions were as follows: reverse transcription at 50°C for 30 min, initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 3 min, final extension at 72°C for 10 min and a pause at 4°C infinitely. Fifty cycles of denaturation-annealing-extension were performed.

The primers were designed to include the XhoI and NheI restriction sites for cloning into the VSVXN2ΔG vector. However, because there is a XhoI restriction site within the LASV GPC gene sequence, a BsmBI restriction site was added at the 5' end of the forward primer in order to perform subcloning prior to cloning into the VSVXN2ΔG vector.

The following primers were used to amplify the LASV GPC gene:

LASJGPCFBsmBI 5' AATCGTCTCCTCGAGCACCATGGGACAAATAGTGACATTCTTCC
BsmBI XhoI Kozak

LASJGPCRNheI 5' AATGCTAGCTCATCTCTTCCATTTCACAGGC

Amplicons were run on a 1% agarose gel containing ethidium bromide to visualize the PCR product. Samples were added to 10X BlueJuice Gel Loading Buffer (Invitrogen) and a 1Kb Plus DNA Ladder (Invitrogen) was used as a molecular weight

marker. The gels were run in Tris/acetate/EDTA electrophoresis buffer and ran for ~45 min at 100 V. DNA was then visualized with a MacroVue UV-25 Hoefer transilluminator or an Alpha Innotech MultiImage Light Cabinet using AlphaEase FC software (V4.1.0, Genetic Technologies, Florida, USA).

3.2.1.2. TOPO TA Cloning

Subcloning using the TOPO TA Cloning strategy (Invitrogen) was performed in order to insert the LASV GPC gene into the VSVXN2ΔG vector. The RT-PCR product was purified prior to the TOPO reaction using the QIAquick PCR Purification Kit (Qiagen) and eluted in 30 μL of sterile ddH_2O. The TOPO TA Cloning protocol was performed according to the manufacturer's instructions using the pCR 2.1-TOPO vector, One Shot Top 10 chemically competent *Escherichia coli* cells and kanamycin (50 μg/mL) as the selective agent.

The transformants were cultured O/N in Luri Broth (LB) medium containing kanamycin (50 μg/mL) and the QIAprep Spin Miniprep Kit (Qiagen) was used to isolate plasmid DNA. Each Miniprep sample was analysed by restriction analysis to confirm the presence of the LASV GPC insert using the NheI and BsmBI restriction enzymes. Positive clones were then sent to the NML DNA Core Facility for sequencing using the LASJGPCFBsmBI, LASJGPCRNheI, and the S31 through S36 primers. The sequences were analyzed and compared to the LASV GPC nucleotide sequence from GenBank (Accession number M15076) using computer programs Chromas (version 1.45, Technelysium Pty Ltd, Minnesota, USA) and Clone Manager 7 (version 7.04, Sci-Ed Software, North Carolina, USA).

S31 through S36 primer sequences:

S31	LassaJosGP 202f	CTT CAG ACT CTG GAA CTA AAC
S32	LassaJosGP 445f	CAG TAT GAG GCA ATG AGC TG
S33	LassaJosGP 673f	ACA ACC TGG GAA GAT CAC TG
S34	LassaJosGP 1003F	CAG TTG ATC AAC AAA GCA G
S35	LassaJosGP 1291F	GTT GAC CTC TTT GTG TTC AG
S36	LassaJosGP 170r	CAA GAC CTA CCA CAC AAC AG

Following the identification of a positive clone, a larger scale purification of plasmid DNA was performed using the QIAfilter Plasmid Maxi Kit (Qiagen) as per the manufacturer's protocol. The DNA concentration of the pCR2.1Topo/LASVGPC plasmid was measured on a Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, DE, USA) according to the manufacturer's instructions.

3.2.1.3. Cloning LASV GPC into the VSVXN2ΔG vector

The VSVXN2ΔG vector contains all VSV genes (nucleoprotein N, phosphoprotein P, matrix protein M and polymerase L), except the VSV G. A unique linker site is present between the VSV M and L genes, allowing the insertion of a foreign glycoprotein gene, in our case LASV GPC. The vector, kindly provided by Alex Silaghi (NML, Canada), was digested with XhoI and NheI restriction enzymes whereas the insert was digested with NheI and BsmBI to overcome the problem of the presence of a XhoI restriction site within the glycoprotein gene sequence, as mentioned above. Both insert and vector were then gel extracted using the QIAquick Gel Extraction Kit (Qiagen) as per the manufacturer's directions. Ligation of the vector and insert was performed at different ratios (1:1; 1:2; 1:3) and a control with no insert was also included. This step was carried out O/N at 14^{o}C using the T4 DNA Ligase HC (Roche, Quebec, Canada).

3.2.1.4. Transformation

The 20 μL ligation mixture was added to 50 μL of One Shot TOP10 Chemically Competent cells (Invitrogen) and incubated on ice for 30 min in a 15 mL tube. The sample was heat shocked at 42^{o}C for 45 sec and then placed on ice for 3 min. One mL of Super Optimal Culture (S.O.C.) medium (Invitrogen) was added and the sample was incubated at RT for 90 min on a rocking platform. The sample was centrifuged at 6,000 rpm for 1 min and the pellet was resuspended in 150 μL of S.O.C medium before being spread on a LB plate containing ampicillin (50 μg/mL) and incubated at RT for two days.

3.2.1.5. Purification of plasmid DNA

The transformants were cultured at RT for two days in LB broth containing ampicillin (50 μg/mL) using the QIAprep Spin Miniprep Kit (Qiagen) to isolate plasmid DNA. The plasmids in each Miniprep sample were analysed by restriction analysis to confirm the presence of the LASV GPC insert using the XhoI and NheI restriction enzymes.

Following the identification of a positive clone, a larger scale purification of plasmid DNA was performed using the QIAfilter Plasmid Maxi Kit (Qiagen) as per the manufacturer's protocol. The DNA concentration of the VSVXN2ΔG/LVGPC plasmid was measured as mentioned above and a double digest followed by agarose gel electrophoresis was performed to visualize the presence of an insert of the appropriate size. A sample of the purified DNA was then sent to the NML DNA Core Facility for sequencing using the LASJGPCFBsmBI and LASJGPCRNheI primers. The sequence was analyzed and compared to the LASV GPC nucleotide sequence from GenBank

(Accession number M15076) using computer programs Chromas (version 1.45, Technelysium Pty Ltd) and Clone Manager 7 (version 7.04, Sci-Ed Software).

3.2.1.6. Virus rescue

For successful virus rescue, accessory plasmids encoding the T7 polymerase and the VSV ribonucleoprotein constituents, VSV N, P and L, are necessary in addition to the full-length VSVXN2ΔG/LVGPC plasmid, which is under the control of the T7 polymerase (Figure 3.1.). The accessory plasmids (pCAGGS/T7, pBluescript SK (+) (pBS)/VSV P, pBS/VSV L and pBS/VSV N) were kindly provided by Kinola Williams (NML, Canada). A large-scale purification of plasmid DNA was performed using the QIAfilter Plasmid Maxi Kit (Qiagen) as per the manufacturer's protocol. The DNA concentration was measured and DNA samples were visualized by agarose gel electrophoresis as above.

293T cells and Vero E6 cells were seeded at a 1:1 ratio in two 6-well plates 24 hr prior to the transfection. The cells were transfected using the Lipofectamine 2000 reagent (LP2000, Invitrogen) according to the manufacturer's instructions with different DNA (μg) to LP2000 (μL) ratios in duplicate. Transfected cells were incubated for 72 hr in a 37°C humidified 5% CO_2 incubator and 1 mL of supplemented DMEM (3% FBS and 2mM L-glutamine) was added to each well 24 hr post-transfection.

The various DNA:LP2000 ratios were as follows:

	VSVXN2ΔG/LVGPC	T7	VSV N	VSV L	VSV P	LP2000
1)	2 µg	2 µg	0.5 µg	0.25 µg	1.25 µg	14 µL
2)	2 µg	2.5 µg	0.5 µg	0.25 µg	1.25 µg	14 µL
3)	2.5 µg	2.5 µg	0.625 µg	0.75 µg	1.56 µg	14 µL
4)	2 µg	2.5 µg	0.5 µg	0.6 µg	1.25 µg	14 µL
5)	-	-	-	-	-	14 µL
6)	-	-	-	-	-	-

A blind passage was performed by transferring 500 µL of supernatant onto confluent Vero E6 cells following centrifugation at 300 x g for 5 min. Supernatant was collected once CPE was apparent and samples were prepared for WB (Section 2.3.) and RT-PCR (Section 3.2.1.). RNA extraction was performed using the QIAamp Viral RNA MiniSpin Kit (Qiagen) prior to the RT-PCR. Once a sample was positive for the presence of a rescued virus, a virus stock was prepared (Section 2.2.1.) by diluting the supernatant sample at 1:1,000.

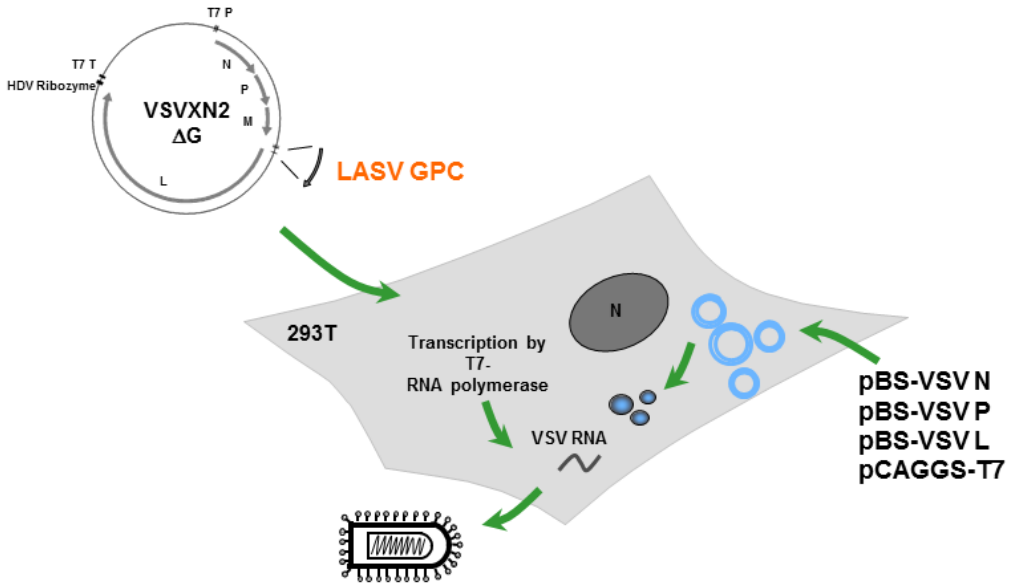


Figure 3.1. Rescue of VSVXN2ΔG/LVGPC.

The VSVXN2ΔG/LVGPC recombinant vaccine was generated by transfecting 293T/Vero E6 cells with plasmids encoding the full-length VSVXN2ΔG/LVGPC genome and the accessory plasmids encoding the T7 polymerase and VSV N, P and L. Supernanants were harvested 72 hr post-transfection and a blind passage was performed in Vero E6 cells before the samples were tested by RT-PCR and WB to confirm successful rescue of the VSVXN2ΔG/LVGPC virus.

Adapted from: Garbutt et al., 2004.

3.3. Results

3.3.1. Cloning and rescue of VSVXN2ΔG/LVGPC

The VSVΔG/LVGPC vaccine was generated using a reverse genetics system. The VSVXN2 vector lacking the VSV G gene (VSVXN2ΔG) is the backbone into which a foreign glycoprotein gene can be inserted to replace the VSV glycoprotein gene. When this vector is transfected into mammalian cells in combination with the accessory plasmids expressing the T7 polymerase and the VSV structural proteins N, P and L, a recombinant virus is generated which resembles a wild-type VSV particle with the exception that the VSV G is replaced by a foreign glycoprotein, such as the LASV GPC in the present study.

The full-length glycoprotein gene from LASV strain Josiah was cloned into the VSVXN2ΔG prior to rescue of the virus. To achieve this, TOPO TA subcloning was first performed using viral RNA from LASV strain Josiah as the template to obtain the GPC gene insert through RT-PCR. The forward and reverse primers were designed to include a forward XhoI and a reverse NheI restriction enzyme site, which are required for cloning into the VSVXN2ΔG vector. However, because there is an XhoI restriction site within the LASV GPC sequence, a BsmBI restriction site was added 5' to the XhoI sequence in the forward primer to overcome this problem.

One of the TOPO TA transformants, clone 15, appeared positive following a double digest of the Miniprep sample. Plasmid DNA from clone 15 was thus sequenced and its sequence was found to perfectly match the LASV GPC strain Josiah nucleotide sequence (GenBank accession number M15076), except for one mismatch at position 1311 AGT→AGC, which results in a silent mutation.

Plasmid DNA from clone 15 was purified in order to isolate the LASV GPC gene needed for cloning into the VSVXN2ΔG backbone vector. After ligation of the LASV GPC gene into VSVXN2ΔG, bacteria were transformed and multiple positive clones identified (Figure 3.2.A). Clone 19 was chosen for large-scale purification of plasmid DNA and further analysis. The same sequence, and silent mutation at position 1311, of the LASV GPC gene was identified as for clone 15 from the subcloning step.

The VSVXN2ΔG/LVGPC plasmid was transfected into mammalian cells along with the accessory plasmids to rescue the VSVΔG/LVGPC virus, which was to be used in the following experiments as a vaccine. Supernatant from the second blind passage was assessed for the presence of the LASV GP2 protein by WB, and RNA was extracted to confirm the presence of the LASV GPC gene through RT-PCR. A band at ~38 kDa, appropriate for LASV GP2 was identified on the Western blot (Figure 3.2.B) and a RT-PCR product with a size of ~1.5 kb, consistent with the expected size of the LASV GPC gene, was visualized by agarose gel electrophoresis (Figure 3.2.C). These findings confirmed a successful rescue of the VSVΔG/LVGPC virus, and a virus stock was therefore prepared using a supernatant sample from the second blind passage.

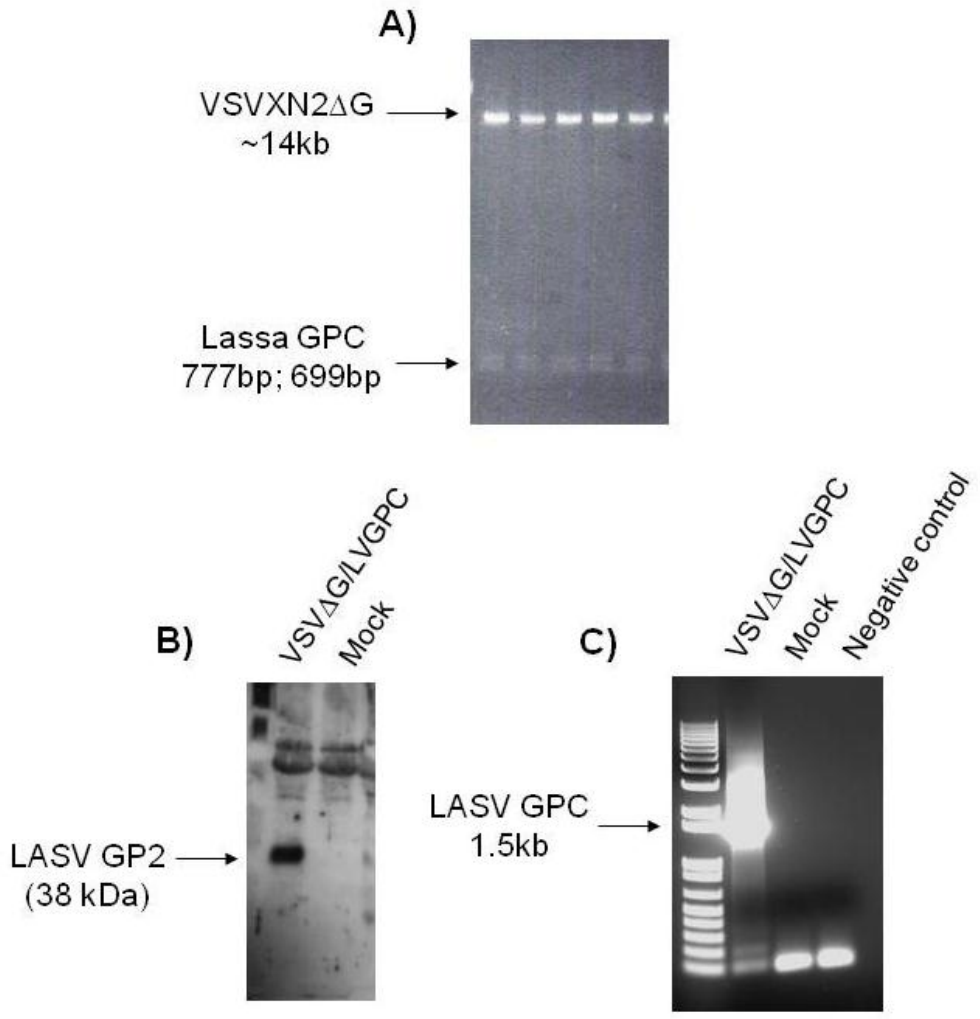


Figure 3.2. Cloning and rescue of VSVΔG/LVGPC strain Josiah.

A) Cloning of the LASV GPC gene insert into the VSVXN2ΔG vector resulted in multiple positive transformants. Two bands are produced for the LASV GPC because it contains an internal site for XhoI. Supernatant was collected from the second blind passage following the rescue of the VSVΔG/LVGPC virus and a B) Western blot was performed to detect the GP2 protein using the anti-GP2 #3 antibody and anti-rabbit IgG-HRP-conjugated secondary antibody (1:1,000; 1:2,000) and C) RT-PCR was performed following RNA extraction to amplify the LASV GPC gene to confirm the presence of the LASV GPC gene.

3.3.2. The immune response to the VSVΔG/LVGPC vaccine

3.3.2.1. Study of the humoral immune response

To study the humoral immune response to the VSVΔG/LVGPC vaccine, three different mouse strains (CBA, BALB/c and C57BL/6) were immunized with three different doses of the vaccine on day 0 (Figure 3.3.). The animals received a booster injection on day 28 and serum samples were collected weekly until day 56 to measure the antibody response by a LASV-infected cell lysate quantitative ELISA.

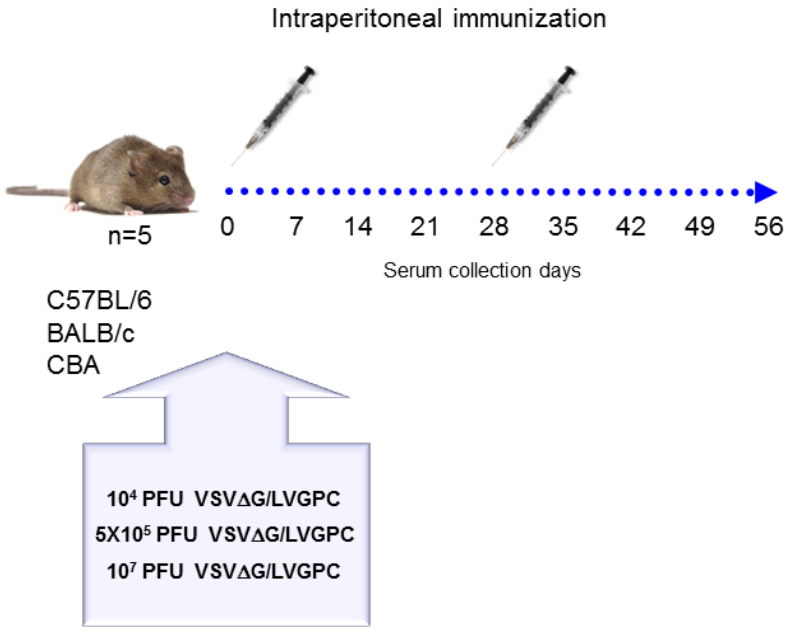
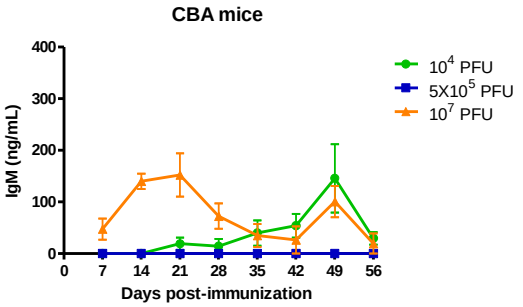


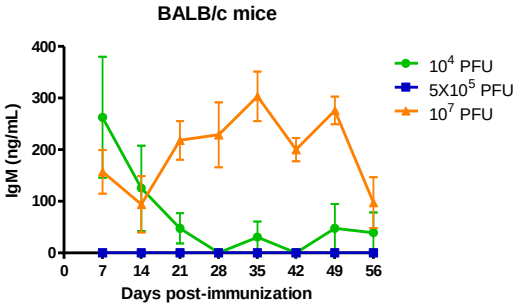
Figure 3.3. Immunization strategy for the study of the humoral immune response to the VSVΔG/LVGPC vaccine.

CBA, BALB/c and C57BL/6 mice (n=5) were immunized on day 0 with 1 mL i.p. of the VSVΔG/LVGPC vaccine (10^4, $5X10^5$ or 10^7 PFU) and received a boost on day 28. Serum samples were collected prior to vaccination on day 0 and then on day 7, 14, 21, 28, 35, 42, 49 and 56. The antibody response to the VSVΔG/LVGPC vaccine was measured on the collected serum samples using a LASV-infected cell lysate quantitative ELISA.

ELISA data showed that the IgM response was the highest in C57BL/6 mice, with a mean titer of 1271 ng/mL 7 days following the boost in the group which received 10^4 PFU of VSVΔG/LVGPC (Figure 3.4.). For the IgG1 subclass, the highest antibody titers were observed for all groups receiving 10^7 PFU of VSVΔG/LVGPC, especially following the booster injection (Figure 3.5.). The IgG1 antibody titers were much higher in BALB/c mice (peak mean concentration of 948 ng/mL on day 35) in comparison to CBA and C57BL/6 mice (peak mean concentration of 146 ng/mL on day 42 and 233 ng/mL on day 49, respectively). Similar trends were observed for the IgG2a subclass (Figure 3.5.), with increased antibody levels following the booster injection, especially in the mice that received 10^7 PFU of VSVΔG/LVGPC. However, the IgG2a antibody levels were the highest in the CBA mouse group (peak mean concentration of 1443 ng/mL on day 56), followed by BALB/c (peak mean concentration of 999 ng/mL on day 49) and then C57BL/6 (peak mean concentration of 156 ng/mL on day 49) mouse groups. The IgG2b antibody levels remained low in all groups but a noticeable increase can be seen 7 days after the boost for the three mouse strains which received 10^7 PFU of VSVΔG/LVGPC, with the highest levels observed in C57BL/6 mice (Figure 3.6.). The IgG3 antibody response was also weak for all groups (Figure 3.6.). Nevertheless, an increase in the IgG3 antibody concentration was observed 7 days after the boost in C57BL/6 mice, with the highest concentrations in mice which received $10^7 > 5X10^5 > 10^4$ PFU of VSVΔG/LVGPC. The levels of IgA were negligible throughout the study (data not shown).

IgM


CBA mice
IgM (ng/mL)
400
300
200
100
0
0 7 14 21 28 35 42 49 56
Days post-immunization
10^4 PFU
5X10^5 PFU
10^7 PFU


BALB/c mice
IgM (ng/mL)
400
300
200
100
0
0 7 14 21 28 35 42 49 56
Days post-immunization
10^4 PFU
5X10^5 PFU
10^7 PFU

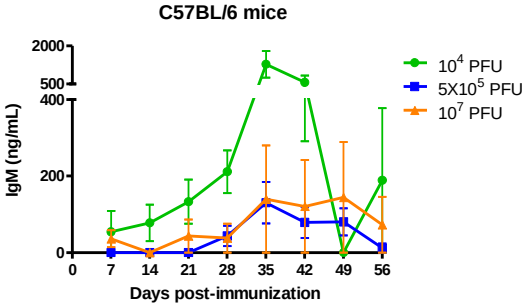

C57BL/6 mice
IgM (ng/mL)
2000
500
400
200
0
0 7 14 21 28 35 42 49 56
Days post-immunization
10^4 PFU
5X10^5 PFU
10^7 PFU

Figure 3.4. IgM antibody response to the VSVΔG/LVGPC vaccine over time in mice with different genetic backgrounds.

CBA, BALB/c and C57BL/6 mice (n=5) were immunized with 10^4 PFU, $5X10^5$ PFU or 10^7 PFU of VSVΔG/LVGPC. Serum samples were collected weekly and anti-LASV antibodies measured with a LASV-infected cell lysate ELISA. Error bars represent SEM.

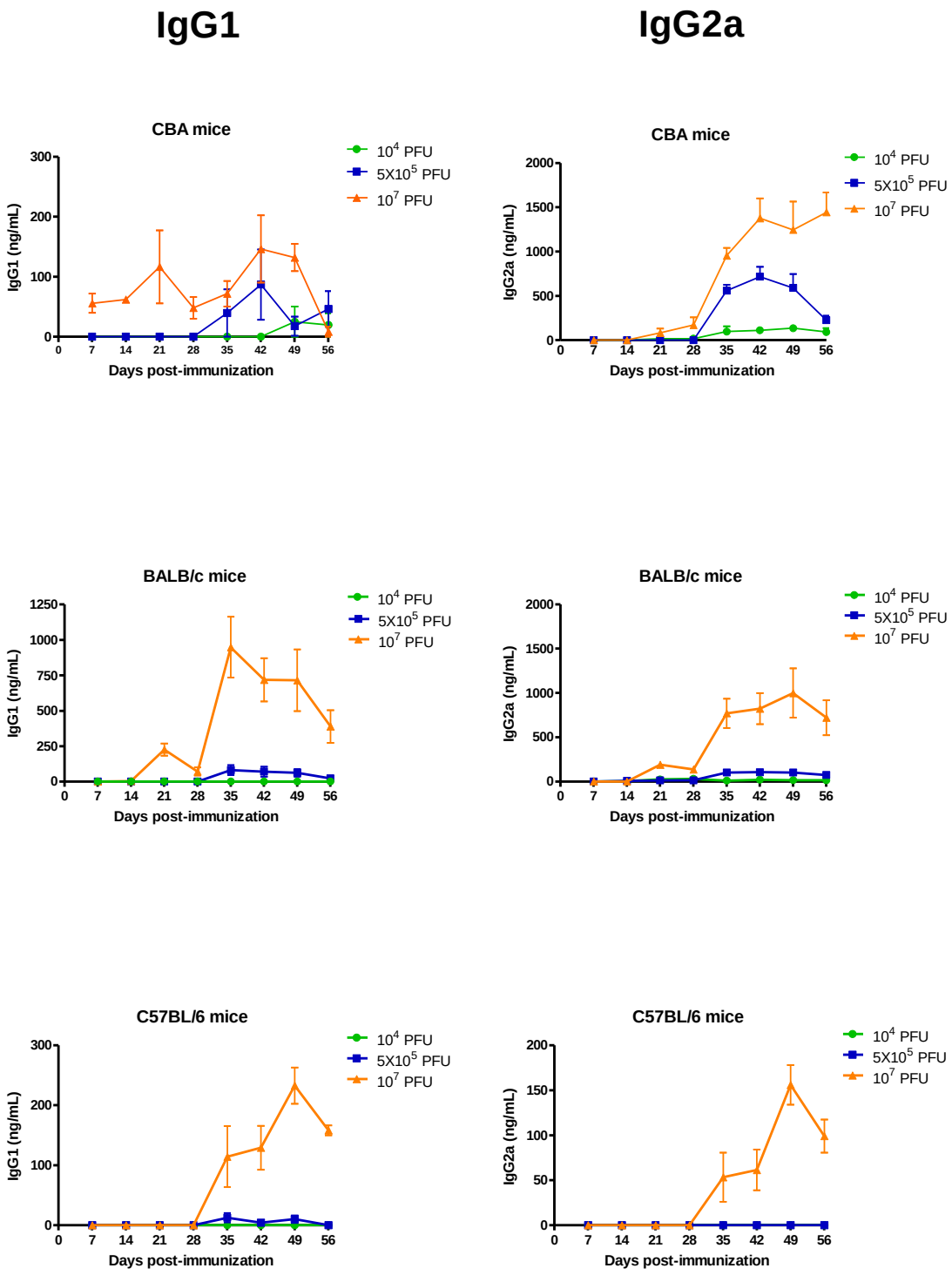

IgG1
IgG2a
CBA mice
BALB/c mice
C57BL/6 mice
IgG1 (ng/mL)
IgG2a (ng/mL)
Days post-immunization
10^4 PFU
5X10^5 PFU
10^7 PFU

Figure 3.5. IgG1 and IgG2a antibody response to the VSVΔG/LVGPC vaccine over time in mice with different genetic backgrounds.

CBA, BALB/c and C57BL/6 mice (n=5) were immunized with 10^4 PFU, $5X10^5$ PFU or 10^7 PFU of VSVΔG/LVGPC. Serum samples were collected weekly and anti-LASV antibodies measured with a LASV-infected cell lysate ELISA. Error bars represent SEM.

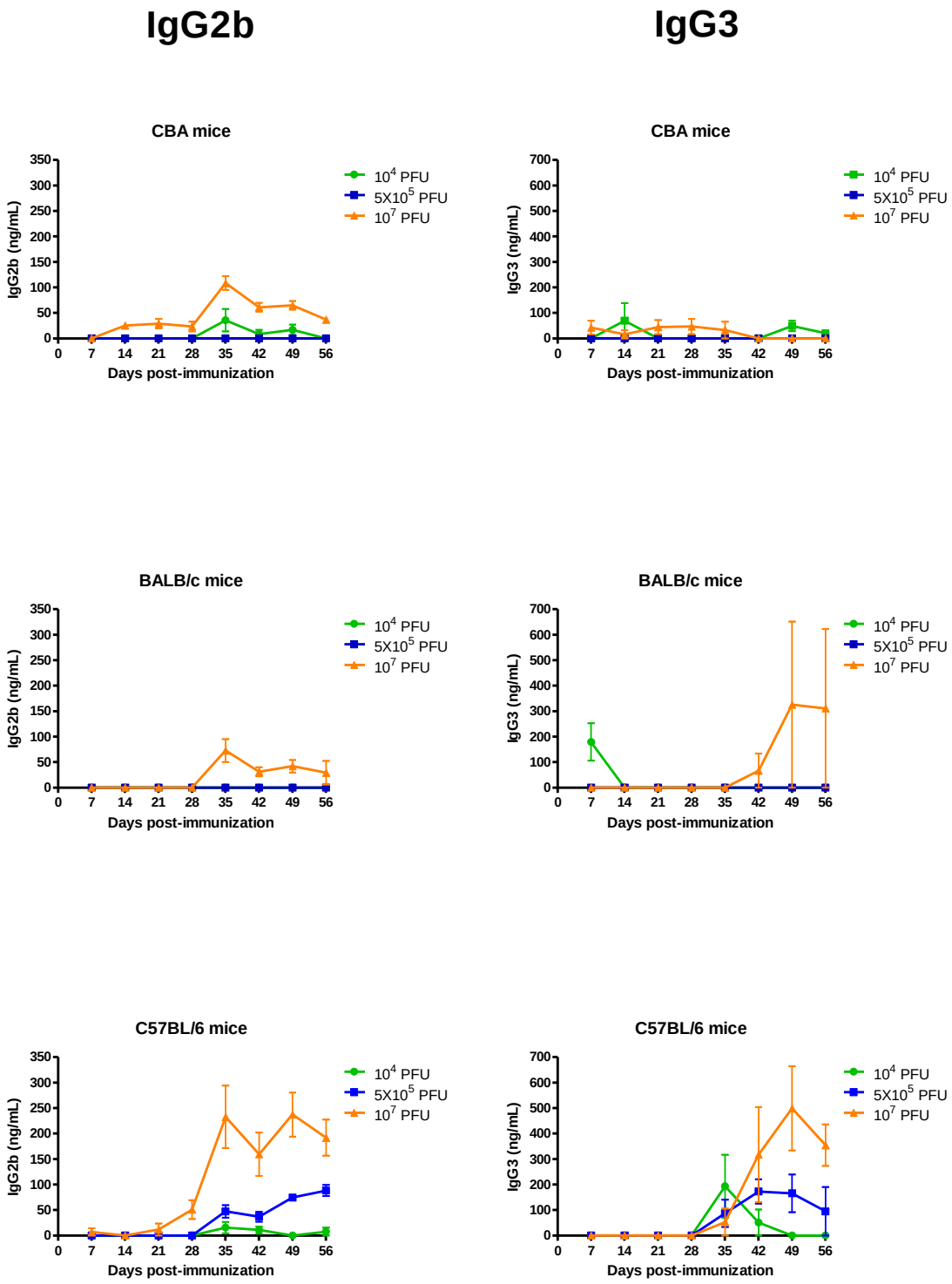

IgG2b
IgG3
CBA mice
BALB/c mice
C57BL/6 mice
IgG2b (ng/mL)
IgG3 (ng/mL)
Days post-immunization
10^4 PFU
5X10^5 PFU
10^7 PFU

Figure 3.6. IgG2b and IgG3 antibody response to the VSVΔG/LVGPC vaccine over time in mice with different genetic backgrounds.

CBA, BALB/c and C57BL/6 mice (n=5) were immunized with 10^4 PFU, $5X10^5$ PFU or 10^7 PFU of VSVΔG/LVGPC. Serum samples were collected weekly and anti-LASV antibodies measured with a LASV-infected cell lysate ELISA. Error bars represent SEM.

3.3.2.2. Study of the T cell response

The cell-mediated immune response was assessed in CBA mice which received a primary injection of the VSVΔG/LVGPC vaccine followed by a booster injection on day 28 post-vaccination (Figure 3.7.). Spleens were harvested ten days after the primary injection or the booster dose, and the splenocytes stimulated with peptide pools spanning the full-length LASV glycoprotein. Cytokine-producing splenocytes were detected by an ELISPOT assay.

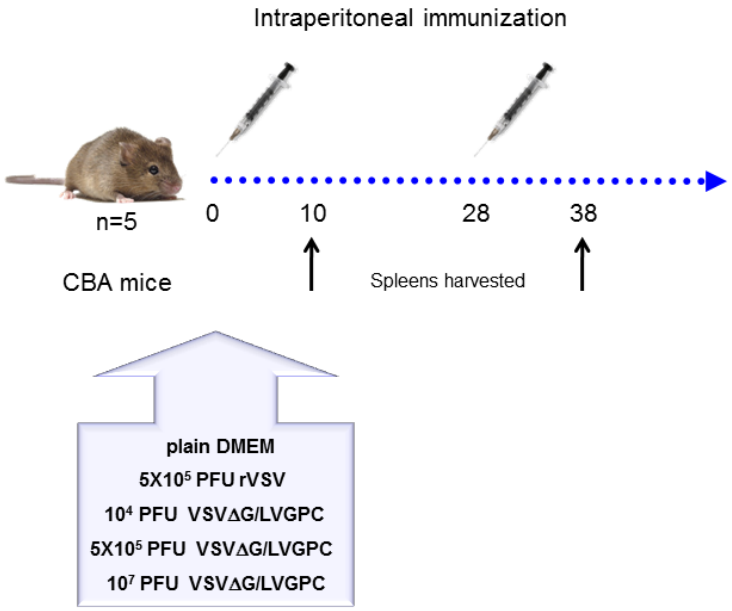


Figure 3.7. Immunization strategy for the study of the T cell immune response to the VSVΔG/LVGPC vaccine.

CBA mice (n=5) were immunized on day 0 with 1 mL i.p. of the VSVΔG/LVGPC vaccine (10^4, $5X10^5$ or 10^7 PFU). Control mice received plain DMEM or rVSV ($5X10^5$ PFU). Half of the mice were euthanized 10 days post-immunization to harvest the spleens for the ELISPOT assay. The second half received a booster injection on day 28 and was euthanized 10 days later to harvest spleens for the ELISPOT assay.

Following the primary injection, the number of spot-forming cells (SFCs) was the highest for IFN-γ > TNF-α > IL-2 > IL-4 > IL-10 > IL-12 cytokines (Figure 3.8.). No statistically significant differences were observed between the control group and groups which received different doses of the VSVΔG/LVGPC vaccine or when comparing the difference between unstimulated and LASV GPC peptide-stimulated samples within a given group. Following one booster injection, the number of SFCs increased for each cytokine and the highest numbers of SFCs were found for IFN-γ > TNF-α > IL-2 > IL-10 > IL-4 > IL-12 cytokines (Figure 3.9.). For the peptide pool A, statistically significant differences were found between the vaccinated mice and control mice for IFN-γ and TNF-α cytokines. For the peptide pool B, statistically significant differences were only found between the vaccinated mice ($5X10^5$ PFU) and control mice for the IFN-γ and IL-2 cytokines. The LASV GPC peptide pool A was thus definitely better at stimulating a cellular immune response for IFN-γ and TNF-α in comparison to peptide pools B and C. In addition, the number of SFCs secreting IFN-γ was higher in mice which received the highest vaccine doses. A mean of 895 SFCs was observed for mice which received 10^4 PFU of VSVΔG/LVGPC whereas a mean of 1512 and 1392 SFCs were measured for mice which received $5X10^5$ and 10^7 PFU, respectively, for the IFN-γ cytokine. In contrast, the number of SFCs secreting TNF-α was higher in the mice that received the lower vaccine doses. A mean of 358 SFCs was observed for mice which received 10^7 PFU of VSVΔG/LVGPC whereas a mean of 790 and 852 SFCs was measured for mice which received 10^4 and $5X10^5$ PFU, respectively.

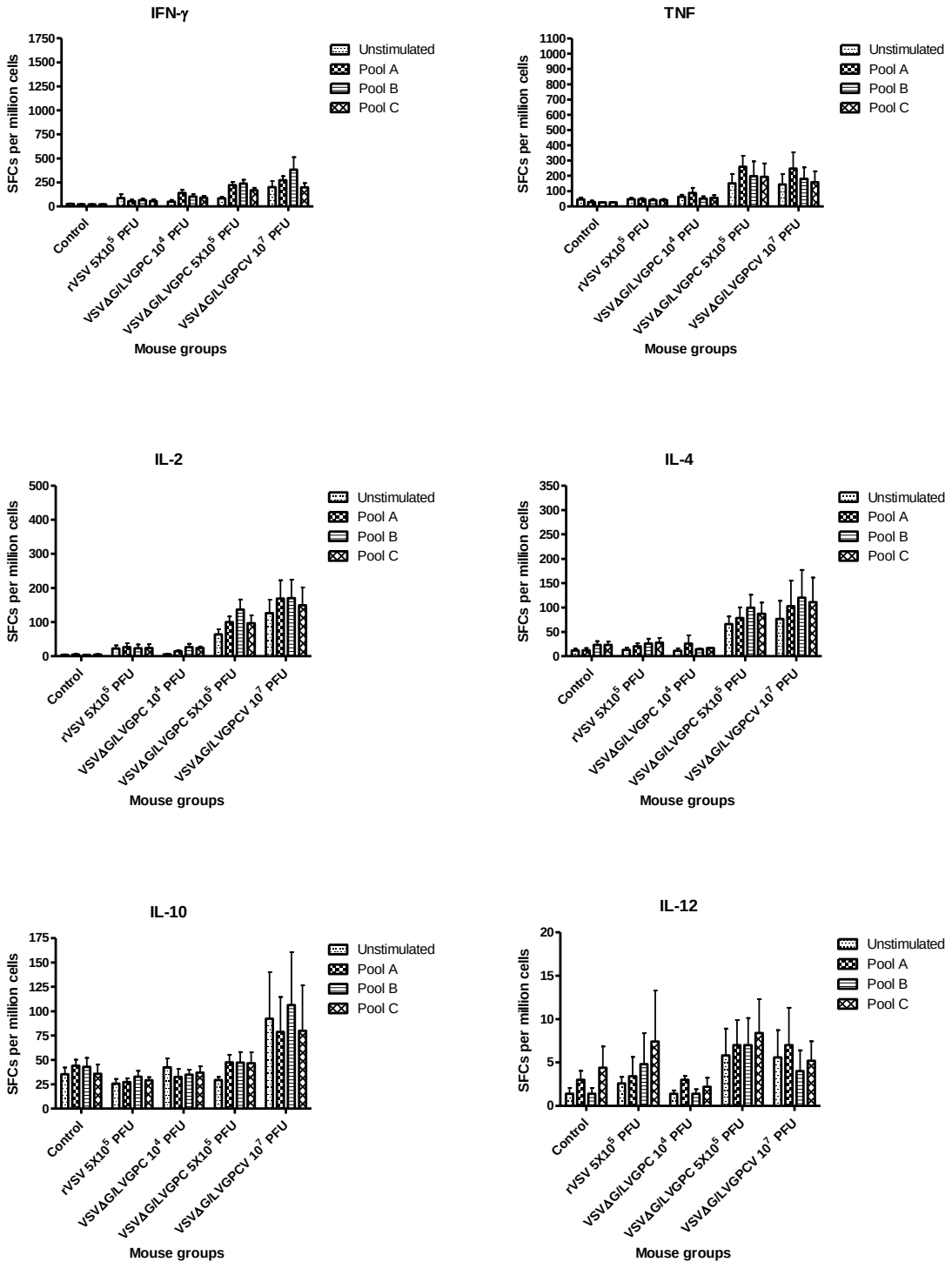

IFN-γ
SFCs per million cells
1750
1500
1250
1000
750
500
250
0
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups
TNF
SFCs per million cells
1100
1000
900
800
700
600
500
400
300
200
100
0
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups
IL-2
SFCs per million cells
500
400
300
200
100
0
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups
IL-4
SFCs per million cells
350
300
250
200
150
100
50
0
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups
IL-10
SFCs per million cells
175
150
125
100
75
50
25
0
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups
IL-12
SFCs per million cells
20
15
10
5
0
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups

Figure 3.8. T cell response to the VSVΔG/LVGPC vaccine prior to boosting.

CBA mice (n=5) were immunized with plain DMEM (negative control), $5X10^5$ PFU of rVSV, 10^4 PFU, $5X10^5$ PFU or 10^7 PFU of VSVΔG/LVGPC. Splenocytes were harvested 10 days post-immunization and stimulated with peptide pools A, B or C spanning the LASV full-length glycoprotein in a cytokine ELISPOT assay. Cytokine production was presented as the number of spot-forming cells (SFCs). Error bars represent SEM.

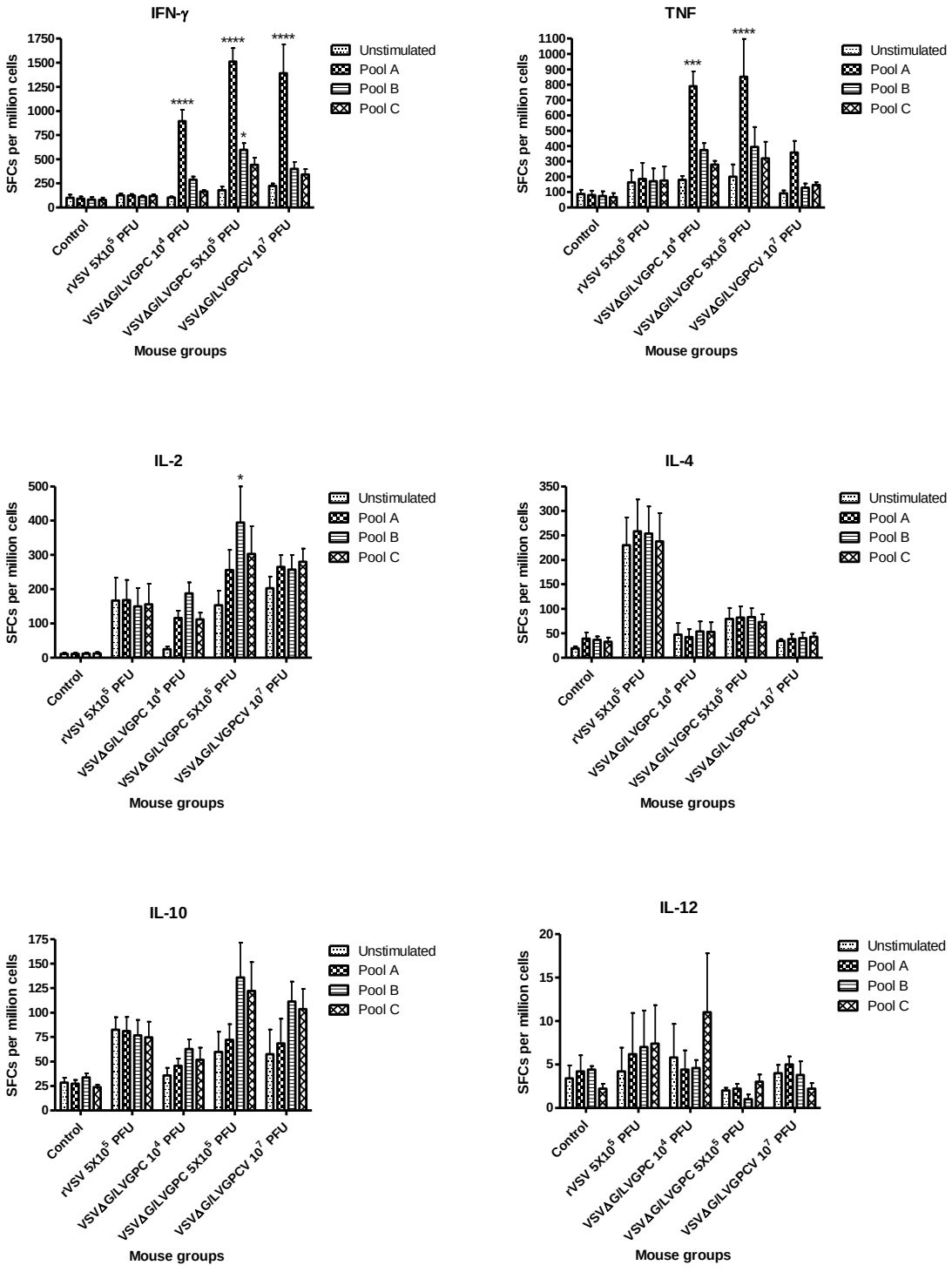
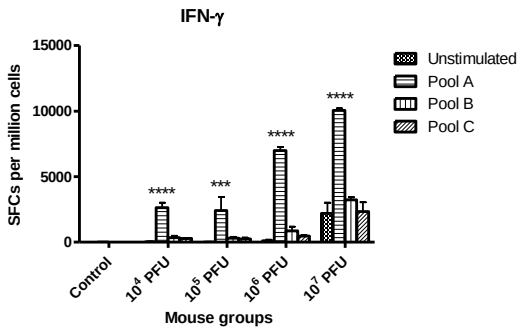

IFN-γ
SFCs per million cells
Unstimulated
Pool A
Pool B
Pool C
Control
rVSV 5X10^5 PFU
VSVΔG/LVGPC 10^4 PFU
VSVΔG/LVGPC 5X10^5 PFU
VSVΔG/LVGPCV 10^7 PFU
Mouse groups
TNF
IL-2
IL-4
IL-10
IL-12

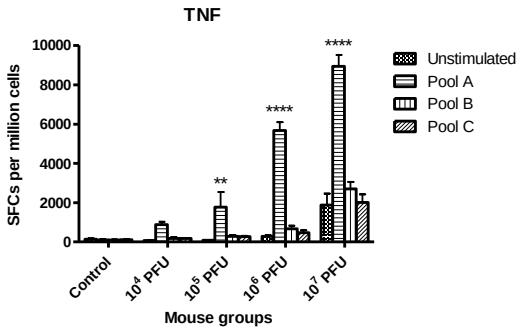
Figure 3.9. T cell response to the VSVΔG/LVGPC vaccine following a boost.

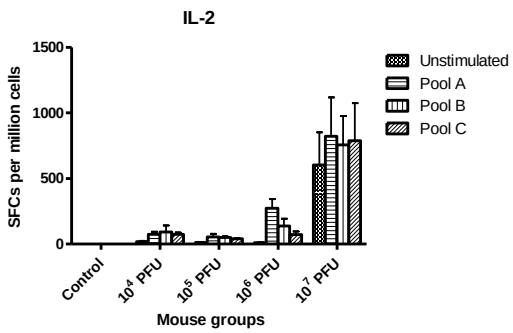
CBA mice (n=5) were immunized with plain DMEM (negative control), $5X10^5$ PFU of rVSV, 10^4 PFU, $5X10^5$ PFU or 10^7 PFU of VSVΔG/LVGPC. Mice received an initial immunization on day 0 and a booster injection on day 28. Splenocytes were harvested 10 days following the booster injection and stimulated with peptide pools A, B or C spanning the LASV full-length glycoprotein in a cytokine ELISPOT assay. Cytokine production was presented as the number of spot-forming cells (SFCs). Error bars represent SEM. P values represent significant changes in cytokine production compared to unstimulated splenocytes within the same group (* $p<0.05$; *** $p<0.001$; **** $p<0.0001$).

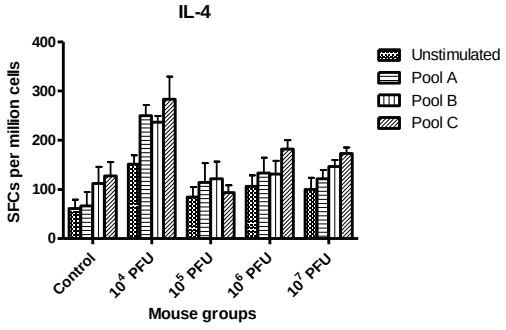
The cytokine response in mice that received one primary immunization dose and two booster injections was also investigated in a separate experiment. CBA mice (n=3) received the initial vaccine dose on day 0 and a booster injection on days 28 and 70. Splenocytes were harvested 10 days after the last immunization for the cytokine ELISPOT assay.

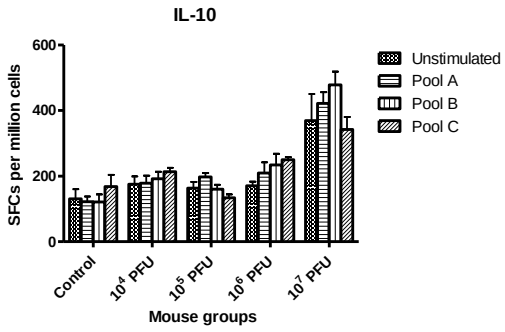
As can be observed in Figure 3.10., differences were found between the control group and certain vaccine groups for the IL-2, IL-4, IL-10 and IL-12p70 cytokines, but the number of SFCs was also high in the unstimulated samples, meaning that the differences were due to high background levels. However, statistically significant differences were found for the IFN-γ and TNF-α cytokines. The number of SFCs was much higher for mice which received two booster injections (Figure 3.10.) than for mice which had received no booster or only one booster injection (Figures 3.8. and 3.9.), and the number of SFCs increased at least 10 fold in comparison to the number of SFCs following a single booster injection for IFN-γ and TNF-α. In mice that received two booster injections, the highest numbers of SFCs were found for IFN-γ > TNF-α > IL-2 > IL-10 > IL-4 > IL-12 cytokines, as was observed in mice which received a single booster injection. The trend of IFN-γ and TNF-α cytokine production towards epitopes contained in peptide pool A versus pools B and C was also observed here, and the number of SFCs were vaccine dose dependent for IFN-γ, TNF-α and IL-2 cytokines.


IFN-γ
SFCs per million cells
15000
10000
5000
0
****
***
****
****
Unstimulated
Pool A
Pool B
Pool C
Control
10^4 PFU
10^5 PFU
10^6 PFU
10^7 PFU
Mouse groups


TNF
SFCs per million cells
10000
8000
6000
4000
2000
0
**
****
****
Unstimulated
Pool A
Pool B
Pool C
Control
10^4 PFU
10^5 PFU
10^6 PFU
10^7 PFU
Mouse groups


IL-2
SFCs per million cells
1500
1000
500
0
Unstimulated
Pool A
Pool B
Pool C
Control
10^4 PFU
10^5 PFU
10^6 PFU
10^7 PFU
Mouse groups


IL-4
SFCs per million cells
400
300
200
100
0
Unstimulated
Pool A
Pool B
Pool C
Control
10^4 PFU
10^5 PFU
10^6 PFU
10^7 PFU
Mouse groups


IL-10
SFCs per million cells
600
400
200
0
Unstimulated
Pool A
Pool B
Pool C
Control
10^4 PFU
10^5 PFU
10^6 PFU
10^7 PFU
Mouse groups

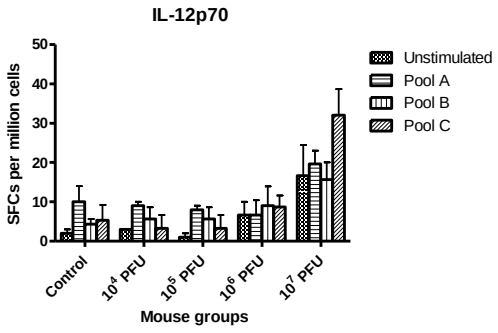

IL-12p70
SFCs per million cells
50
40
30
20
10
0
Unstimulated
Pool A
Pool B
Pool C
Control
10^4 PFU
10^5 PFU
10^6 PFU
10^7 PFU
Mouse groups

Figure 3.10. T cell response to the VSVΔG/LVGPC vaccine following two booster injections.

CBA mice (n=3) were immunized with plain DMEM (negative control), 10^4 PFU, 10^5 PFU, 10^6 PFU or 10^7 PFU of VSVΔG/LVGPC i.p. Mice received an initial immunization on day 0 and a booster injection on days 28 and 70. Splenocytes were harvested 10 days following the last booster injection and stimulated with peptide pool A, B or C spanning the LASV full-length glycoprotein in a cytokine ELISPOT assay. Cytokine production was presented as the number of spot-forming cells (SFCs). Error bars represent SEM. P values represent significant changes in cytokine production compared to unstimulated splenocytes within the same group (** $p<0.01$; *** $p<0.001$; **** $p<0.0001$).

3.4. Discussion

We previously demonstrated that immunization of NHPs with the VSVΔG/LVGPC vaccine provides protection against an otherwise lethal challenge with LASV (Geisbert et al. 2005). In the current study, we found that the VSVΔG/LVGPC vaccine induced a humoral immune response in the mouse, with antibody levels increasing in animals receiving a higher vaccine dose, and also following a booster injection. The T cell response also increased following a vaccine boost, and a shift towards the production of cytokines against LASV GPC peptides from the pool A over pools B and C was observed, notably for IFN-γ and TNF-α.

The antibody response was measured in three mouse strains to determine whether the host genetic background would influence the induction of the different immunoglobulin isotypes. CBA and C57BL/6 mice are believed to induce a T_{H1}-type immune response whereas BALB/c mice stimulate a T_{H2}-type immune response (Mosmann and Coffman 1989; Soares, David, and Titus 1997). CBA mice were included since they are frequently used by other laboratories for LASV vaccine studies. IgG1 concentrations were higher in BALB/c mice in comparison to C57BL/6 and CBA mice. On the other hand, IgG2a antibody levels were the highest in CBA mice followed by BALB/c and C57BL/6 mice. The IgG2b concentration was comparable among the three mouse strains, except for a two-fold increase in C57BL/6 mice at the highest vaccine dose after the boost. These results are in accordance with the fact that IgG1 is considered a T_{H2}-type IgG subclass, whereas IgG2a and IgG2b are believed to be T_{H1}-type IgG subclasses (Nimmerjahn and Ravetch 2005). Serum concentrations of IgG3 remained low in most groups, which is not unexpected since this IgG subtype is known to be induced

primarily by T-independent carbohydrate antigens (Nimmerjahn and Ravetch 2005). In addition, the antibody concentrations generally increased following the booster injection and were vaccine dose-dependent, which was expected.

The fact that IFN-γ and TNF-α were the predominant cytokines induced by the VSVΔG/LVGPC vaccine is in accordance with our previous study in NHPs, in which the same cytokines were shown to be produced by T cells (Geisbert et al. 2005). IFN-γ is a cytokine produced by NK and T cells, and plays a role in macrophage activation, antigen processing and presentation through MHC molecules, promotes T helper cell differention towards a T_{H1}-type phenotype and favors isotype switching to IgG2a, IgG2b and IgG3 in the mouse (Schreiber and Schreiber 2003). TNF-α, on the other hand, is an inflammatory mediator which can be secreted by many cell types, including MO/MP, B and T cells (Wang, Czura, and Kacey 2003). It plays a role in host defense responses, fever and septic shock, as well as being toxic for many tumors (Wang, Czura, and Kacey 2003).

We also observed that the IFN-γ and TNF-α cytokine production was directed mainly towards LASV GPC epitopes from the peptide pool A, and each booster injection dramatically shifted the response further towards this peptide pool. Peptide pool A includes 40 overlapping peptides spanning the N-terminus proximal one-third of the LASV GPC strain Josiah, from amino acids 1 to 170 of the GP1 protein (Figure 2.1., Chapter 2). Our results suggest that the immunodominant epitope(s) of the VSVΔG/LVGPC vaccine lie within the N-terminal region of the LASV GPC protein. This is consistent with the known immunodominant epitopes within the LASV GPC which have been identified in GP1, although immunodominant epitopes have also been found in GP2 (Boesen, Sundar, and Coico 2005; Botten et al. 2006). Further fine mapping

studies could be performed by stimulating splenocytes with individual peptides rather than peptide pools to determine the exact immunodominant epitopes of this vaccine, and determine whether they match the epitopes previously identified. Another option would be to generate synthetic peptides using the sequences of the known LASV immunodominant epitopes and then stimulate splenocytes to measure the cytokine response in the context of the VSVΔG/LVGPC vaccine.

One important characteristic of the VSV-based vaccines is postexposure prophylaxis, which has been shown to be successful for EBOV and MARV infections. Five out of five rhesus macaques infected with a lethal dose of MARV survived when given VSVΔG/MARVGP 20 to 30 min post-challenge (Daddario-DiCaprio et al. 2006). Partial protection was achieved in MARV-infected rhesus macaques when VSVΔG/MARVGP was given 24 h (5/6 animals; 83% survival) or 48 h post-challenge (2/6 animals; 33% survival) (Geisbert et al. 2010). All BALB/c mice (n=5) challenged with a lethal dose of mouse-adapted ZEBOV also survived when vaccinated with VSVΔG/ZEBOVGP 24 hours prior to challenge, 30 min or 24 hr post-challenge (Feldmann et al. 2007). However, in the same study, only partial protection was achieved in guinea pigs and NHPs. Four out of six Hartley guinea pigs immunized with VSVΔG/ZEBOVGP 24 hr prior to challenge with guinea pig-adapted ZEBOVsurvived whether 5/6 and 3/6 survived when vaccinated 1 or 24 hr post-challenge, respectively (Feldmann et al. 2007). Fifty percent (4/8) survival was achieved in rhesus macaques when VSVΔG/ZEBOVGP was administered 20 to 30 min after receiving a lethal dose of ZEBOV (Feldmann et al. 2007). Complete postexposure protection was demonstrated to be possible in *Sudan ebolavirus*-infected rhesus macaques (n=4) when a VSV vaccine

expressing the *Sudan ebolavirus* GP was administered 20 to 30 min post-challenge (Geisbert et al. 2008b). These data suggest that these two vaccines are promising for post-exposure treatment of EBOV and MARV infections. This approach might also be possible for LASV and should be investigated in the future.

One important issue regarding VSV-based vaccines is the fact that they are live attenuated and thus could result in disease in immunocompromised individuals, which is particularly important for vaccines targeting populations where HIV is endemic. The VSVΔG/ZEBOVGP vaccine was shown to be safe when administered to rhesus macaques infected with simian-human immunodeficiency virus, which did not show any signs of illness (Geisbert et al. 2008a). Similar results were obtained in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice immunized with VSVΔG/ZEBOVGP or VSVΔG/MARVGP (Jones et al. 2007). Additionally, the VSVΔG/ZEBOVGP vaccine has been used in a laboratory worker who had an accidental needdlestick injury, that contained *Zaire ebolavirus,* while working in the BSL-4 (Gunther et al. 2011). The worker received $5X10^7$ PFU of the VSVΔG/ZEBOVGP vaccine 48 hr later as postexposure treatment. VSVΔG/ZEBOVGP viremia was detected for two days post-administration and the laboratory worker remained asymptomatic, except for a fever and myalgia 12 hr post-vaccination. The PCR remained negative for EBOV RNA and no antibodies against EBOV were detected, except against the EBOV GP. Altogether these data suggest that VSV-based vaccines would be safe for use in humans, including immunocompromised individuals.

In conclusion, the VSVΔG/LVGPC vaccine can induce a humoral and cellular immune response in both the mouse and NHP. Additionally, this vaccine can protect

against a lethal challenge with LASV in NHPs. The predominant antibody isotypes induced following immunization with the VSVΔG/LVGPC vaccine varies in the mouse depending on the genetic background, emphasizing the importance of host factors in promoting a useful and protective immune response in vaccination studies. IFN-γ and TNF-α are the two predominant cytokines produced in both animal models. Whether production of these cytokines is essential for protection against LASV challenge remains to be investigated.

4. Characterization of the immune response to the Lassa virus-like particle vaccine

4.1. Introduction

In addition to the VSVΔG/LVGPC vaccine, a subunit vaccine in the form of virus-like particles was generated. The LASV genome encodes five proteins: an RNA polymerase, L; a matrix protein, Z; the nucleoprotein, NP; and two envelope glycoproteins, GP1 and GP2. The LASV matrix protein Z plays an essential role in virus assembly and budding (Eichler et al. 2004; Perez, Craven, and de la Torre 2003; Salvato et al. 1992; Strecker et al. 2003). Expression of the LASV Z protein in transfected cells results in budding of Z-containing enveloped particles in the absence of other viral proteins (Eichler et al. 2004; Perez, Craven, and de la Torre 2003; Strecker et al. 2003). VLP release is mediated by two late domains which are present in the C-terminus domain of the Z protein. Mutation of these motifs drastically reduces the efficient release of VLPs (Strecker et al. 2003). It is currently unknown whether solitary expression of the LASV glycoproteins or NP can result in the release of enveloped particles or whether co-expression of LASV proteins can result in the generation of multi-protein VLPs.

The creation of multi-protein VLPs could be an important contribution to the Lassa virus field, since it will permit further study of the virus without resorting to the complexities associated with BSL-4 facilities, which are currently required to conduct research on LASV. They can be used as a tool to study certain aspects of the Lassa virus life cycle, such as virus assembly and release, as has been done for several viruses, including VSV and EBOV (Jasenosky et al. 2001; Noda et al. 2002; Schmitt and Lamb 2004). Another important potential application of the Lassa VLPs would be to use them as a vaccine, as has been shown for other viruses, such as Human Papillomavirus (HPV)

(Ghim, Jenson, and Schlegel 1992; Kirnbauer et al. 1992; Rose et al. 1993; Zhou et al. 1991), EBOV and MARV (Swenson et al. 2004; Swenson et al. 2005; Warfield et al. 2003; Warfield et al. 2004; Warfield et al. 2007b; Warfield et al. 2007a; Ye et al. 2006). The HPV VLP vaccine is currently commercially available for the prevention of cervical cancer and has been shown to be safe, immunogenic and protective (Einstein et al. 2011; Haupt and Sings 2011). We also had an interest in comparing the VLP vaccine platform to the VSVΔG/LVGPC vaccine. One limitation of the VSVΔG/LVGPC vaccine is that it is a live-attenuated virus. Subunit vaccines, such as VLP-based vaccines, are generally considered safer than live vector vaccines because they are replication-incompetent, and are therefore preferred. They have also been found to be immunogenic, to protect against disease and are preferred over other types of subunit vaccines because they mimic the structure of authentic virus particles (Noad and Roy 2003; Roy and Noad 2009). Another advantage of a VLP vaccine is that the VLPs are made of various Lassa proteins, ensuring that the immune response will be targeted to multiple proteins of the virus, whereas the VSV-based vaccine only contains the Lassa glycoproteins.

Therefore, we examined by biochemical and electron microscopy techniques whether multi-protein VLPs could be generated in a eukaryotic system upon co-expression of various combinations of LASV Z, GPC and NP. Additionally, we determined whether immunization of mice with these multi-protein VLPs could induce an adaptive immune response, by measuring serum levels of different immunoglobulin isotypes by ELISA and the T cell response by ELISPOT.

4.2. Materials and methods

4.2.1. Cells and viruses

LASV strain Josiah was grown in Vero E6 cells. Cells were infected at a 1:100 dilution from a virus stock of $1.23X10^5$ PFU/mL and supernatant was harvested on day 8 for sucrose cushion purification. Huh7 cells (obtained from Michael Carpenter, NML, Winnipeg, Canada) were cultured as described for Vero E6 cells and 293 cells (ATCC CRL-1573) were cultured as described for 293T cells in Section 2.1. CHO-K1 cells (ATCC CCL-61) were cultured in F-12K Nutrient Mixture Kaighn's Modification (Invitrogen) supplemented with 10% FBS.

4.2.2. Expression constructs and cell transfection

Full-length cDNAs encoding LASV Josiah GPC, Z or NP cloned into the mammalian expression vector pCAGGS were kindly provided by Alex Silaghi (University of Manitoba and NML, Winnipeg, Canada), Josh Adams (University of Manitoba and NML, Winnipeg, Canada) and Thomas Strecker (Institute of Virology, Philipps University Marburg, Marburg, Germany), respectively.

For the transfections performed with the FuGENE6 transfection reagent (Roche), 70% confluent cells were plated 24 hr prior to transfection in 6-well plates pre-coated with poly-D-lysine (Sigma-Aldrich) to prevent the cells from detaching. FuGENE6 (6 μL) and plasmid DNA were added to Opti-MEM (Invitrogen) in a 1.5 mL tube to obtain a total volume of 100 μL. The transfection mixture was incubated for 5 min at RT. The medium was then removed from the wells and the cells were washed with plain DMEM prior to adding 900 μL of Opti-MEM per well. The transfection mixture was then added dropwise to each well and the plates were incubated for 72 hr, unless otherwise stated, in

a 37°C humidified 5% CO_2 incubator. Supernatants were collected and cleared of cellular debris by centrifugation at 3000 xg for 10 min at 4°C before preparing the samples for WB as described in Section 2.3.

For transfection with the Lipofectamine 2000 reagent (Invitrogen), cells were also plated 24 hr prior to transfection in 6-well plates pre-coated with poly-D-lysine (Sigma-Aldrich). Confluent 293T cells were transfected using LP2000 according to the manufacturer's instructions with a DNA (μg) to LP2000 (μL) ratio of 3:10 unless otherwise specified. Equal amounts of DNA of each plasmid encoding the LASV proteins were used for transfection and when only one or two of these plasmids were used, an empty pCAGGS vector was used to keep the total amount of transfected DNA constant. LP2000 was added to 250 μL of Opti-MEM in a 1.5 mL tube and plasmid DNA was added to 250 μL of Opti-MEM in a second 1.5 mL tube. The tubes were incubated for 5 min at RT before the plasmid DNA was combined to the LP2000. The transfection mixture was incubated for 15 min at RT. Medium was then removed from the wells and the 90% confluent cells were washed with plain DMEM prior to adding 500 μL of Opti-MEM per well. The transfection mixture was then added dropwise to each well and the plates were incubated for 72 hr, unless otherwise stated, in a 37°C humidified 5% CO_2 incubator. Supernatants were collected and cleared of cellular debris by centrifugation at 3000 xg for 10 min at 4°C before preparing the samples for WB as described in Section 2.3.

When triple flasks were used instead of 6-well plates to produce VLPs, the amount of DNA and transfection reagent were upscaled proportionally to the surface area of a triple flask versus a 6-well plate. The supernatants were harvested and cleared from

cellular debris by two centrifugations at 1000 xg for 10 min at 4°C and stored at 4°C if needed.

4.2.3. Purification and proteinase K protection assay

Cleared supernatants were overlaid onto a 20% sucrose cushion prepared in PBS and ultracentrifuged at 28,000 rpm for 2 h at 4°C using a SW-28 rotor (Beckman Optima L-70K), followed by a 30 min PBS wash at 28,000 rpm at 4°C. Pelleted material was resuspended in 1 mL PBS per triple flask and stored at 4°C. For the proteinase K assay, purified VLPs were either left untreated or treated with 300 µg/mL proteinase K (Biochemical Corporation, Worthington, Lakewood, NJ, USA), with or without 1% Triton X-100 for 2 hr at 37°C (Figure 4.1.).

4.2.4. Electron microscopy (EM)

Non-purified VLPs or virus were prepared for negative stain as previously described (Hazelton and Coombs 1999). Briefly, cleared supernatants were fixed with 0.1% glutaraldehyde for 10 min at RT and the fixative was then quenched by adding 0.1 M glycine. LASV samples prepared in BSL-4 were fixed with 4% paraformaldehyde and 2% glutaraldehyde (final concentration) and were γ-irradiated (2 Mrad) on wet ice. Samples were stored at 4°C until they were processed for EM. Samples were centrifuged directly onto Formvar-coated, carbon-stabilized 400-mesh copper electron microscopy grids (Airfuge EM-90 rotor; Beckman, Palo Alto, CA) at 26 lb/in^2 for 30 min. Negative staining was performed with 1.2 mM phosphotungstic acid, pH 7.0. Samples were viewed with a Philips model 201 electron microscope. Pictures were taken using Direct Positive film 5302 (Kodak, New York, USA) and printed on Kodak Polycontrast III paper.

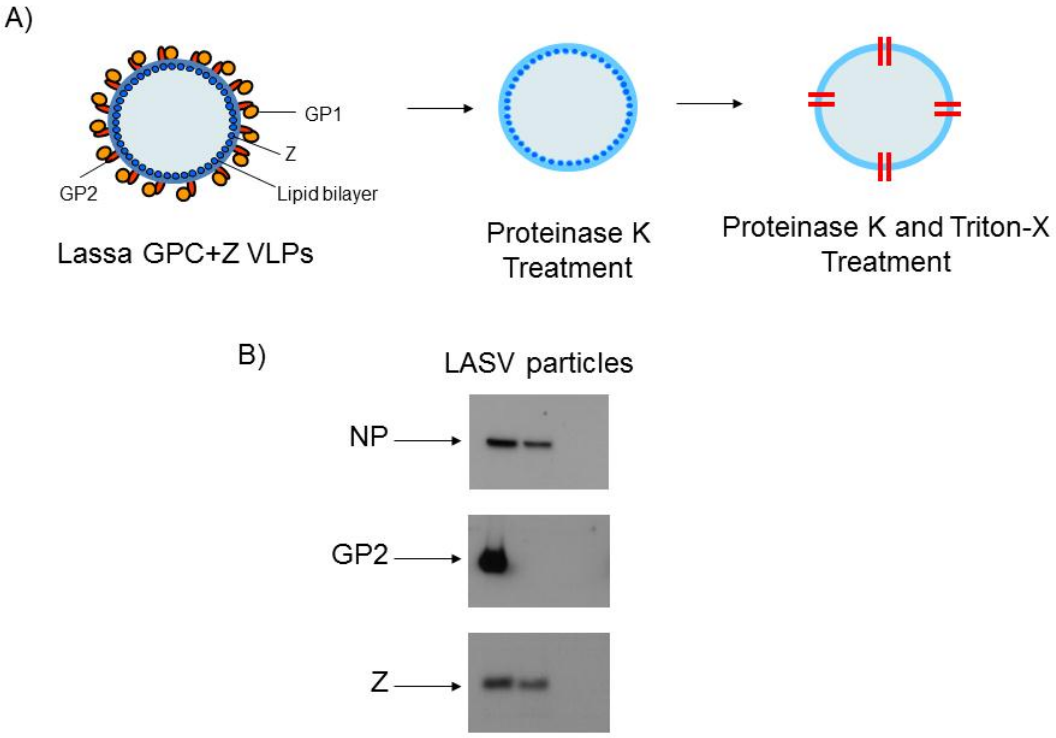


Figure 4.1. Proteinase K protection assay.

A) The proteinase K enzyme can only digest proteins that are exposed on the surface of a lipid bilayer. Proteins protected by a lipid bilayer will only be digested if the sample is concomitantly treated with a detergent, such as Triton-X. Triton-X will dissolve the lipid bilayer, exposing internal proteins to the action of the proteinase K enzyme. B) With LASV or Lassa VLPs, the glycoproteins are exposed on the surface of the viral envelope, whereas the Z and NP proteins are protected by it. The glycoprotein band would thus be expected to disappear upon treatment with the proteinase K, whereas the Z and NP protein bands would only disappear in the samples containing both proteinase K and Triton-X in Western blotting.

4.2.5. Vaccine preparation for immunization

LASV VLPs containing GPC and Z were generated by transfecting 293T cells in triple flasks as described above (Sections 4.2.2. and 4.2.3.). Following sucrose cushion purification, the samples were further purified through on a 20 to 60% OptiPrep gradient. The OptiPrep Density Gradient Medium (Sigma-Aldrich) was diluted to obtain the desired concentrations of 20, 30, 40, 50 and 60% in PBS and each dilution was layered on top of the next starting with the 60% solution at the bottom of the ultracentrifuge tube. Samples were ultracentrifuged at 28,000 rpm (SW-28 rotor, Beckman Optima L-70K) with a slow acceleration and no brake for 16 hr at 4°C. Two mL fractions were collected from top to bottom and analyzed by WB to determine which fractions contained both the glycoproteins and Z before pooling the positive samples together. The pooled fractions were washed in PBS to remove the OptiPrep Density Gradient Medium at 28,000 rpm for 1 hr at 4°C. The pelleted VLPs were resuspended in Thermo Scientific HyClone PBS (Fisher Scientific) and stored at 4°C. The protein concentration of each sample was determined by the DC Protein Assay (Bio-Rad) according to the manufacturer's protocol. A serial dilution of BSA served as the protein standard. Different VLP preparations were pooled together prior to the vaccination of the mice and the presence of both LASV GPC and Z proteins was confirmed again by WB analysis. The samples were also tested for presence of endotoxin using the PYROGENT Plus Gel Clot LAL assay (Lonza, MD, USA).

Group receiving VLPs alone

CBA mice received 50 μg of LASV GPC+Z VLPs in 200 μL i.p. The VLPs were diluted in HyClone PBS.

Group receiving VLPs and Alum

CBA mice received 200 µL containing 50 µg of LASV GPC+Z VLPs with Alum adjuvant s.c. The VLPs were diluted in HyClone PBS and combined at a 1:1 ratio with Thermo Scientific Imject Alum Adjuvant (Pierce Biotechnology, IL, USA) and mixed for 30 min in a syringe to emulsify the preparation.

Group receiving VLPs in Freund's adjuvant

For the first dose, CBA mice received 200 µL containing 50 µg of LASV GPC+Z VLPs in Complete Freund's adjuvant s.c. The VLPs were diluted in HyClone PBS and combined at a 1:1 ratio with Complete Freund's adjuvant (Sigma-Aldrich) and mixed for 30 min in a syringe to emulsify the preparation. For the two boosting doses, CBA mice received 200 µL containing 50 µg of LASV GPC+Z VLPs in Incomplete Freund's adjuvant (Sigma-Aldrich) s.c. The VLPs were diluted in HyClone PBS and combined at a 1:1 ratio with Incomplete Freund's adjuvant (Sigma-Aldrich) and mixed for 30 min in a syringe to emulsify the preparation.

Group receiving VLPs and Sigma adjuvant

CBA mice received 200 µL containing 50 µg of LASV GPC+Z VLPs in Sigma Adjuvant System s.c. The Sigma Adjuvant System (Sigma-Aldrich) was reconstituted with 1 mL HyClone PBS and warmed to 40-45°C. The VLPs were diluted in HyClone PBS and mixed at a 1:1 ratio with Sigma Adjuvant System previously warmed at 37°C. The mixture was vortexed to obtain an emulsion.

Group receiving VLPs and TiterMax

CBA mice received 200 µL containing 50 µg of LASV GPC+Z VLPs with TiterMax adjuvant s.c. The TiterMax Gold Adjuvant (Sigma-Aldrich) was warmed at

37°C and vortexed. The VLPs were diluted in HyClone PBS and combined at a 0.5:1 ratio with TiterMax Gold Adjuvant (Sigma-Aldrich) and mixed for 2 min in a syringe to emulsify the preparation. The second half the the VLP preparation was added to the mixture and emulsified for 2 min in a syringe.

Group receiving media only

CBA mice received 200 μL of plain DMEM i.p.

Group receiving VSVΔG/LVGPC

CBA mice received $5X10^5$ PFU of VSVΔG/LVGPC in 200 μL i.p. The virus was diluted in plain DMEM to obtain the desired concentration.

4.3.Results

4.3.1. Generation of Lassa virus-like particles

Lassa VLPs were generated by transient expression of LASV structural proteins GPC, Z and NP in different combinations in 293T cells (Figure 4.2.). Plasmid vectors expressing GPC, Z or NP were transfected in various combinations into 293T cells and supernatants were collected 72 hr post-transfection for VLP purification and analysis through EM and WB.

4.3.1.1. Optimization of the transfection protocol

Four different cell lines, 293T, 293, CHO-K1 and Huh7, were transfected with the pCAGGS-LASV GPC and Z Josiah plasmids to determine which cell line would be the most suitable for the production of Lassa VLPs. Using the FuGENE6 transfection reagent, it was found that although the Z protein was present in the supernatant of the transfected 293 and CHO-K1 cells, GP2 was absent or poorly expressed in those cell lines as compared to the 293T cell line (Figure 4.3.). In addition, GP2 and Z were not detected

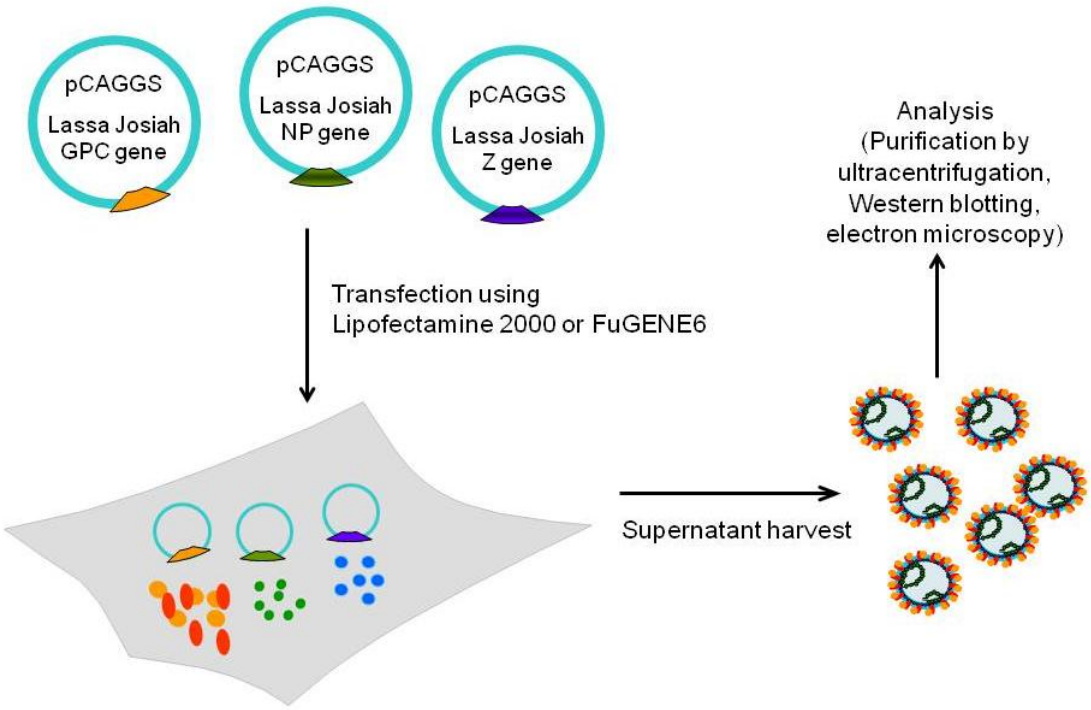


Figure 4.2. Generation of Lassa VLPs by transfection of 293T cells.

The pCAGGS vectors expressing LASV GPC, NP or Z were transfected in different combinations in 293T cells. Harvested supernatants were purified and analyzed by Western blotting or electron microscopy.

in the supernatants from transfected Huh7 cells (data not shown). The subsequent experiments were thus performed with 293T cells.

Further optimization of the transfection protocol was thus performed using 293T cells. However, since the yield of Lassa VLPs remained low, as observed by EM, despite trying different ratios of FuGENE6 to plasmid DNA and ratios of GPC DNA to Z DNA, we decided to repeat the experiments with another transfection reagent, Lipofectamine 2000, to determine if this would increase the VLP yield. As can be seen in Figure 4.4., the VLP yield is much higher when the LP2000 transfection reagent is used in comparison to FuGENE6, both by WB and EM. The intensity of the GP2 and Z protein bands in the WB were the highest at 96 hr post-transfection with LP2000 (lanes 4 and 5, Figure 4.4.). However, because cellular debris was more pronounced when analyzing the 96 hr samples by EM (data not shown), 72 hr was chosen as the ideal time point to harvest supernatant for VLP purification and analysis. In addition, a DNA (μg) to LP2000 (μL) ratio of 3:10 (lane 3, Figure 4.4.) was chosen for further studies. As mentioned above, further transfections were conducted in 293T cells. The full length GPC is detected in these samples because the samples were not purified through a 20% sucrose cushion.

4.3.1.2. Formation of VLPs by individually expressed viral proteins

Since it has been previously shown that solitary expression of the Z protein is sufficient for the release of enveloped particles (Eichler et al. 2004; Strecker et al. 2003), we examined whether the glycoproteins or NP could also generate lipid-containing particles when expressed individually. Cells were transfected with individual plasmids encoding GPC, Z or NP, and harvested supernatants were subjected to a proteinase K protection assay, as well as EM analysis, to show VLP formation.

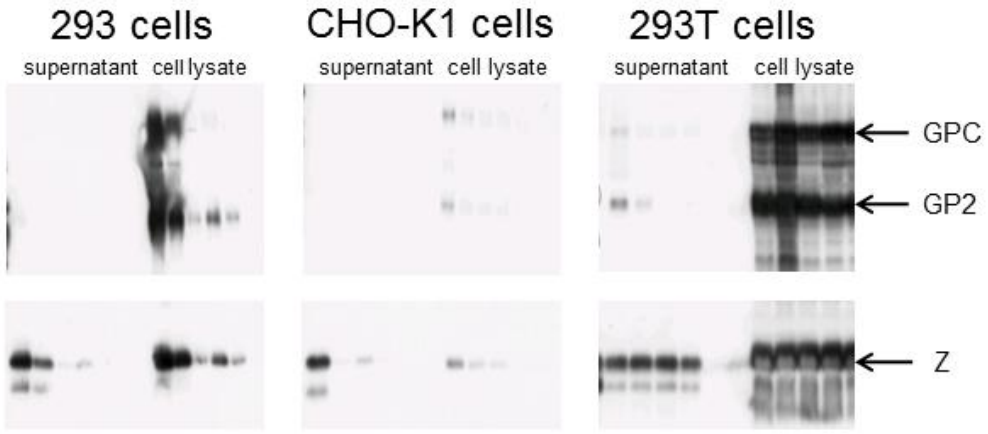


Figure 4.3. Determination of the optimal cell line for VLP production.

293, CHO-K1 or 293T cells were transfected with individual pCAGGS vectors encoding GPC and Z using the FuGENE6 transfection reagent. Culture supernatants were harvested 72 hr post-transfection, and then purified by ultracentrifugation over a 20% sucrose cushion. Presence of Lassa VLPs in the supernatant and protein expression in the cell lysate samples were determined by WB (#4 anti-GP2 (1:2,000)/anti-rabbit IgG-HRP (1:3,000) and anti-Z (1:10,000)/anti-rabbit IgG-HRP (1:20,000).

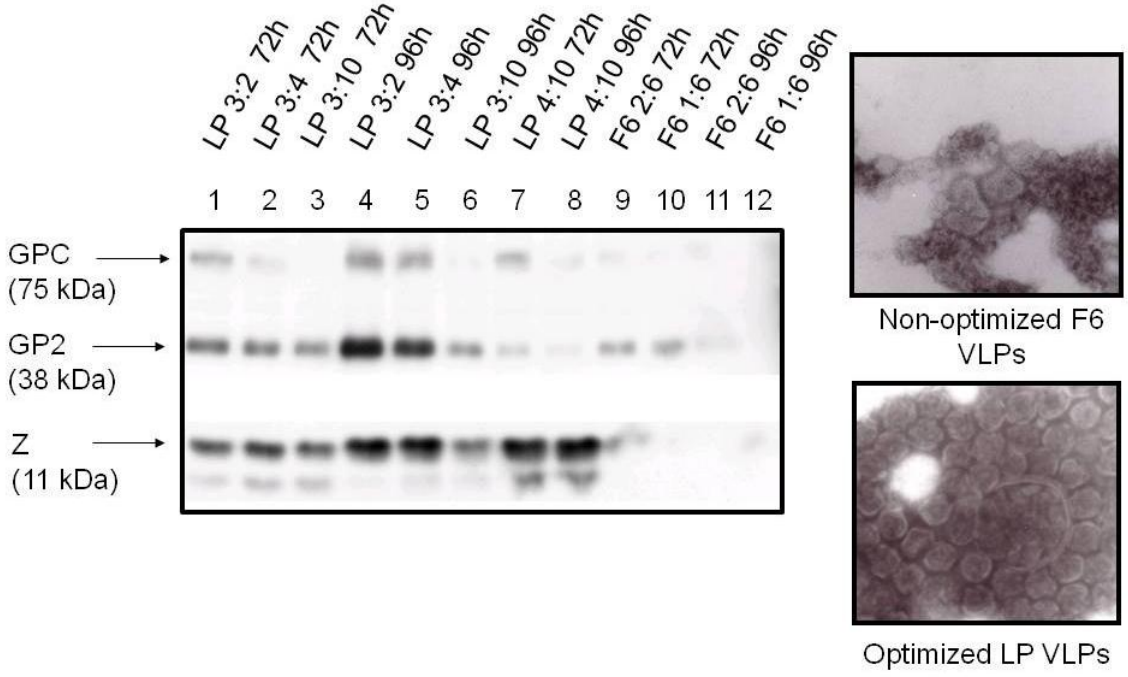


Figure 4.4. Optimization of the VLP protocol.

Two transfection reagents, FuGENE6 and Lipofectamine 2000, were compared for efficiency regarding the yield of VLPs in 293T cells, as determined by WB (#4 anti-GP2 (1:2,000)/anti-rabbit IgG-HRP (1:3,000) and anti-Z (1:10,000)/anti-rabbit IgG-HRP (1:20,000) and EM (x100,000). LP: Lipofectamine 2000; F6: FuGENE6; ratio of DNA (μg) to transfection reagent (μL) is shown.

The proteinase K protection assay was first performed with native LASV. When purified virus is incubated with the proteinase K alone, only the glycoproteins are digested since they are exposed on the surface of the lipid-containing envelope of the viral particle (Fig. 4.5.A). However, when Triton-X is present, the Z and NP proteins are also digested because the integrity of the envelope has become impaired, allowing proteinase K access to these proteins (Fig. 4.5.A).VLPs containing individual LASV proteins were then compared to authentic LASV particles. When the glycoproteins were expressed alone, enveloped particles with visible glycoprotein spikes were observed by EM (Fig 4.6.A). The full-length glycoprotein was detected by WB in the supernatant but disappeared after sucrose cushion purification (data not shown), in accordance with previous results that only cleaved glycoproteins are incorporated in virus particles (Lenz et al. 2001). Conversely, although the NP protein was detected in the purified supernatant, it was not incorporated into lipid-containing particles, as shown in the proteinase K protection assay (Fig. 4.5B), and no particles were visualized by EM (Fig. 4.6C). As expected, expression of the Z protein alone was sufficient for the release of enveloped particles (Fig. 4.5.B and Fig. 4.6.B).

4.3.1.3. Formation of multi-protein VLPs by coexpression of various viral proteins

To determine whether the different viral proteins could interact together to form multi-protein VLPs, the LASV glycoproteins, Z and NP were coexpressed in various combinations and purified supernatants were analyzed as described above. All combinations resulted in the generation of multi-protein VLPs (GPC+Z, Z+NP and GPC+Z+NP) (Fig. 4.5.C and Fig. 4.6.D E and G), with the exception of the glycoproteins

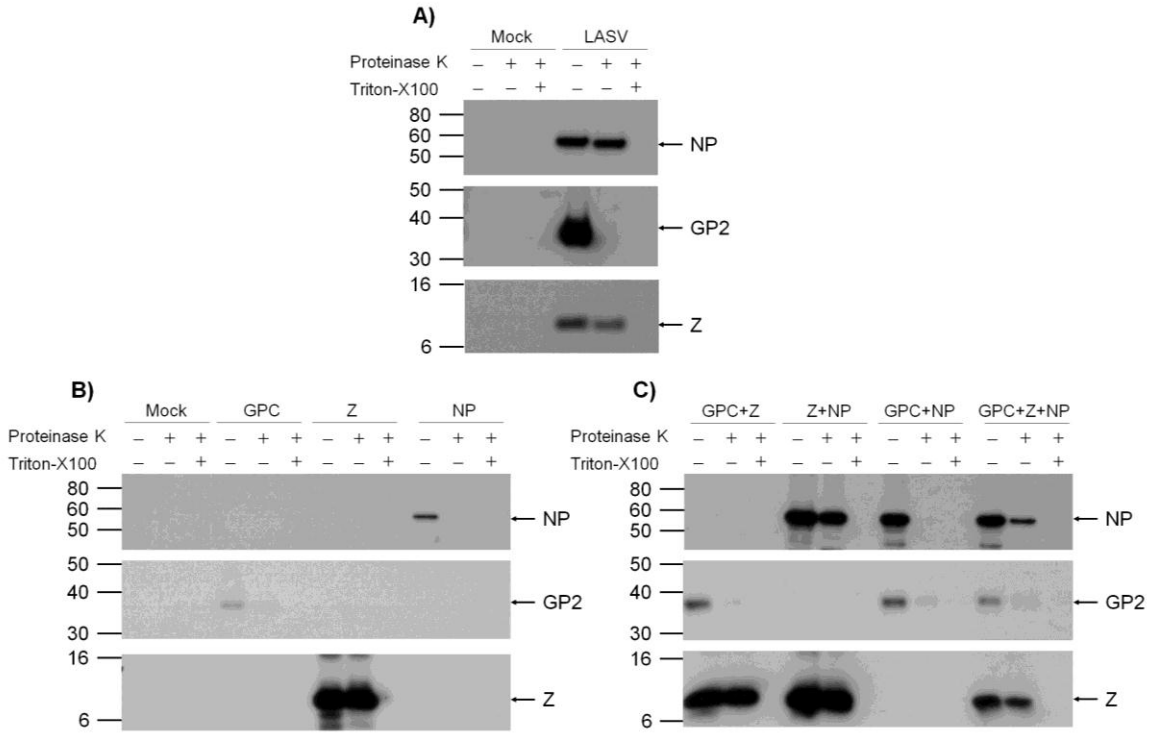


Figure 4.5. Analysis of the viral proteins released into culture supernatant.

(A) 293T cells were infected with LASV or plain medium only (Mock). Culture supernatants were harvested on day 8 post-infection, purified by ultracentrifugation over a 20% sucrose cushion, subjected to a proteinase K protection assay and analyzed by Western blot. (B) Formation of VLPs by individually expressed LASV GP, Z or NP. 293T cells were transfected with pCAGGS encoding GPC, Z or NP, and as a control, empty pCAGGS (Mock). Culture supernatants were harvested 72 hr post-transfection, and then treated as described in (A). (C) Formation of multi-protein VLPs by coexpression of GP, Z and NP. Samples were prepared as described in (A) and (B). Masses are given in kiloDaltons.

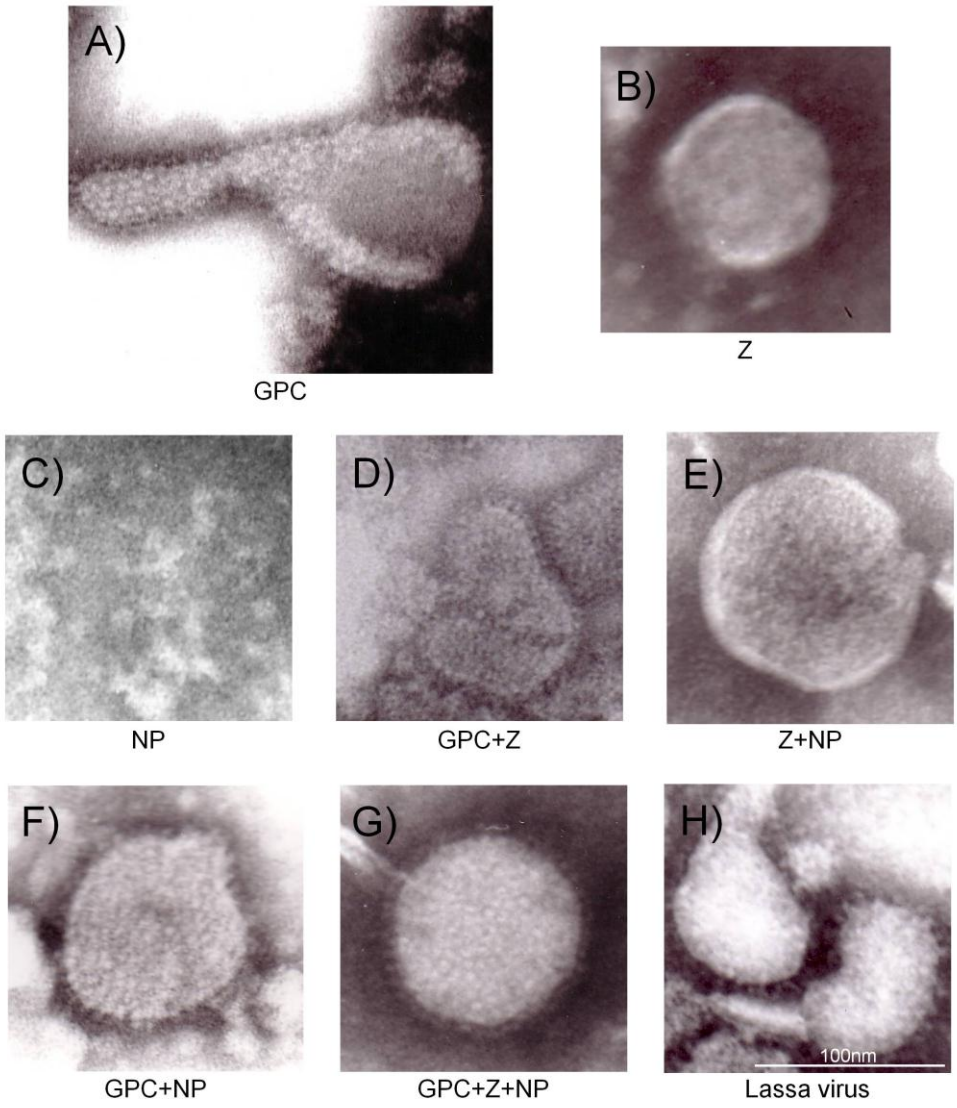


Figure 4.6. Electron microscopic analysis of the different virus-like particles.

293T cells were transfected with different combinations of pCAGGS vectors encoding GPC, Z or NP. LASV is also shown for comparison purposes. Culture supernatants were harvested 72 hr post-transfection, fixed with 0.1% glutaraldehyde and the fixative was quenched by 0.1M glycine (4% paraformaldehyde/2% glutaraldehyde for LASV). Particles were visualized by electron microscopy after negative staining. Bar, 100 nm. The scale is the same for each image in this figure.

and NP combination (GPC+NP). In the latter case, NP was not incorporated inside the VLPs, in contrast to the other combinations in which Z was present (Z+NP and GPC+Z+NP) (Fig. 4.5.C). This implies that Z is required for the incorporation of NP inside the VLPs. EM analysis of VLPs in the supernatant of transfected cells illustrates a remarkable resemblance to native LASV particles, both in morphology and size. VLPs of various sizes were visible, and the glycoprotein spikes were clearly apparent on the surface of the VLPs in the GPC positive samples (GPC, GPC+Z, GPC+NP and GPC+Z+NP). Visualization of the Lassa VLPs supports the successful generation of LASV GPC+Z, Z+NP and GPC+Z+NP VLPs.

4.3.2. Immune response to the LASV virus-like particle vaccine

To determine whether Lassa GPC+Z VLPs could be used as a novel vaccine strategy against Lassa fever, CBA mice were immunized with VLPs, and the immune response it elicits was followed (Figure 4.7.). GPC was chosen as a well-known immunogenic protein and Z because it is the driving force for the budding of the VLPs. NP was excluded because current evidence suggests that it might be a deleterious antigen in vaccines against Lassa fever (Fisher-Hoch et al. 2000). A quantitative ELISA was used to measure the antibody response to viral proteins and the T cell response was measured by a cytokine ELISPOT assay. The VSVΔG/LVGPC vaccine was used as a positive control in this experiment (data not shown). Since VLP vaccines are non-replicating and thus often poorly immunogenic by themselves, we decided to include groups for which the VLPs were combined with an adjuvant to determine whether this would increase the immunogenicity of the vaccine. Four different adjuvant systems were chosen based on their distinct properties.

Alum is an aluminium salt adjuvant. Although there is no consensus on the mechanism of action of aluminium salt adjuvants, the main hypothesis is the formation of depots of antigen in tissue, which causes local inflammation, resulting in APC activation (Hunter 2002). It induces a T_{H2}-type immune response and is not effective for inducing cell-mediated immunity, or for use with peptide antigens (Baylor, Egan, and Richman 2002; Hunter 2002).

In contrast to incomplete Freund's adjuvant, complete Freund's adjuvant contains heat killed *Mycobacterium tuberculosis*, but both preparations are a water-in-oil emulsion. The initial injection is performed with complete Freund's adjuvant and the subsequent booster injections with the incomplete preparation. Although Freund's adjuvant is only used in experimental conditions due to safety issues, complete Freund's adjuvant is often considered the gold standard of adjuvants for testing new vaccine candidates and was thus included in our study. It should be noted nevertheless that evidence has shown that in certain conditions, such as a vaccine based on whole killed Plasmodium yoelii parasites, complete Freund's adjuvant did not induce protection, although other adjuvants will (Hunter 2002). Freund's adjuvant can elicit both T_{H1} and T_{H2} responses (Brunner, Jensen-Jarolim, and Pali-Scholl 2010). TiterMax Gold is a water-in-oil emulsion, like Freund's adjuvant, and is well-known for inducing high antibody titers, without the toxic side effects of Freund's adjuvant.

The Sigma Adjuvant System, the equivalent of the Ribi Adjuvant System, which is used in humans (GlaxoSmithKline Biologicals), is an oil-in-water emulsion derived from bacterial and mycobacterial cell wall components. More specifically, it contains a

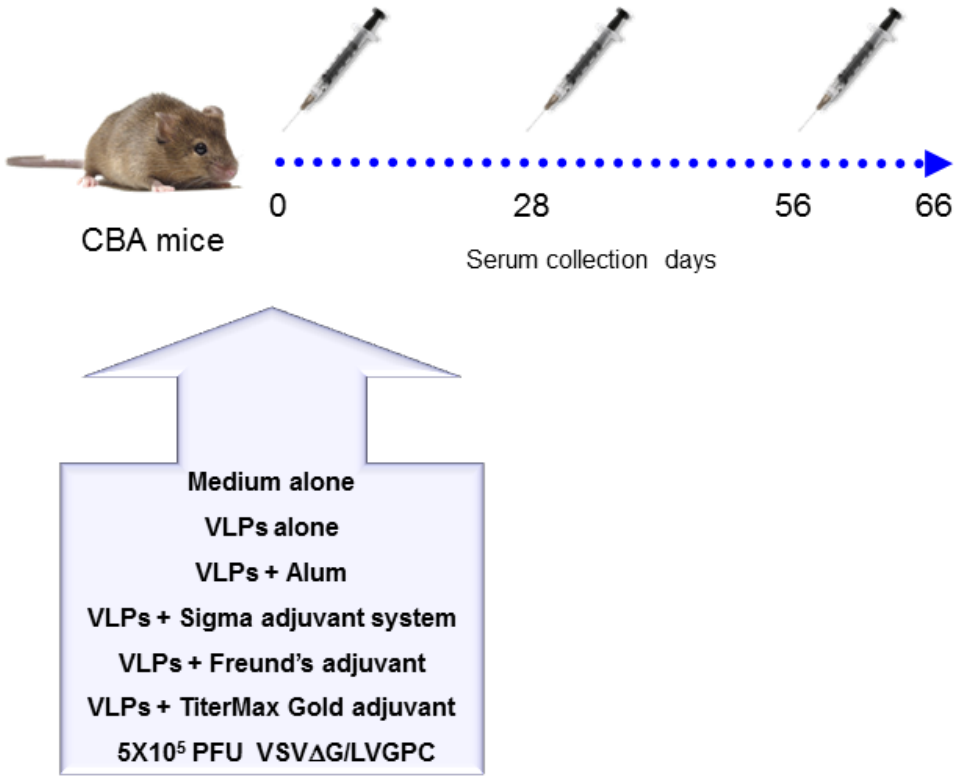


Figure 4.7. Immunization strategy.

CBA mice were immunized on day 0 with either medium alone as a negative control (n=4), 50 µg VLPs alone (n=5), 50 µg VLPs in combination with an adjuvant (n=5, except for the VLPs + Sigma Ad group for which n=4) or VSVΔG/LVGPC ($5X10^5$ PFU) as a positive control (n=4). Mice received boosts on days 28 and 56 and were sacrificed on day 66 at which point spleens were harvested for the ELISPOT assay. Serum samples were collected prior to vaccination on day 0 and then on days 28, 56 and 66 for antibody detection through ELISA. The VSVΔG/LVGPC vaccine was used as a positive control in this experiment (data not shown).

detoxified endotoxin, Monophosphoryl Lipid A (MPL), isolated from *Salmonella minnesota* and synthetic dicorynomycolate. MPL has been shown to induce predominantly a T_{H1}-type immune response, and to a lesser extent T_{H2}-type cytokines and antibody isotypes (Baldridge et al. 2000; Brunner, Jensen-Jarolim, and Pali-Scholl 2010; Casella and Mitchell 2008). MPL is currently used as an adjuvant in several vaccine clinical trials and is used in commercially available vaccines such as the HPV vaccine Cervarix (Casella and Mitchell 2008).

Lassa GPC+Z VLPs were shown to be poorly immunogenic in our hands. Only 3 of the 4 mice vaccinated with Lassa GPC+Z VLPs without adjuvant demonstrated an antibody response (Table 4.1.). One mouse had detectable levels of IgG1 and IgG2a and two more mice had IgG2a antibodies. One out of five mice from the VLPs+Alum group had IgG2a antibodies whereas 3/5 mice in the VLPs+Freund's and 4/5 mice in the VLPs+TiterMax group had detectable antibody levels. The best humoral immune response was observed in the VLPs+Sigma Ad group. All mice in this group had detectable levels of IgG1. Additionally, 3/4 had IgG2a and 2/4 had IgG2b antibodies against LASV antigen. None of the mice had detectable antibody levels against LASV antigen prior to receiving the booster injection of VLPs. Overall, when considering all of the immunization protocols, IgG2a was the dominant isotype produced followed by IgG1 and IgG2b. The levels of IgA were negligible throughout the study (data not shown).

None of the Lassa GPC+Z VLP vaccine preparations was shown to induce a significant production of cytokines following the two booster injections, except for the VLPs+Freund group (Figure 4.8.). The only significant change was seen with the IFN-γ and TNF cytokines in the VLPs+Freund group, where the number of cells secreting IFN-γ

and TNF increased for pool B in comparison to the unstimulated samples. For all remaining IFN-γ and TNF samples and all other cytokines, no significant differences were observed between the number of SFCs from unstimulated samples and samples stimulated with either Pool A, B or C. One complication that was observed was the high background level of cytokine production. This appears to be a non-specific cytokine production and the results were thus mostly non-significant.

4.4. Discussion

In addition to the VSV-based vaccine, which is a live attenuated vaccine, we have developed a type of recombinant subunit vaccine based on Lassa virus-like particles. Upon expression of GPC, Z and NP in a eukaryotic system, we have shown the production of enveloped virus particles lacking the viral genome; and are thus replication-incompetent. The morphology and size of the observed VLPs did not differ from native LASV particles. The particles revealed pleomorphic architecture, varied greatly in size and harboured glycoprotein spikes on their surface when glycoproteins were included. Mice were immunized with GPC+Z VLPs and the elicited humoral and cell mediated immune responses were examined.

It was previously demonstrated that solitary expression of the LASV Z matrix protein results in the formation and budding of membrane-bound particles with the Z protein located on the cytoplasmic side of the envelope (Eichler et al. 2004; Perez, Craven, and de la Torre 2003; Strecker et al. 2003). We have now shown that solitary expression of the glycoproteins results in the presence of vesicles in the culture supernatant with visible glycoprotein spikes on the surface. In contrast to solitary

Antibody, day after immunization	Serum antibody level, ng/mL																							
	Media only				VLPs alone				VLPs + Alum				VLPs + Freund				VLPs + Sigma Ad				VLPs + Titermax			
IgG1																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	42	0	0	0	0	0	0	0	0	173	46	151	0	0	0	0	0
66	0	0	0	0	0	0	0	46	0	0	0	0	0	0	0	0	205	99	432	80	0	0	0	0
IgG2a																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	98	0	0
56	0	0	0	0	0	25	0	0	57	0	0	0	0	283	178	0	111	0	0	0	0	0	0	0
66	0	0	0	0	31	0	0	32	50	0	0	0	0	221	125	0	119	41	50	0	13	21	0	19	33
IgG2b																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0	0	74	105	0	94	0	80	0	0	0	0	0
66	0	0	0	0	0	0	0	0	0	0	0	0	0	41	103	0	96	0	178	0	0	0	0	0
IgG3																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	330
IgM																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	189	0	791	0	0	0	510	0	0	0

Table 4.1. Antibody response to the LASV VLP vaccine over time in mice.

CBA mice were immunized with media only (negative control, n=4), VLPs alone (n=4), VLPs + Alum (n=5), VLPs + Freund (n=5), VLPs + Sigma adjuvant (n=4) or VLPs + Titermax (n=5). Serum samples were collected on day 0, 28, 56 and 66, and anti-LASV antibodies measured with a LASV-infected cell lysate ELISA. Columns represent the result for each mouse in the group. The rows represent the day after immunization. Error bars represent SEM.

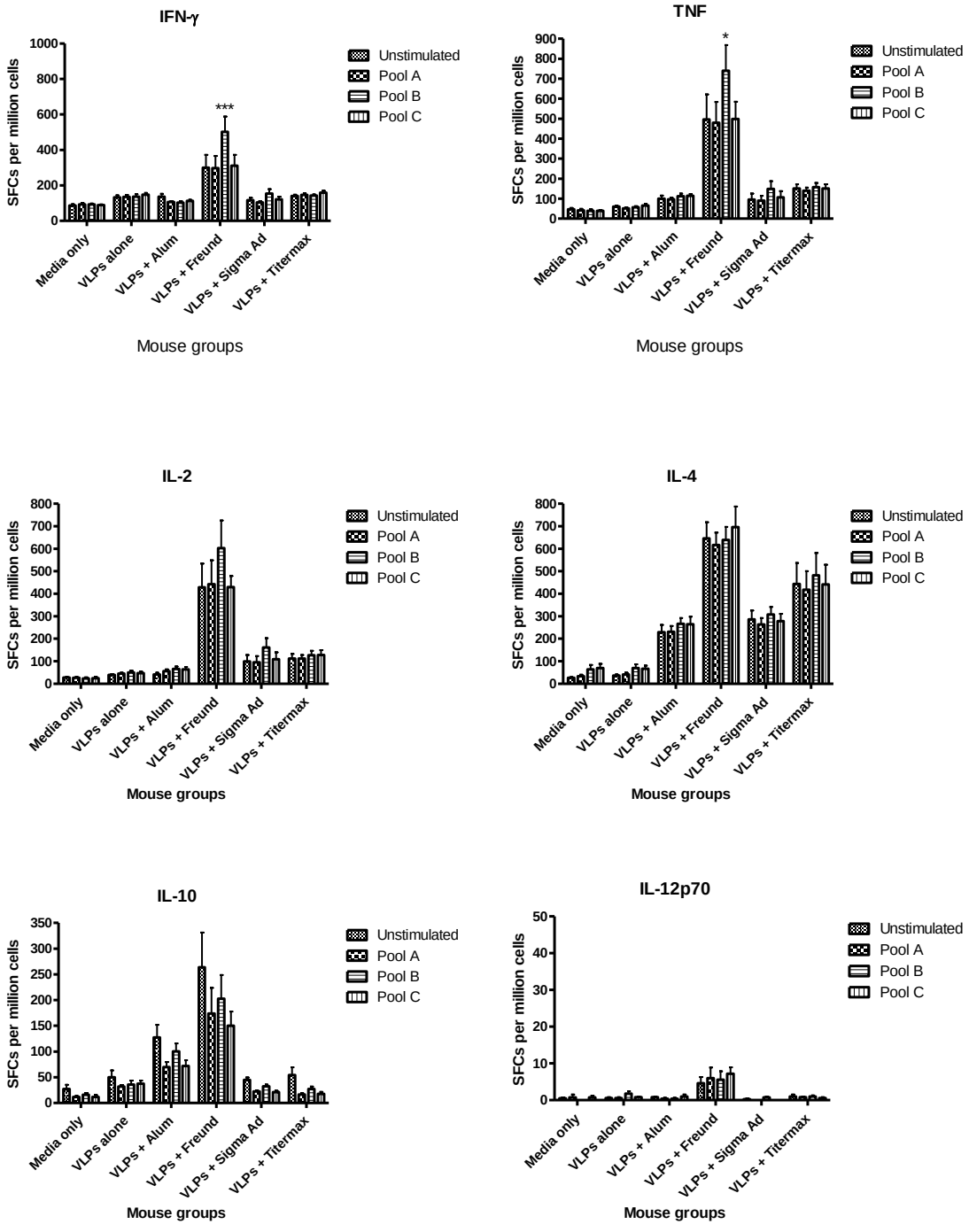

IFN-γ
SFCs per million cells
1000
800
600
400
200
0
***
Unstimulated
Pool A
Pool B
Pool C
Media only
VLPs alone
VLPs + Alum
VLPs + Freund
VLPs + Sigma Ad
VLPs + Titermax
Mouse groups
TNF
900
800
700
600
500
400
300
200
100
0
*
Unstimulated
Pool A
Pool B
Pool C
Media only
VLPs alone
VLPs + Alum
VLPs + Freund
VLPs + Sigma Ad
VLPs + Titermax
Mouse groups
IL-2
SFCs per million cells
800
700
600
500
400
300
200
100
0
Unstimulated
Pool A
Pool B
Pool C
Media only
VLPs alone
VLPs + Alum
VLPs + Freund
VLPs + Sigma Ad
VLPs + Titermax
Mouse groups
IL-4
SFCs per million cells
800
700
600
500
400
300
200
100
0
Unstimulated
Pool A
Pool B
Pool C
Media only
VLPs alone
VLPs + Alum
VLPs + Freund
VLPs + Sigma Ad
VLPs + Titermax
Mouse groups
IL-10
SFCs per million cells
350
300
250
200
150
100
50
0
Unstimulated
Pool A
Pool B
Pool C
Media only
VLPs alone
VLPs + Alum
VLPs + Freund
VLPs + Sigma Ad
VLPs + Titermax
Mouse groups
IL-12p70
SFCs per million cells
50
40
30
20
10
0
Unstimulated
Pool A
Pool B
Pool C
Media only
VLPs alone
VLPs + Alum
VLPs + Freund
VLPs + Sigma Ad
VLPs + Titermax
Mouse groups

Figure 4.8. T cell response to the LASV VLP vaccine in mice.

CBA mice were immunized with media only (negative control, n=4), VLPs alone (n=4), VLPs + Alum (n=5), VLPs + Freund (n=5), VLPs + Sigma adjuvant (n=4) or VLPs + Titermax (n=5). Splenocytes were harvested 10 days following the last immunization and stimulated with peptide pools A, B or C spanning the LASV full-length glycoprotein. Cytokine production was visualized as the number of spot-forming cells (SFCs). Error bars represent SEM. P values represent significant changes in cytokine production compared to unstimulated splenocytes within the same group (* $p<0.05$; *** $p<0.001$).

expression of the glycoproteins or Z, NP expression did not result in the formation of membrane-bound particles in the culture supernatant. We also demonstrated that co-expression of different LASV proteins results in the generation of multi-protein VLPs. Specifically, NP is always incorporated into VLPs when Z is present, independently of glycoprotein expression. As well, it appears that the glycoproteins, Z and NP can be incorporated conjointly in enveloped particles. All together, our results suggest that all combinations of these three viral proteins (the glycoproteins, Z and NP) can generate multi-protein VLPs, with the exception of GPC+NP. Our results are in accordance with findings from Schlie et al. who also recently reported the generation of GPC VLPs, Z+NP VLPs and GPC+Z+NP VLPs (Schlie et al. 2010).

We found that NP was incorporated into the VLPs only when the Z protein was expressed, suggesting that NP needs to interact with Z to be incorporated inside viral particles. The finding that the LASV Z protein, the driving force for particle budding, acts as a bridge interacting with the glycoproteins on one side and the nucleoprotein on the other correlates with a previous study based on an LCMV minigenome system (Perez, Craven, and de la Torre 2003) in which it was determined that Z and NP, but not the glycoproteins, were required to detect RNA in the supernatant of transfected cells. A direct interaction between the Z protein and the nucleoprotein NP is supported by a previous study demonstrating their colocalization by IFA and co-immunoprecipitation in cells transfected with vectors expressing LASV Z and NP (Eichler et al. 2004), by MOPV, TACV and JUNV VLP studies (Casabona et al. 2009b; Shtanko et al. 2010) and by crosslinking studies of LCMV Z and NP (Salvato et al. 1992). Our results support the hypothesis that the Z protein would incorporate the viral RNA indirectly through

interaction with NP, rather than by requiring a pre-formed viral RNA-NP complex. However, the influence of having NP in the form of the RNP complex on the efficiency of NP and viral RNA incorporation inside viral particles cannot be ruled out at this point.

Upon solitary expression of the Lassa glycoproteins (GPC VLPs), we visualized membrane-bound particles with glycoprotein spikes on the surface by electron microscopy. These results suggest that the glycoproteins might have autonomous exocytosis activity. Several viruses from distinct families have been shown to have this property and in some cases, the GP was even revealed to be the driving force for budding (Schmitt and Lamb 2004). However, budding is believed to be mediated through the presence of late domain motifs within the viral protein responsible for this process (Bieniasz 2006; Freed 2002; Welsch, Muller, and Krausslich 2007), and are lacking in the Lassa GPC sequence. GPC VLPs could thus simply be an artifact representing cellular debris due to the cellular damage during the transfection. Nevertheless, Schlie et al., who have also observed the presence of vesicles harbouring glycoprotein spikes upon solitary expression of GPC , believe that the LASV glycoproteins have the property of inducing particle budding in a similar fashion to the Z protein (Schlie et al. 2010).

Lassa GPC+Z VLPs were used to immunize CBA mice alone or in combination with adjuvant with a prime + 2 boosts scheduled at 4-week intervals. Mice received 50 μg i.p. or s.c. of the vaccine preparation. Although the immune response was weak, all mice which received VLPs in combination to the Sigma Adjuvant, containing the MPL antigen, produced an IgG response. In the other groups which received VLPs, only some mice raised an antibody response to Lassa antigen. Regarding the T cell response, cytokine production was also poorly induced, and only the VLPs+Freund's adjuvant

group produced a significantly increased level of IFN-γ and TNF-α producing cells in comparison to the control group. Interestingly, it is the LASV GPC peptide pool B which elicited a significant number of SFCs secreting the IFN-γ and TNF-α cytokines, whereas pool A was the most immunogenic with the VSVΔG/LVGPC vaccine. However, a high background level of cytokine production was noted, especially for the Freund's adjuvant group. Therefore, although statistically significant results were observed for IFN-γ and TNF-α cytokines for pool B in the Freund's adjuvant group, this experiment should be repeated and compared to control mice vaccinated with adjuvant alone, without VLPs.

Branco et al. have recently shown the generation of Lassa multi-protein VLPs and studied the immunogenicity of GPC+Z versus GPC+Z+NP VLPs in BALB/c mice (Branco et al. 2010a) by looking at the IgG response. They found that 10 μg i.p. of VLPs, a dose five fold lower than ours, with no adjuvant, was sufficient to induce an antibody response in BALB/c as determined by an ELISA using both VLPs and soluble GP as the antigen. In addition, it was determined in their study that the IgG response was better when GPC+Z+NP VLPs were used in comparison to GPC+Z VLPs. This could be explained by the fact that NP has been reported in the literature to enhance the incorpotation of the glycoproteins into New World arenavirus VLPs (Casabona et al. 2009a; Groseth et al. 2010). However, Schlie and colleagues have observed no difference in the efficiency of glycoprotein incorporation when NP was present in LASV VLPs (Schlie et al. 2010). The results obtained by Branco et al. suggest that either our ELISA assay is less sensitive than the ones they used, or that the incorporation of the viral proteins into their VLPs was more efficient. Moreover, their production of VLPs itself appears more efficient than ours since they were able to visualize a protein band after

sucrose gradient purification, whereas we never obtained enough VLPs to visualize one. There are thus several experimental differences between the two studies, including the use of different ELISA protocols and mouse strain, which could explain the better antibody response observed by Branco et al.

In conclusion, we have demonstrated successful generation of LASV multi-protein VLPs, in which the Z matrix protein acts as a bridge between the glycoproteins and the nucleoprotein to induce the formation of multi-protein VLPs. These results reemphasize the crucial role of the LASV Z protein during particle assembly and budding. Although the humoral and cellular immune response to Lassa VLPs was weak in our hands, others have demonstrated that a strong antibody response to Lassa VLPs can be induced in the mouse, even without the addition of an adjuvant. Overall, study of the formation of multi-protein VLPs provided insights into the assembly and budding of LASV. Furthermore, LASV VLPs could potentially be used as a novel vaccine platform against Lassa fever, but protective efficacy in experimental animals remains to be determined.

5. Establishment of a new mouse model for Lassa virus infection

5.1. Introduction

Three different animal species are known to be susceptible to LASV infection: the mouse, the guinea pig, and the non-human primate (Section 1.2.6). However, LASV is not lethal in immunocompetent mice, unless the challenge is performed through the intracranial route. This results in a central nervous system disease which does not reflect pathogenesis in humans (Lukashevich 1985; Lukashevich 1992; Lukashevich et al. 2005; Peters et al. 1987). While LASV infection of strain 13 guinea pigs more closely resembles the disease process occurring in humans (Jahrling et al. 1982), this model is not commercially available and the limited range of immunological reagents available for this animal species prevents its use in immunological studies. Consequently, the lack of an adequate small animal model for LASV infection has made studies of the pathogenesis and the immune response to this virus very difficult.

The mouse, if susceptible, would offer great possibilities with the panoply of immunological tools and knock-out (KO) mouse strains available; there is significant potential to dissect the immune response which protects normal mice from LASV infection and thereby shed light on the reasons that LASV infection can be so devastating in humans. For this reason, we used various mouse strains with defects impairing innate or adaptive immunity with the aim of establishing a new mouse model for LASV infection. This would allow us to study novel antiviral compounds and vaccine candidates against LASV infection and to provide us with insights into the immune mechanisms involved in Lassa fever pathogenesis.

5.2. Materials and methods

5.2.1. Mice

Mice deficient in CD4 (helper T cell$^{-/-}$, cat. No 2663), CD8 (cytotoxic T cell$^{-/-}$, cat. No 2665), μMT (B cell$^{-/-}$, cat. No 2288), IFN-γ (cat. No 2287), C57BL/6 control mice (cat. No 0664), NOD/SCID (cat. No 1303), NOD/SCID/IL2Rγ (cat. No 5557) and NOD mice (cat. No 1976) were obtained from The Jackson Laboratory (Maine, USA) (Table 5.1.). 129S6/SvEv-*Stat1*^{tm1Rds} mice (signal transducer and activator of transcription 1, STAT1$^{-/-}$, cat. No 2045), control 129/S6 mice (cat. No 129SVE-F) and Rag-2$^{-/-}$ (cat. No 461) were purchased from Taconic (Table 5.1.). All mice were females, 3-5 weeks of age, except for the Rag-2$^{-/-}$, NOD/SCID/IL2Rγ$^{-/-}$ and the STAT1$^{-/-}$ mice for the LD_{50} experiment which were males. Upon arrival, mice were placed in the BSL-2 where subdermal transponders to monitor body temperature (Biomedic Data Solutions, Seaford, DE, USA) were implanted. Following implantation, the animals were kept in BSL-2 or moved into the BSL-4 for LASV experiments and allowed to acclimate for 7 days before LASV inoculation. Mice were kept in the BSL-2 and BSL-4 under barrier conditions in environmentally enriched sterile housing with food and water *ad libitum*. All procedures were performed in the BSL-2 and BSL-4 facilities according to the Canadian Science Centre for Human and Animal Health Animal Care Committee guidelines, and were in accordance to the principles of the Canadian Council on Animal Care.

KO mouse strain name	Ordering information	Genetic background	Immunodeficiencies	Selected reference(s)
CD4 B6.129S2-*Cd4*^{tm1Mak}/J	Jackson labs # 002663	C57BL/6	CD4 KO mice have a deficiency in helper T cells	(Rahemtulla et al. 1991)
CD8 B6.129S2-*Cd8a*^{tm1Mak}/J	Jackson labs # 002665	C57BL/6	CD8 KO mice have a deficiency in cytotoxic T cells	(Fung-Leung et al. 1991)
μMT or Bcell$^{-/-}$ B6.129S2-*Ighm*^{tm1Cgn}/J	Jackson labs # 002288	C57BL/6	These mice lack mature B cells and there is no expression of membrane-bound IgM	(Kitamura et al. 1991)
IFN-γ B6.129S7-*Ifng*^{tm1Ts}/J	Jackson labs # 002287	C57BL/6	These mice lack the IFN-γ cytokine.	(Dalton et al. 1993)
NOD NOD/ShiLtJ	Jackson labs # 001976	NOD (non-obese diabetic)	These mice develop diabetes at ~12 weeks of age in females and several weeks later in males. They also exhibit multiple innate immunity defects (marked decrease of NKT cells, lack of functional NK cells, no circulating complement and abnormal APCs).	(Godfrey et al. 1997; Makino et al. 1980; Serreze et al. 1997)
NOD/SCID NOD.CB17-*Prkdc*^{scid}/J	Jackson labs # 001303	NOD	SCID mice in the NOD background are considered to be less leaky than other SCID mice because of the innate immunity deficiencies that brings the NOD background in addition to the lack of T and B cells.	(Shultz et al. 1995)
NOD/SCID/ IL2Rγ NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ	Jackson labs # 005557	NOD	These mice bear immunodeficiencies associated with the NOD and SCID backgrounds and lack the gamma subunit of the IL-2 receptor.	(Shultz et al. 2005)
STAT1 129S6/SvEv-*Stat1*^{tm1Rds}	Taconic # 2045	129S6/SvEv	Lack of the STAT1 protein results in unresponsiveness of target cells to type I and type II IFN	(Durbin et al. 1996; Meraz et al. 1996)
Rag-2 B6.SJL(129S6)-*Ptprc*^{a}/BoyCrTac-*Rag2*^{tm1Fwa} N10	Taconic # 461	B6.SJL	These mice lack mature B, NKT and T cells because the Rag-2 mutation prevents VDJ recombination	(Shinkai et al. 1992)

Table 5.1. Description of each immunodeficient mouse strain used in this study.

5.2.2. Virus infection

Virus dilutions were prepared in unsupplemented DMEM and the volume of inoculum adjusted to 200 µL for administration i.p. using a 28G needle.

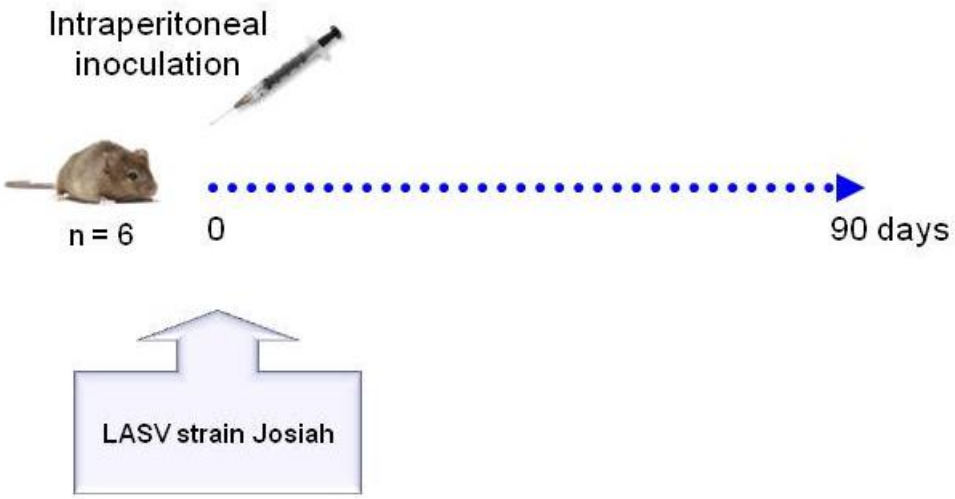


Figure 5.1. Overall experimental plan: Susceptibility of various immunodeficient mice to LASV infection.

Groups of 6 mice, unless indicated otherwise, were inoculated intraperitoneally with LASV strain Josiah. Weight loss and body temperature were monitored for 2 weeks and survival for 90 days.

5.2.3. Calculation of the median lethal dose

The median lethal dose (LD_{50}) was calculated from the experimental data based on the Reed-Muench method (Welkos and O'Brien 1994).

5.3. Results

5.3.1. Susceptibility of immunodeficient mice to LASV infection

To assess the susceptibility of immunodeficient mice to LASV infection and lethality, Rag-2, CD4, CD8, B cell deficient mice, C57BL/6 control mice, NOD/SCID and NOD mice (n=6) were infected with 10^4 PFU of LASV strain Josiah. None of these mouse strains showed any signs of clinical disease, as assessed by the monitoring of body temperature and weight of the animals over a period of 14 days (Figure 5.2.). Additionally, all survived over the 3-month period in which they were followed (data not shown). Rag-$2^{-/-}$ mice were also infected with a higher dose of LASV, 10^6 PFU and NOD/SCID/IL2Rγ KO mice were inoculated with 10^4 or 10^6 PFU of LASV Josiah. All survived and no change in body temperature or weight were noted (data not shown).

STAT1 KO mice as well as control 129S6 mice (n=6) were also infected with 10^4 PFU of LASV to determine whether this mouse strain was susceptible to LASV infection since the interferons are known to play a pivotal role in the host' antiviral immunity (Bray 2001; Durbin et al. 1996; Johnson and Roehrig 1999; Takaoka and Yanai 2006). STAT1 KO mice, unlike the other mouse strains, showed clinical signs of illness by day 6 p.i., as demonstrated by physical signs such as ruffled fur and decreased spontaneous activity, weight loss and drop in body temperature (Figure 5.3.). Some animals developed neurological signs such as paresis and convulsions; hemorrhages from the nose were also noted. Four out of the 6 STAT1 KO mice were dead by day 10 p.i. (33% survival rate) whereas all of the wild-type 129S6 control mice survived.

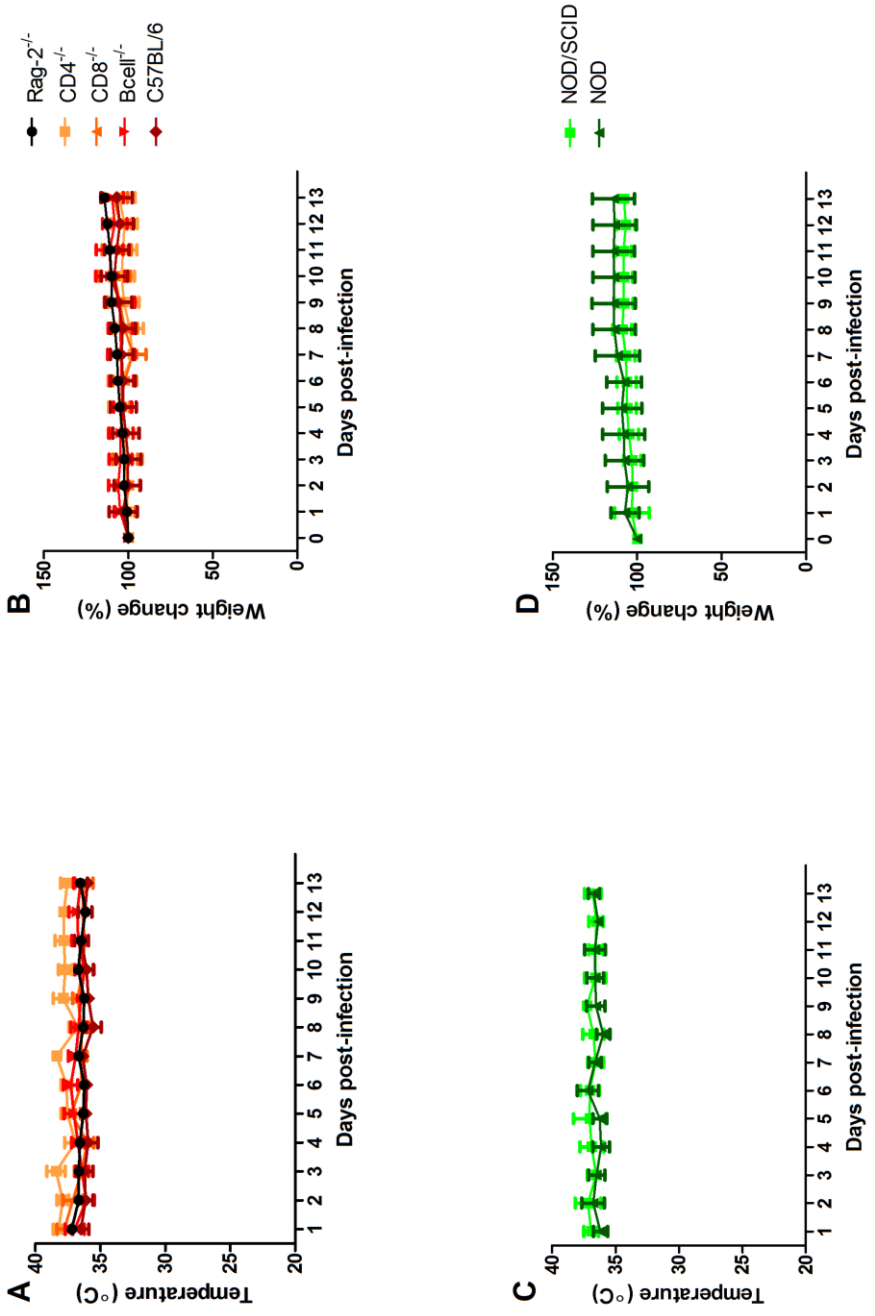


Figure 5.2. Susceptibility to LASV infection of mice with various defects in immunity.

Rag-2$^{-/-}$, CD4$^{-/-}$, CD8$^{-/-}$, B cell$^{-/-}$, wild-type C57BL/6, NOD/SCID and NOD mice (n=6) were infected i.p. with 10^4 PFU of LASV strain Josiah. All infected animals survived for 90 days. There was no change in (A&C) body temperature and (B&D) weight over the 14 days that they were monitored.

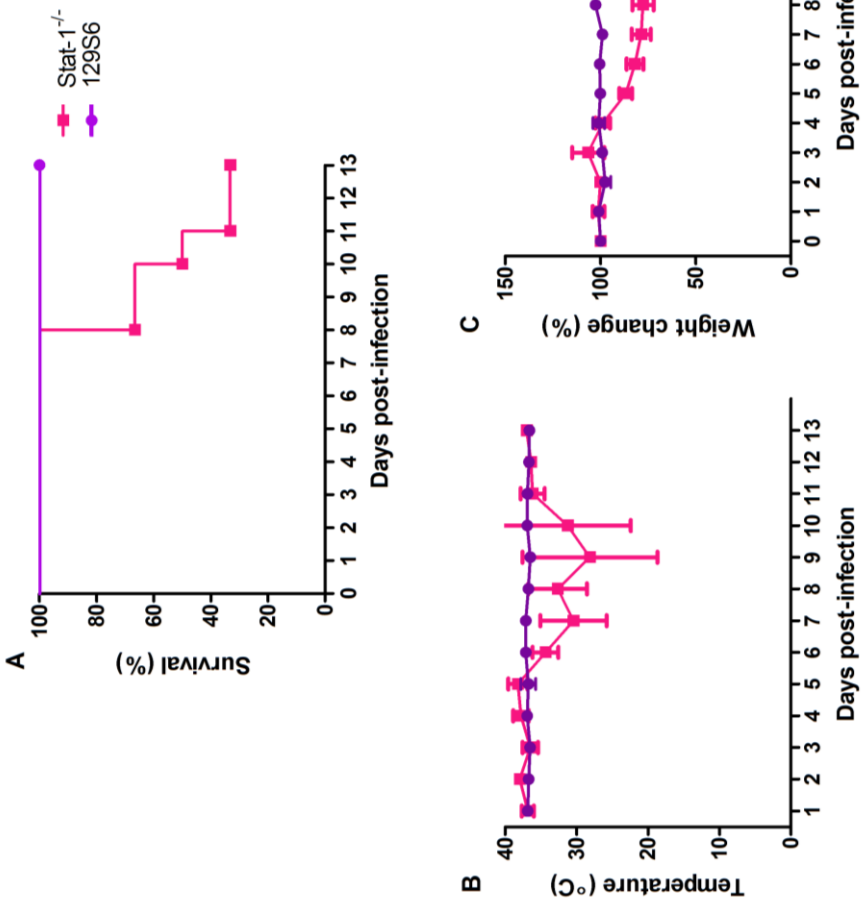


Figure 5.3. Susceptibility to LASV infection of Stat-1$^{-/-}$ mice.

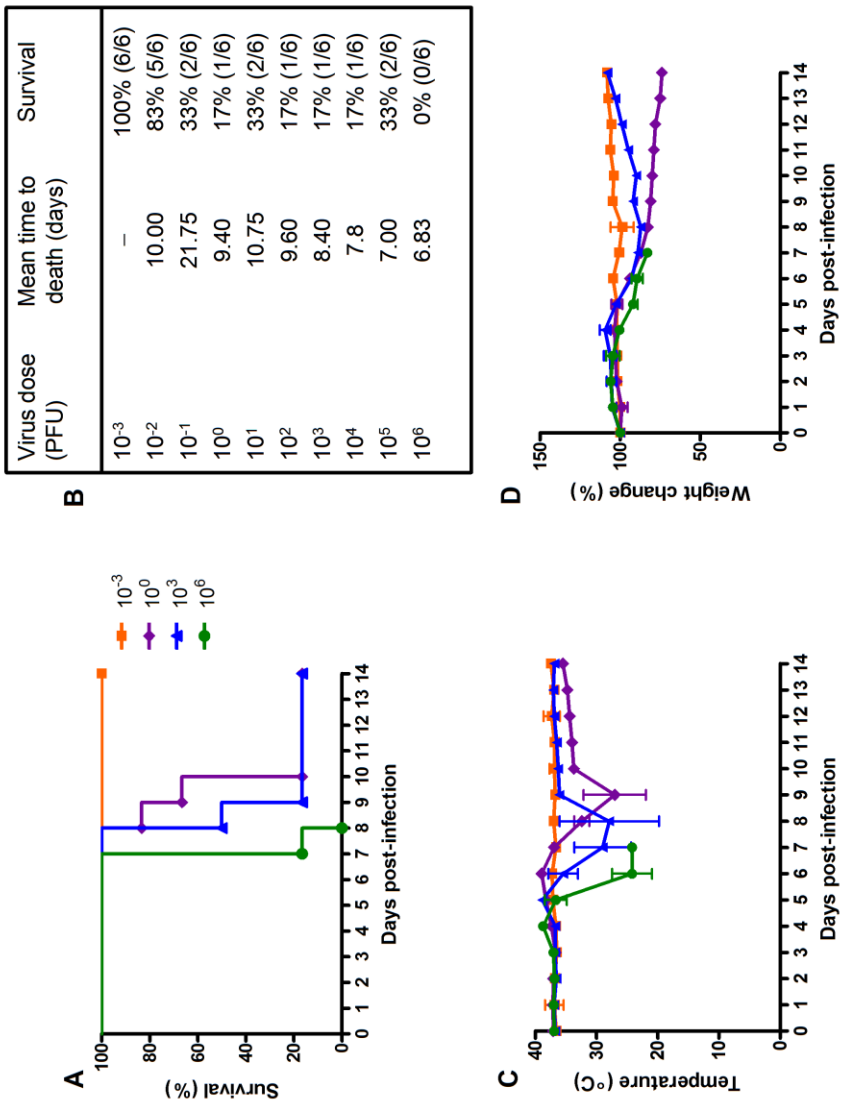
Stat-1$^{-/-}$ and wild-type 129S6 mice (n=6) were infected i.p. with 10^4 PFU of LASV strain Josiah. Survival (A) was monitored over a period of 90 days whereas body temperature (B) and weight (C) of infected animals were monitored daily over a period of 14 days.

5.3.2. Determination of the LD_{50} in STAT1 KO mice

Following the discovery that LASV was lethal to STAT1 KO mice upon i.p. inoculation of 10^4 PFU, an LD_{50} experiment in this mouse strain was performed to determine whether 100% lethality could be achieved in this model. STAT1 KO mice (n=6) were infected with LASV strain Josiah at doses between 10^{-3} and 10^6 PFU (10-fold serial dilutions) and monitored over a period of 15 days for survival, weight and body temperature. All of the animals survived at the lowest dose (10^{-3} PFU) with no significant change in weight and body temperature, whereas 100% lethality was achieved with the highest virus dose (10^6 PFU) (Figure 5.4.). All other doses resulted in partial survival with the LD_{50} calculated to be 0.56 PFU.

5.3.3. Susceptibility of IFN-γ KO mice to LASV infection

Since STAT1 KO mice are defective in their response to Type I as well as Type II interferons, the susceptibility of IFN-γ KO mice to LASV infection was also investigated in order to determine whether it was the lack of responsiveness to Type I or Type II interferons which played a role in this model. In this experiment, IFN-γ KO mice (n=6) in the C57BL/6 background (this deficiency was not available to us in the same genetic background as the STAT1 deficiency) were infected with 10^4 or 10^6 PFU of LASV strain Josiah and monitored for 14 days for survival, weight change and body temperature (Figure 5.5.). Although 100% survival was observed in the group which received the lowest virus dose (10^4 PFU), their weight started to drop by day 7 p.i., as well as their body temperature by day 9. However, all the mice recovered by day 13 p.i. and survived until the end of the study at 90 days (Figure 5.5.). On the other hand, it was noticed on



Virus dose (PFU)	Mean time to death (days)	Survival
10^{-3}	–	100% (6/6)
10^{-2}	10.00	83% (5/6)
10^{-1}	21.75	33% (2/6)
10^{0}	9.40	17% (1/6)
10^{1}	10.75	33% (2/6)
10^{2}	9.60	17% (1/6)
10^{3}	8.40	17% (1/6)
10^{4}	7.8	17% (1/6)
10^{5}	7.00	33% (2/6)
10^{6}	6.83	0% (0/6)

Figure 5.4. Determination of the LD_{50} in Stat-$1^{-/-}$ mice.

Stat-$1^{-/-}$ mice (n=6) were infected i.p. at doses of 10^{-3}; 10^{-2}; 10^{-1}; 10^{0}; 10^{1}; 10^{2}; 10^{3}; 10^{4}; 10^{5}; or 10^{6} PFU of LASV strain Josiah. Survival was monitored over a period of 90 days whereas body temperature and weight of infected animals were monitored daily over a period of 15 days.

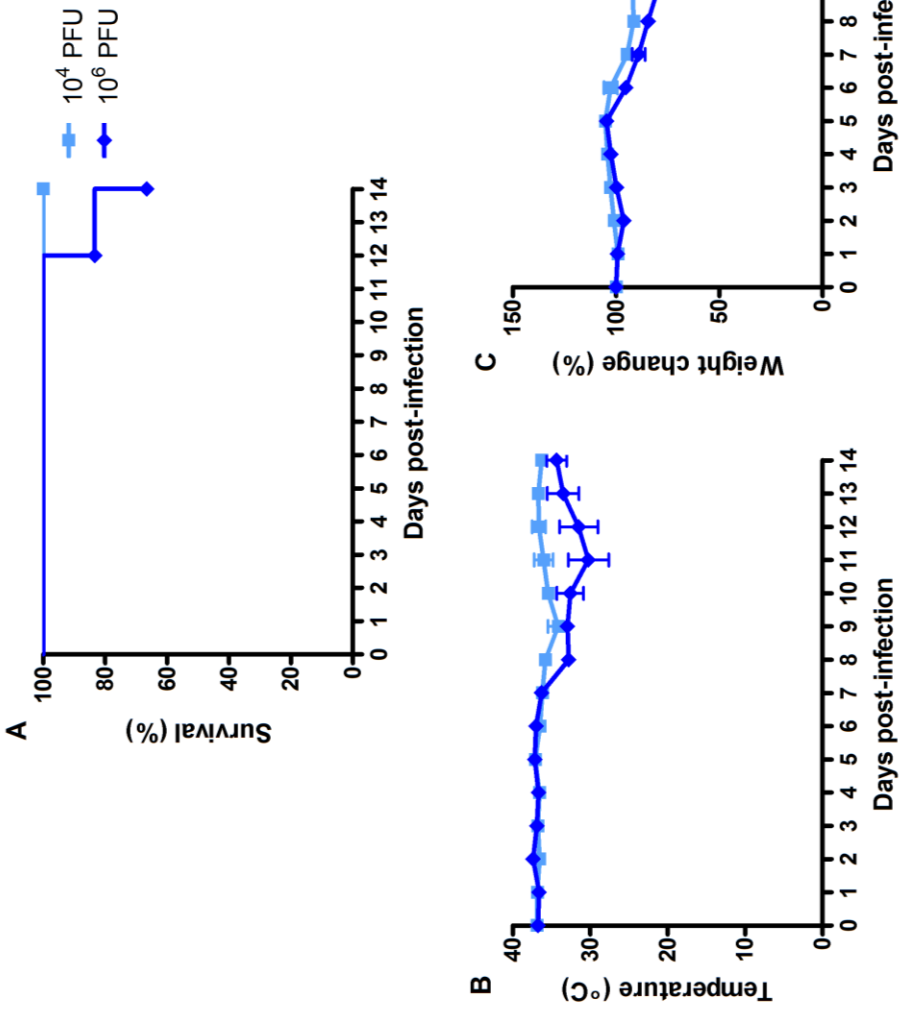


Figure 5.5. Susceptibility to LASV infection of IFN-$\gamma^{-/-}$ mice.

IFN-$\gamma^{-/-}$ (n=6) were infected i.p. with 10^4 or 10^6 PFU of LASV strain Josiah. A) Survival was monitored over 90 days whereas B) body temperature and C) weight of infected animals were monitored daily over a period of 14 days.

day 6 p.i. that the mice which received the highest virus dose (10^6 PFU) were showing signs of illness as observed by weight loss and drop in body temperature (Figure 5.5. B and C). Their level of activity was also diminished by day 8 and one mouse died on day 12 and a second one on day 14 while the remaining four recovered, with an overall survival rate of ~67% (Figure 5.5. A).

5.3.4. Evaluation of the VSVΔG/LVGPC vaccine in the STAT1 KO mouse model of LASV infection

Given that the primary goal of the development of a lethal small animal model for LASV was to evaluate vaccine efficacy, our VSVΔG/LVGPC vaccine was administered to STAT1 KO mice to determine whether this vaccine would protect these mice against LASV challenge. STAT1 KO mice were thus immunized with 10^2, 10^3, 10^4, 10^5 or 10^6 PFU of the VSVΔG/LVGPC vaccine i.p. and compared to a control group which only received plain DMEM. Unfortunately, most of the mice succumbed to the live-attenuated vaccine (Figure 5.6. A). One mouse from the 10^4 PFU group and 4 mice from the 10^2 PFU group survived past day 14 post-immunization; however, no mice from the 10^4 PFU group and only two mice from the 10^2 PFU group survived until day 28 post-immunization. The immunization was then also performed i.m. at 3 different doses (10^1, 10^2 and 10^4 PFU) to determine whether a different injection route would result in lower virulence and survival of the mice. However, 100% lethality was observed for all doses when the i.m. immunization route was used (Figure 5.6. B).

5.3.5. Susceptibility of STAT1 KO mice to the recombinant VSV and VSVΔG/ZEBOVGP viruses

Following the finding that STAT1 KO mice were highly susceptible to VSVΔG/LVGPC, their susceptibility to the recombinant VSV (rVSV) and VSVΔG/ZEBOVGP viruses was also investigated**.** Mice were inoculated with 10^2 or 10^6 PFU i.p. of the recombinant VSV and VSVΔG/ZEBOVGP viruses and it was found that these two viruses were highly lethal in these mice, resulting in significant haemorrhaging from mucosal areas such as the nose and genital area and death of the animals by 2 to 4 days p.i. (Figure 5.7. A and B).

5.1. Discussion

In the present study, susceptibility to LASV disease was observed in mice with immune defects affecting the interferon response. Specifically, STAT1 KO mice were completely susceptible to LASV lethality whereas partial lethality was observed in IFN-γ KO mice inoculated intraperitoneally. In contrast, mice lacking major components of the adaptive immune system, as well as wild-type mice, did not show any apparent sign of disease following infection with LASV. Vaccine testing in STAT1 KO mice was also evaluated using the recombinant VSVΔG/LVGPC vaccine but STAT1 KO mice were found to be highly sensitive to this virus and all mice but two succumbed following i.p. or i.m. immunization.

Studies in STAT1 KO mice and *in vitro* culture of cells originating from these mice suggest that the STAT1 protein is specific to the IFN pathway, including Type I, II and III IFNs (Durbin et al. 1996; Meraz et al. 1996). Although other cellular factors (epidermal growth factor, platelet-derived growth factor, leukemia inhibitory factor, IL-6,

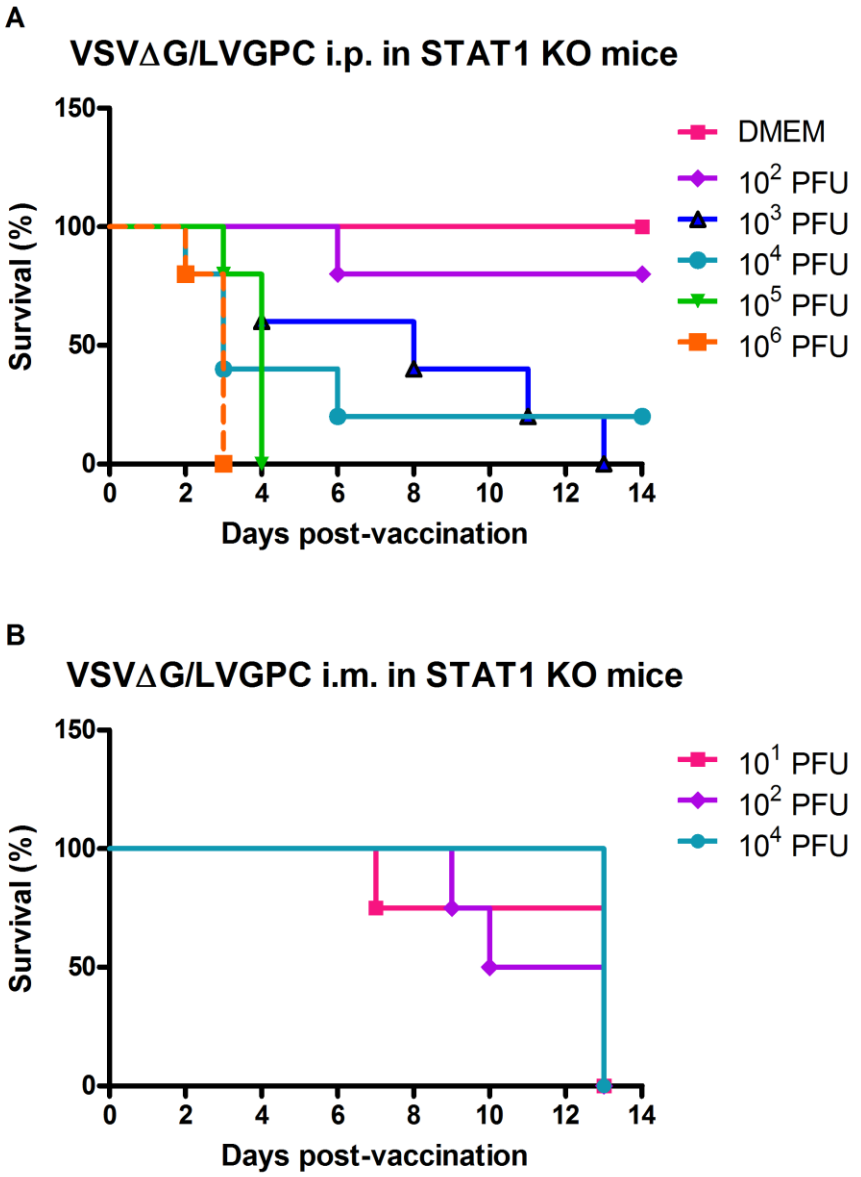


Figure 5.6. The VSVΔG/LVGPC vaccine is lethal to Stat-1$^{-/-}$ mice.

A) Stat-1$^{-/-}$ mice (n=5) were immunized i.p. with DMEM as control or 10^2, 10^3, 10^4, 10^5 or 10^6 PFU of the VSVΔG/LVGPC vaccine. B) Stat-1$^{-/-}$ mice (n=4) were immunized i.m. with 10^1, 10^2, or 10^4 PFU of the VSVΔG/LVGPC vaccine. Animals were monitored daily for survival.

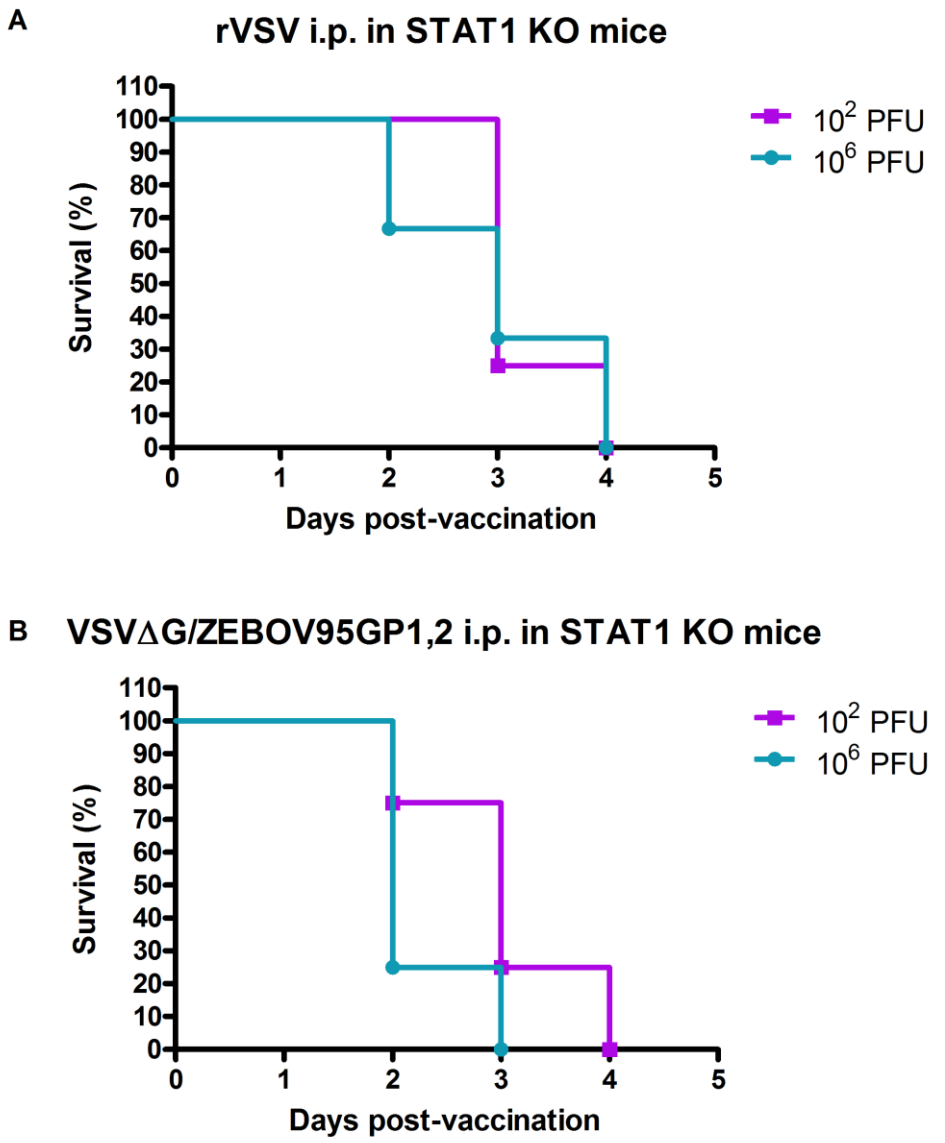


Figure 5.7. Recombinant VSV and VSVΔG/ZEBOV viruses are lethal to STAT1$^{-/-}$ mice.

A) STAT1$^{-/-}$ mice were immunized i.p. with 10^2 (n=4) or 10^4 (n=3) PFU of the rVSV. B) STAT1$^{-/-}$ mice (n=4) were immunized i.p. with 10^2 or 10^4 PFU of the VSVΔG/ZEBOV95GP1,2 vaccine. Animals were monitored daily for survival.

colony stimulating factor 1 and angiotensin), in addition to the IFNs, have previously been associated with STAT1 phosphorylation under *in vitro* conditions, no noticeable defects have been observed in STAT1 KO mice in regard to these molecules (Durbin et al. 1996; Meraz et al. 1996). This suggests that STAT1 activation does not play an essential role in the intracellular pathways activated by these other cellular factors, and that they can use redundant pathways for their signaling. Nevertheless, there have been some recent reports of other molecules signalling through STAT1, such as IL-7 and fibroblast growth factor (Lee et al. 2000; Lee, Gimeno, and Levy 1999; Sahni et al. 1999). It should be emphasized that although STAT1 appears to be relatively specific to the IFN signaling cascade, the reverse does not hold: STAT1-dependent and -independent pathways have been shown to exist in IFN-α/β and IFN-γ signaling (Gimeno et al. 2005; Platanias 2005; Ramana et al. 2002) and either cooperate or function independently of the predominant JAK-STAT pathways. The IFN system was also shown to mediate protection against Dengue virus infection through a Stat-1 independent pathway in the mouse (Shresta et al. 2005). Overall, these data imply that the lethality of STAT1 KO mice infected with LASV is most likely due to the lack of response of these mice to IFNs through the STAT1-dependent pathway. However, it will be important in the future to compare the susceptibility of STAT1 KO mice to IFN receptor KO mice to ensure that it is not the impairment of another molecule signalling through STAT1 which results in lethality.

One limitation of the present study is that the various immunodeficient mice originated from different genetic backgrounds. The mouse genotype is known to influence the death outcome when mice are inoculated with LASV through the

intracranial route (Peters et al. 1987). In addition, although STAT1 KO mice in the 129S6 background die when infected with severe acute respiratory syndrome coronavirus (SARS-CoV) (Frieman et al. 2010), no lethality was observed in STAT1 KO mice from the C57BL/6 background (unpublished data, personnal communication with Steven Jones). These observations stress the importance of comparing mice from the same genetic background. To rule out the possibility that the observed susceptibility of STAT1 KO versus resistance of the other immunodeficient mice is due a different mouse genotype, it will thus be of primary importance in a future study to test the susceptibility of Rag-$2^{-/-}$ mice in the 129S6 background or STAT1 KO mice in the C57BL/6 background to ensure that resistance of mice deficient in T and B cells to LASV disease is truly due to this immune deficiency. Additionally, this raises an issue regarding the results observed in STAT1 KO mice, which are deficient in their cellular response to Type I, II and III IFNs, in comparison to the IFN-γ KO mice, which only have a non-functional IFN-γ gene. Since STAT1 KO mice were from the 129S6 background and the IFN-γ KO mice were from the C57BL/6 genetic background, no conclusions can currently be drawn concerning the importance of the IFN-γ response in comparison to the Type I IFN response. It will be very interesting in the future to dissect in greater detail the relevance of the different IFNs to the immune response to LASV. This could be achieved by looking at IFN-α/β receptor (A129), IFN-γ receptor (G129) and both IFN-α/β and IFN-γ receptors (AG129) deficient mice, all originating from the same mouse genetic background, and which are available in certain research laboratories (Huang et al. 1993; Muller et al. 1994). Another option would be to treat immunocompetent mice with antibodies against murine IFNs.

Although other factors may play a role in the resistance of mice to LASV, the fact that partial lethality can be achieved in IFN-γ KO mice implies that this cytokine is involved, at least partially, in resistance of wild-type mice to LASV disease. The antiviral activities of IFN-γ are mediated through intrinsic and extrinsic mechanisms. Intrinsic mechanisms occur in the infected cell itself, and include the induction of antiviral molecules such as inducible nitric oxide (iNOS), PKR and 2',5'-oligoadenylate synthetase family members (OASs) (Schreiber and Schreiber 2003). Extrinsic mechanisms, on the other hand, refer to the induction of the adaptive immune response through upregulation of MHC class I and II antigen presentation, macrophage activation and control of the differentiation of naïve $CD4^+$ T cells into T_{H1} cells (Schoenborn and Wilson 2007). Potential sources of IFN-γ are NK cells, NKT cells, and defined subsets of T cells (Schoenborn and Wilson 2007). Interestingly, in our study, various KO mouse strains deficient in T cells and/or NKT cells, did not show any apparent signs of disease following inoculation with LASV. In addition, the NOD mutation present in NOD and NOD/SCID mice has been reported to result in a functional deficit in NK cells. However, the NOD mutation also causes defects in APCs, which could interfere with LASV replication in macrophages and DCs. Depletion of cell types known to produce IFN-γ in wild-type mice would thus be an important experiment to perform in the future in order to clarify their role during LASV infection of mice.

Another mouse strain has recently been reported to be susceptible to LASV lethality. Flatz et al. (Flatz et al. 2010) have found that HHD mice, which express a human/mouse-chimeric HLA-A2.2 molecule rather than the murine MHC class I gene products, are susceptible to LASV infection and disease (~22% lethality, n=23). In

contrast, wild-type C57BL/6 control mice, as well as C57BL/6 x HHD F1 mice (heterozygous at the relevant MHC loci), were shown to clear the infection by day 7 p.i. and did not show any obvious signs of disease. Interestingly, MHC-I KO mice and HHD mice depleted of $CD4^+$ and $CD8^+$ cells were found to have a persistent high-level viremia but no apparent clinical disease. These data suggest that the murine MHC is involved in the control of LASV replication whereas murine T cells may play a role in LASV pathogenesis in the presence of human MHC class I molecules, at least in this model. Histological and cytokine studies also demonstrated that monocytes/macrophages were activated in HHD mice and produced IL-12p40 and nitric oxide. The authors thus hypothesized that murine T cells most likely cause immunopathology by overstimulation of monocytes/macrophages, which in turn secrete inflammatory mediators, resulting in disease.

Our results with mice harboring deficiencies in B and/or T cells indicate that an adaptive immune response is not required to protect against LASV disease and lethality in the mouse, at least in the presence of a functional IFN response. This is in accordance with the current knowledge on the immunity to LASV, which suggests that it is a lack of an efficient innate immune response which results in disease (Baize et al. 2004; Baize et al. 2009; Lukashevich et al. 1999; Mahanty et al. 2003; Pannetier et al. 2011). On the other hand, Flatz et al. have demonstrated that T cells can cause disease in the presence of a certain human MHC genotype. The role of T cells versus IFNs in the pathogenesis of Lassa fever remains to be clarified. The contradiction between our results could be explained in different ways. We hypothesize that the protective host factors and pathogenesis might be different in humans and mice. Immunocompetent mice would be protected from lethal LASV infection by the induction of a strong innate antiviral defense

through IFNs whereas human Lassa fever would be triggered by an immunopathological response of T cells. An alternative hypothesis would be that in the absence of the induction of IFNs, the immune response is skewed towards the activation of immune cells in a way that immunopathology is induced rather than protective immunity, resulting in disease. In this case T cells would be the mediators of disease in both mice and humans. Since LASV NP has been shown to antagonize the IFN response, it would be interesting to determine whether this inhibitory effect occurs only in human cells and not murine cells. This information could help clarify the difference between mice and humans concerning the role of the IFNs in LASV pathogenesis.

Since the main goal to the development of a novel mouse model of Lassa fever was to assess vaccine efficacy, STAT1 KO mice were vaccinated with the VSVΔG/LVGPC vaccine. Unfortunately, the vaccine was highly lethal, as well as the recombinant VSV and VSVΔG/ZEBOV GP viruses. It has been reported in the literature that wild-type VSV is lethal in STAT1 KO mice (Takaoka and Yanai 2006). However, the recombinant VSV vaccines are thought to be less virulent than the wild-type VSV since it has been demonstrated previously that NOD/SCID mice immunized with VSVΔG/ZEBOV GP or VSVΔG/MARV GP were still alive 79 days post-immunization (Jones et al. 2007), but not with wild-type Ebolavirus (Bray 2001). These data thus emphasize the primordial role of IFNs when dealing with a viral infection. Furthermore, these results bring up the safety concern of using a live VSV-based vaccine in humans harbouring an IFN deficiency. Complete deficiency of STAT1 has been reported, but the infants died of severe viral infections, whereas IFN-γ receptor deficiencies resulted in less severe viral infections (van de Vosse, van Dissel, and Ottenhoff 2009). Acquired cytokine

deficiencies have also been reported, but are also a rare event and occur as autoantibodies following an infection (van de Vosse, van Dissel, and Ottenhoff 2009). Nevertheless, although the literature suggests that innate immunodeficiencies in humans are rare, the prevalence of immunodeficiencies in the general population is unknown and therefore this safety issue should not be ignored when performing clinical trials of live vaccines. Even though the STAT1 KO mouse model was not effective for vaccine testing with the live VSVΔG/LVGPC vaccine platform, its use for vaccine and antiviral drug testing might still be possible. Protection against Dengue virus following vaccination with a live vaccine was successful in the AG129 mouse model, which is deficient in both IFN-α/β and IFN-γ receptors (Johnson and Roehrig 1999). Furthermore, partial survival and delayed time to death for the fatalities were observed when STAT1 KO mice were infected with a lethal dose of Crimean-Congo hemorrhagic fever virus (Bente et al. 2010).

In summary, this study demonstrates, using mouse strains deficient in various immune cells and molecules, that defects in the IFN response, but not in other immune components including T and B cells, play a role in protection against LASV disease and lethality in the mouse. This opens the door to future studies to dissect in greater detail the immune correlates of protection in regard to Lassa fever. It will also be important to determine whether the STAT1 KO mouse model has a potential application in antiviral compound and vaccine development testing. Although this was not successful in our hands with the recombinant VSV vaccine platforms, which are live vaccines, there is still hope that success could be achieved with non-replicating agents. Additionally, the finding that IFNs play a role in resistance of mice to Lassa disease and lethality raises the question of whether IFNs could be used as a treatment for Lassa fever in humans.

6. General Discussion

6.1. Summary

LASV is the causative agent of Lassa fever and is endemic in West Africa. Over 200,000 infections are estimated to occur every year, resulting in 3000 to 5000 deaths annually (Fisher-Hoch and McCormick 2004). The virus is transmitted to humans through the rodent host *Mastomys natalensis*. Rodent control is currently unrealistic and the antiviral agent ribavirin is far too expensive for routine use in Africa. In addition, the factors responsible for the disease manifestations and ultimately death, which occurs in a small proportion of infected individuals, are still mainly unknown. Progress in the understanding of the pathogenesis and development of vaccines and treatments against Lassa fever has been hampered by the lack of a suitable small animal model. The establishment of a novel small animal model of LASV infection and disease could allow a better understanding of the immune response to LASV. As well, the development of a successful vaccine would diminish the public health burden of Lassa fever in West Africa.

Two vaccine candidates were presented in this thesis. One of them, the VSVΔG/LVGPC vaccine, is a live attenuated vaccine which has already been shown to protect non-human primates against a lethal challenge with LASV. The second one is a novel vaccine platform based on LASV virus-like particles. Both offer distinct advantages. The VSVΔG/LVGPC vaccine is thought to be able to induce a stronger and longer lasting immune response based on its replicating ability whereas the VLP vaccine is believed to be safer because it is replication-incompetent. We have shown in the present study that both vaccine platforms can induce an adaptive immune response in

immunocompetent mice, although the response was stronger with the VSVΔG/LVGPC vaccine than the Lassa VLP vaccine candidate. Mice immunized with the VSVΔG/LVGPC vaccine produced mainly IgG antibodies of the IgG1, IgG2a and IgG2b subclasses. IgG1 titers were higher in BALB/c mice than C57BL/6 and CBA mice whereas IgG2a titers were the highest in CBA mice. These data emphasize the influence of the host genetics on the immune response that will be triggered. The antibody titers increased with higher vaccine doses and the number of booster injections. IFN-γ and TNF-α were shown to be the predominant cytokines produced following immunization of CBA mice with VSVΔG/LVGPC. This cytokine response was mainly directed towards epitopes from the N-terminal portion of the LASV GPC, and this was especially apparent in mice which received two booster injections. This suggests that the immunodominant epitopes lie within the proximal third of LASV GPC. LASV GPC+Z VLPs were also tested as a novel vaccine candidate in CBA mice, alone or in combination with an adjuvant. Our results demonstrated that VLPs combined with the Sigma adjuvant was the most efficient combination to induce an IgG response. The cytokine response was weak in all groups, although an IFN-γ and TNF-α response to peptides from the middle portion of LASV GPC was observed in the VLPs+Freund's adjuvant group.

The lack of an adequate small animal model for LASV infection has resulted in the immune response to this virus being poorly understood. LASV is not lethal in immunocompetent mice, unless the challenge is performed through the intracranial route, leading to a central nervous system disease which does not reflect pathogenesis in humans. While LASV infection of strain 13 guinea pigs more closely resembles the disease process occurring in humans, there are significantly more reagents available for

studying the immune response in mice. In addition, many KO mouse strains have been developed, allowing us to carefully dissect the immune response which protects normal mice from LASV infection. In the present study, we investigated the role of the immune system during LASV infection using various KO mice with defects impairing innate ($STAT1^{-/-}$, IFN-$\gamma^{-/-}$) or adaptive (B $cell^{-/-}$, $CD4^{-/-}$, $CD8^{-/-}$, NOD/SCID, NOD/SCID/IL2R$\gamma^{-/-}$) immunity. Complete lethality was observed in $STAT1^{-/-}$ mice, which are unresponsive to the interferons, and partial lethality was achieved in IFN-$\gamma^{-/-}$ mice upon LASV challenge through the intraperitoneal route. In contrast, mice lacking major components of the adaptive immune system, as well as wild-type mice, did not show any apparent signs of disease following challenge with LASV. Our results demonstrate that the innate interferon response plays an important role in resistance to LASV infection in the mouse.

6.2. Future studies

6.2.1. Vaccine development

Of the various vaccine approaches that have been developed against Lassa fever, the most appealing ones are the ML29 reassortant, the chimeric Yellow fever, the VSVΔG/LVGPC and VLP vaccine candidates. Only the VSVΔG/LVGPC and ML29 vaccines have been shown to confer protection in a NHP model of Lassa fever (Carrion, Jr. et al. 2007a; Geisbert et al. 2005). The stability of the chimeric Yellow fever vaccine needs to be improved before it can be considered a realistic vaccine candidate, and the safety of the ML29 vaccine is still a concern as MOPV itself has not yet been proven to be non-pathogenic in humans. The VLP vaccine platform is interesting because non-replicating vaccines are generally believed to be safer than live vaccines. However, to determine if the LASV VLP vaccine candidate is an interesting alternative, demonstration

of its efficacy in protecting experimental animals against LASV challenge will be an important step in the near future. Moreover, determination of the number of doses required to confer protection remains to be determined. The immune correlates of protection are still unknown for all of these vaccines, except for the ML29 vaccine which appears to require T cells but not B cells to provide protective efficacy in the mouse (Lukashevich et al. 2005). IFN-γ and TNF-α were the predominant cytokines induced in mice vaccinated with the VSVΔG/LVGPC and VLP vaccine candidates. These cytokines have been shown to be secreted at higher levels in non-fatal cases of human Lassa fever in comparison to fatal cases (Mahanty et al. 2001). *In vitro*, LASV was shown to be susceptible to the antiviral activity of IFN-γ, but not TNF, in Huh7 and Vero cells (Asper et al. 2004). In contrast, IFN-γ was shown to have no effect on human APCs (Baize et al. 2006). The relevance of IFN-γ and TNF-α during LASV infection thus remains unclear, and the importance of these cytokines in mice vaccinated with the VSVΔG/LVGPC and VLP vaccine candidates is yet to be demonstrated. Additionally, the cytokine response was targeted towards epitopes from distinct regions of the LASV GPC for the VSVΔG/LVGPC and VLP vaccine candidates. Determination of whether this results in a difference in protective efficacy requires further studies. Antibody levels were higher in mice immunized with the VSVΔG/LVGPC vaccine in comparison to the VLP vaccine. Although current evidence suggests that T cells play an important role in the immune response during Lassa fever, this does not exclude the possibility that efficient induction of an antibody response by a vaccine could be responsible for conferring protection against LASV. Moreover, combining the VLPs to an adjuvant could potentially skew the immune response towards the desired one. Altogether, available evidence suggests that

the VSVΔG/LVGPC vaccine currently appears to be the most promising vaccine candidate against Lassa fever. However, improvements in the VLP vaccine design and production could transform this vaccine into an attractive alternative to the other potential vaccine candidates, which are live attenuated vaccines.

One area of vaccine studies against Lassa fever that is deficient is whether protection can be obtained against heterologous challenge. This has not been investigated yet for the VSVΔG/LVGPC vaccine, which contains the GPC gene from LASV strain Josiah. More and more strains of LASV are discovered, and the ability of a monovalent vaccine to protect against multiple LASV strains remains mostly unknown. IFA studies have shown broad cross-reactivity among the Old World arenaviruses LASV, MOPV, LCMV and MOBV (Peters et al. 1987). In contrast, a plaque-reduction neutralization test clearly distinguished the different viruses as the $\log_{10}$ neutralization index titers were higher for homologous than for heterologous viruses (Jahrling and Peters 1986; Peters et al. 1987). Additionally, passive antibody therapy was shown to be more effective when the plasma was matched to homologous virus infection in animal experiments (Jahrling and Peters 1984). On the other hand, two Old World arenavirus species, MOPV and LCMV, are known to confer protection against LASV (Kiley, Lange, and Johnson 1979; Peters et al. 1987). Vaccines containing LASV antigen(s) have also been shown to protect animals against lethal challenge with LCMV and PICV (Rodriguez-Carreno et al. 2005). However, there have been conflicting data concerning the ability of a LASV vaccine containing antigen from one Lassa strain to protect against heterologous LASV strains. The ML29 reassortant (containing GPC and NP from LASV strain Josiah) was shown to protect strain 13 guinea pigs against heterologous LASV challenge (strain

80321/NIG/74/H) (Carrion, Jr. et al. 2007b). In contrast, heterologous challenge with different LASV strains has been shown to be unsuccessful in the case of the YFV17D/LASV-GPC vaccine (Bredenbeek et al. 2006). Moreover, most studies were performed with LASV strain Josiah, a now laboratory strain that has been through multiple cell passages and thus might not reflect the LASV genetic diversity currently circulating in countries where LASV is present. Thess issues thus need clarification as a LASV vaccine will need to provide good coverage against the virus strains responsible for endemicity in West Africa. Nevertheless, this problem could be overcome by blending multiple constructs together to obtain a multivalent vaccine. This approach has been successful experimentally with the VSV-based vaccines expressing either MARV or EBOV GP (Geisbert et al. 2009) and in humans with the HPV vaccines (Einstein et al. 2011), and could thus be used with either the VSVΔG/LVGPC vaccine or VLP vaccine.

In addition, it would be interesting in the future to use the VSVΔG/LVGPC vaccine platform to determine whether the addition of other viral proteins, i.e. Z and NP, could result in better protection, especially against heterologous challenge. This could possibly be investigated since the VSV-based vector has the ability to incorporate additional genes. The VLP vaccine could also be used to this effect. The only study which investigated this issue showed that GP1 or GP2 alone did not confer protection, whereas vaccination with NP only partially protected (3/11 NHPs survived) and appeared to cause more severe disease (Fisher-Hoch et al. 2000). Better protection was obtained when NP was added in combination with GPC and 7/8 monkeys survived (Fisher-Hoch et al. 2000). The comparison of vaccine candidates that include the NP antigen or not will help clarify whether the incorporation of NP in a vaccine is detrimental.

One interesting finding in my studies is the fact that homologous prime-boosting with the VSVΔG/LVGPC vaccine in mice resulted in a stronger cytokine immune response. Homologous prime-boosting is known to be a efficacious strategy to boost the humoral immune response but heterologous prime-boosting is generally believed to be required to increase the number of antigen-specific $CD8^+$ T cells as well as selection of high-affinity $CD8^+$ T cells (Nolz and Harty 2011; Woodland 2004). The correlation between the increased cytokine response and protective efficacy in animal models of Lassa fever will thus be important to determine in the future. Comparison of homologous and heterologous prime-boosting strategies with the VSVΔG/LVGPC and LASV VLP vaccines could also be explored.

The ideal vaccine against Lassa fever would be safe, effective with a single dose and provide lomg-term immunity, would prevent disease and death, but also prevent major sequalae such as sensorineural deafness. The neurologic sequelae associated with Lassa fever have been hypothesized to be a result of an immunopathologic mechanism. It will therefore be important to determine whether vaccine candidates cause these sequelae, which might be difficult to assay in animal models but will definitely be required in eventual human clinical trials. Neurologic deficits have not been directly evaluated so far in animal vaccine studies.

6.2.2. Establishment of a novel small animal model for LASV infection and disease

We have established a novel mouse model for LASV based on interferon system-defective mice. However, further studies are required to characterize this model in greater detail. For example, a time course experiment to determine viremia levels, organ tropism

and immunologic analysis has yet to be performed. The role of the Type I versus Type II versus Type III interferons, as well as the correlation between the interferon response and pathogenesis in humans also remain to be determined. Numerous mouse strains with defects in molecules essential to the IFN pathway could be used in order to pin-point the type(s) of IFNs playing a role in LASV infection in the mouse. Our data also suggest that the IFNs might potentially be used as an antiviral drug against LASV infection and this should be investigated in the future. Altogether, our studies in immunodeficient mice have brought new insights into the interaction of LASV with the host's immune system, indicating that innate immunity is more important than adaptive immunity, at least in the mouse. Whether this reflects the pathogen-host interaction between LASV and its natural rodent host or accidental human host remains unknown as of now but will be interesting to investigate in future studies. This novel mouse model of LASV infection will certainly broaden our knowledge on the immune response to this virus.

Further studies are required to determine whether STAT1 KO mice could be used as a model for vaccine and antiviral agent testing. The STAT1 KO mouse model would not be suitable for testing of antiviral agents targeting the immune system but it might be possible to test antiviral drugs interfering with virus replication for example. Although vaccine testing was unsuccessful in our hands with the recombinant VSV-based vaccines due to the high susceptibility of STAT1 KO mice to these viruses, this mouse model could still potentially be used since mice with defects in the interferon pathway have been successfully used in the past for vaccine and drug testing for other viral agents (Bente et al. 2010; Johnson and Roehrig 1999). Nevertheless, ideally drug and vaccine testing should be performed in immunocompetent animals. The fact that lethality can be achieved in wild-type mice through intracranial inoculation and in immunodeficient mice

through intraperitoneal inoculation opens the door to the development of novel mouse models, such as mouse-adapted strains of LASV. This approach has been used for several other viruses, including Dengue virus and EBOV (Bray et al. 1998; Yauch and Shresta 2008) and might thus be an achievable aim for LASV.

6.3. Conclusion

In conclusion, recent findings in the field of the immunology of Lassa fever suggest that an efficient innate immune response is necessary to prevent severe disease and lethality following infection with LASV. Current evidence suggests that cellular immunity is more important than humoral immunity in natural Lassa fever infection, although this evidence is mostly indirect. Further work is thus necessary to confirm these data. Our findings that lethality is observed in mice with defects in the interferon pathway but not in mice with no adaptive immune system support the hypothesis that innate immunity plays a major role in protecting the host against LASV disease. Further studies are necessary to determine whether the interferon response is essential for the human accidental host, rodent natural host, or both. Additionnally, we have presented two vaccine platforms in this thesis. VSVΔG/LVGPC is a live attenuated vaccine whereas Lassa VLPs present a potentially safer alternative since they are non-replicating. However, protective efficacy of the VLP vaccine candidate remains to be demonstrated. Moreover, the VLP vaccine might necessitate booster injections to achieve efficacy, which might not be suitable for mass vaccination in West Africa. Future studies are thus required to determine whether protection can be achieved with a single injection. Further work is also necessary to establish whether the humoral or cellular arms of the adaptive

immune system are required for protection for the VSVΔG/LVGPC and VLP vaccine candidates.

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