Nonspreading Rift Valley fever virus:

A potent and flexible vaccine platform

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Nonspreading Rift Valley fever virus: A potent and flexible vaccine platform

Niet-spreidend Rift Valley fever virus: een potent en flexibel vaccinplatform

(met een samenvatting in het Nederlands)

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Every moment is precious

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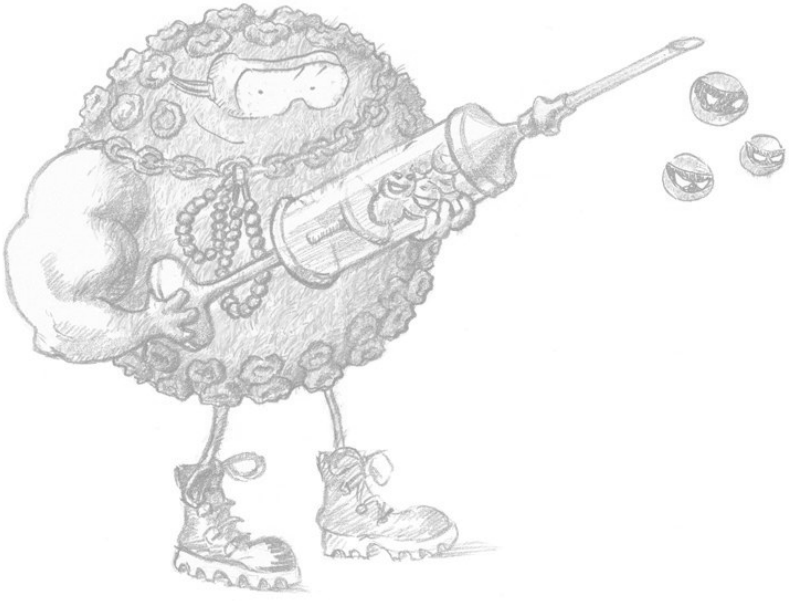
General introduction



Rift Valley fever

Rift Valley fever virus (RVFV) is a mosquito-borne pathogen that affects ruminants and humans. In herds of sheep, which are the most susceptible to disease, the virus causes characteristic abortion storms and mortality of up to 20% among adult animals and 100% among newborns. Cattle, goats and wildlife ruminants are somewhat less susceptible, but also in these species considerable morbidity can be observed [1-5]. In humans, the disease generally manifests as a febrile illness that lasts several days and may be accompanied by other symptoms, such as severe headache, malaise, muscular pains, joint pains and gastro-intestinal disorders [6-8]. These symptoms, often referred to as flu-like or dengue-like in the past, mostly resolve within few days [1, 9]. In some cases however, complications develop that may include retinal damage, hepatitis, jaundice, neurological disease and hemorrhagic fever [10-14]. Historically the disease is reported to be lethal for less than 1% of infected individuals. Fatal cases most often occur among patients that suffer from encephalitis or hemorrhagic fever.

Emergence and spread

The virus was identified for the first time by Daubney *et al*. in 1931 after investigation of an outbreak on a farm near Lake Naivasha in the Rift Valley of Kenya in the previous year [1]. The disease emerged after exceptionally heavy rainfall and caused massive abortions among sheep and high mortalities among newborn lambs. Daubney and his co-workers identified an undescribed virus that was associated with the observed outbreak, and stated that similar outbreaks had been noted at least 20 years earlier. Until the 1950's the disease was restricted to East Africa. In 1951, a major outbreak was registered for the first time in South Africa that was associated with a hundred thousand deaths and half a million abortions amongst sheep [15]. The causative agent was ultimately recognized to be RVFV, but only after veterinary personnel developed severe disease after performing necropsy on a dead animal [16]. In the following decades, RVFV infections were recorded in several countries in the sub-Saharan region either during outbreaks or retrospectively by detecting RVFV-specific antibodies in humans or animals (Fig. 1).

In 1977, a massive RVF outbreak was recorded for the first time north of the Sahara desert, in Egypt. The outbreak lasted for two years and still remains the largest epidemic on record. An estimated 200,000 people were effected and 598 deaths were recorded among hospitalized patients [17, 18]. Similar to the

outbreak in South Africa in 1951, the disease was first recognized in humans and subsequently in livestock. This delay in recognition is believed to have contributed to the extensive losses for the livestock sector, estimated at more than $115 million at that time [19]. The acknowledgement that humans have acted as sentinels during past RVF outbreaks calls for an increase in awareness and improved surveillance programs.

Along with recurrent outbreaks in endemic areas, RVFV continued conquering new territories. In 1987, a human outbreak was reported in two countries in West Africa, Mauritania and Senegal [20, 21], although circulation of the virus was detected in the area before that time [22, 23]. Three years later, an outbreak was reported in Madagascar [24, 25] and in the year 2000 the virus caused outbreaks in Saudi Arabia and Yemen [26, 27]. Currently, most of the countries on the African continent have registered circulation of the virus or have experienced outbreaks, affecting humans and livestock (Fig. 1). The virus has shown a remarkable capability of crossing large territories and geographical barriers including the Sahara desert and the Red sea. Although RVFV is still largely confined to the African continent and the Arabian Peninsula, its spread in the past century calls for awareness for future incursions into yet unaffected territories.

Transmission

Among ruminants, RVFV is transmitted by mosquito vectors. In humans, most infections are attributed to direct contact with blood or tissues of diseased or dead animals or aborted fetuses. Infections are believed to occur through small skin abrasions, via the conjunctival mucosa or via inhalation [28-30]. Consumption of raw milk and meat has been suggested as a possible route of human infection, although experimental evidence is lacking [13, 31, 32]. However, the massive number of human cases in the Egyptian outbreak of 1977 (more than 200,000) implies involvement of mosquito vectors [33]. Mosquitoes are also believed to have played a role in the epidemic in Saudi Arabia in the year 2000, as around 20% of the RVF cases could not be attributed to contact with animals or animal products [14].

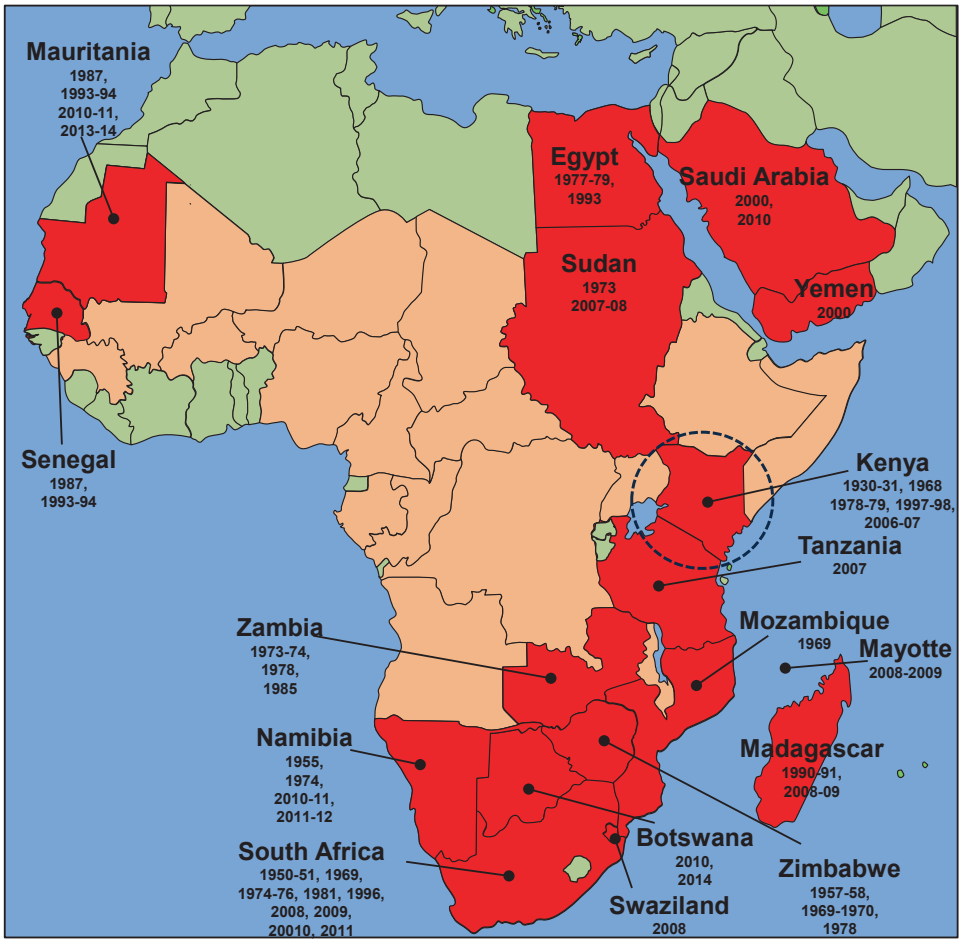


Figure 1. Geographic distribution of RVFV. Countries that have experienced outbreaks are depicted in red and the years when outbreaks occurred are shown. Countries that have reported presence of the virus based on serological evidence or sporadic virus isolations are depicted in pink. Adapted from Bird *et al.*, 2009 [34].

An association between RVF outbreaks and heavy rainfalls facilitating the amplification of mosquito vectors was already proposed in the first record about RVFV in 1931 [1] and was supported by reports of subsequent outbreaks. Evidence supporting the mosquito vector transmission hypothesis was first provided by Smithburn *et al.* in 1948, who isolated the virus from mosquitoes caught in the wild [35], followed by successful experimental infections of lambs and mice [36]. Later, Linthicum *et al.* (1985) performed an elegant study in which he investigated mosquitoes, hatched from field-collected larvae for the presence of the virus. He successfully isolated virus from the larvae of *Aedes*

mcintoschi mosquitoes, which have drought-resistant eggs capable of preserving the virus during dry seasons [37]. This first evidence of transovarial transmission provided an explanation for the survival of the virus during interepidemic periods when no evidence of circulation among vertebrate or mosquito hosts could be found for long periods of time.

Two cycles of RVFV circulation in nature can be distinguished: enzootic/endemic and epizootic/epidemic. In the former cycle, the virus circulates between mosquitoes and ruminants and perhaps small rodents [38-41]. Its dissemination is restricted to limited numbers of mosquito vectors and susceptible animals [34]. In the latter cycle, floods resulting from excessive rainfall play a major role. Flood water is retained in certain lay structures with difficult to drain soil, collectively known as "dambos". Thereby, favorable conditions are created for explosive amplification of mosquitoes, as well as massive hatching of drought-resistant eggs of floodwater *Aedes* mosquitoes, some of which may be infected with RVFV [42]. After these eggs have hatched and the corresponding mosquitoes have transmitted the virus to susceptible animals, several *Aedine*, *Culicine* and, to a lesser extent, *Anopheline* mosquito species may contribute to further spread of the virus [43] (Fig. 2).

RVFV has been isolated from a variety of mosquito species, belonging to six genera – *Aedes, Culex, Anopheles, Eretmapodites, Mansonia, and Coquillettidia,* although only members of the first two genera have been shown to play a significant role in virus transmission [44]. The capability of RVFV to be transmitted by multiple mosquito species, together with the global distribution of mosquitoes that have been associated with RVFV transmission in endemic areas, explains the fear for future incursions outside the current habitat of the virus. This risk may be augmented by globalization and climate change. In view of the devastating disease caused in domestic ruminants as well as the high zoonotic potential of RVFV, united efforts of veterinary and human health services are needed to provide diagnostics, treatment and prophylactic tools to control the disease.

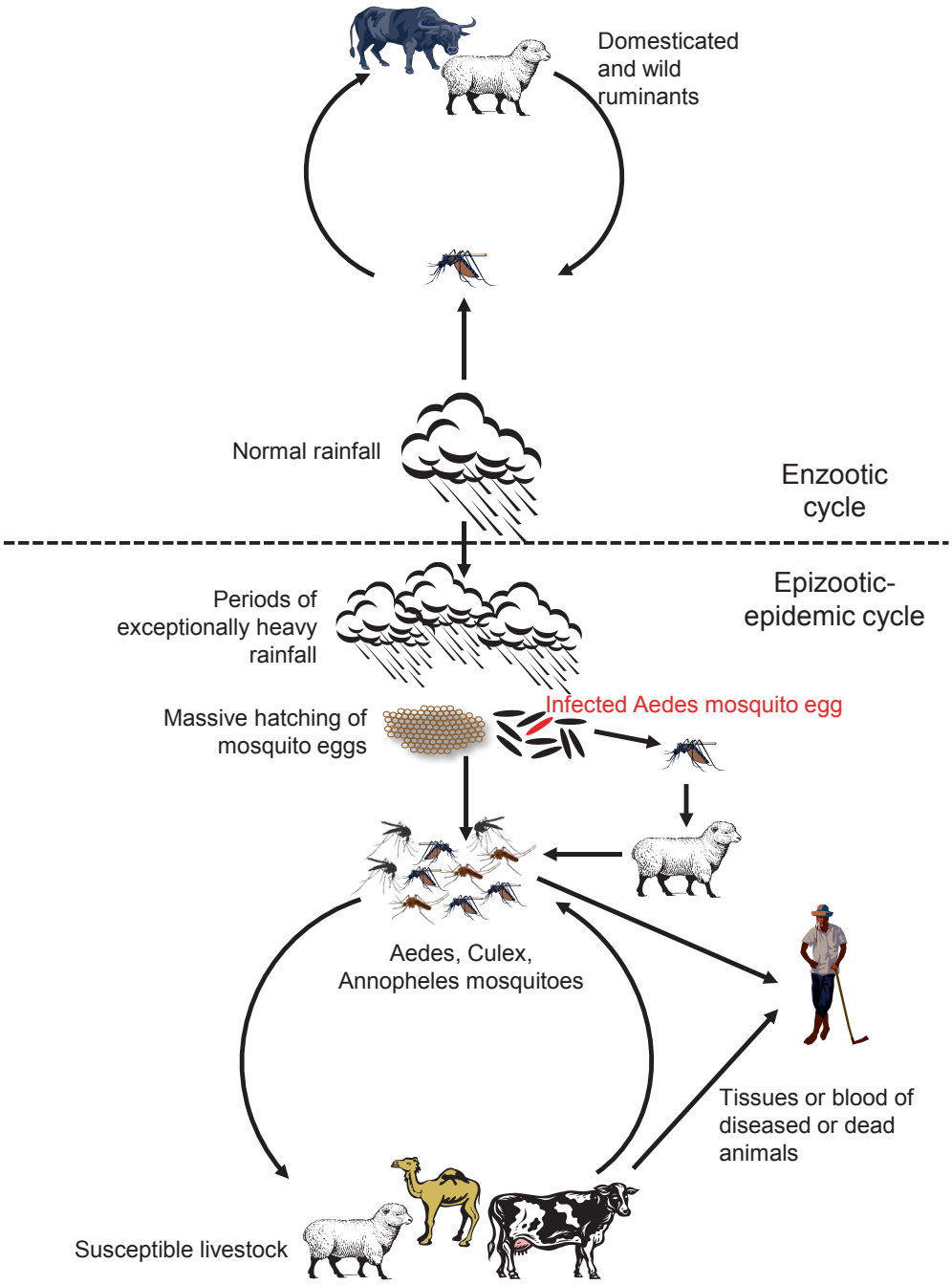


Figure 2. Enzootic and epizootic circulation of RVFV.

Taxonomy

RVFV belongs to the genus *Phlebovirus* of the largest virus family, *Bunyaviridae*. The family comprises five genera - *Orthobunyavirus*, *Nairovirus*, *Phlebovirus, Tospovirus* and *Hantavirus*. All viruses that belong to the first four genera are arboviruses, which means that they replicate in, and are transmitted by arthropod vectors. *Orthobunya-, Nairo- and Phleboviruses* infect animals and *Tospoviruses* infect plants [45]. *Hantaviruses* are the only exception in the family, having rodents as a natural reservoir, and are transmitted to humans via aerosolized rodents' excreta [46]. The most important properties of selected bunyaviruses are listed in Table 1.

Table 1. Characteristic features of bunyaviruses relevant to animal, human or plant health [47-51].

Genus/virus	Host	Disease	Vector	Distribution
Orthobunyavirus				
Akabane	Cattle	Abortion and congenital defects	Midge	Africa, Asia, Australia
Cache Valley	Sheep, cattle	Congenital defects	Mosquito	North America
La Crosse	Human	Encephalitis	Mosquito	North America
Ngari	Human	Hemorrhagic fever	Mosquito	Africa
Oropouche	Human	Febrile illness	Midge	South America
Tahyna	Human	Febrile illness	Mosquito	Europe
Hantavirus				
Hantaan	Human	Severe hemorrhagic fever with renal syndrome (HFRS)	Field mouse	Eastern Europe, Asia
Seoul	Human	Moderate HFRS	Rat	Worldwide
Puumala	Human	Mild HFRS	Bank vole	Western Europe
Sin Nombre	Human	Hantavirus pulmonary syndrome	Deer mouse	North and South America
Nairovirus				
Crimean–Congo hemorrhagic fever	Human	Hemorrhagic fever	Tick	Eastern Europe, Africa, Asia
Nairobi sheep disease	Sheep, goats	Fever, hemorrhagic gastroenteritis, abortion	Tick	Africa, Asia
Phlebovirus				
Rift Valley fever	Human	Encephalitis, hemorrhagic fever, retinitis	Mosquito	Africa, Arabian Peninsula
	Ruminants	hepatic necrosis, abortion		
Sandfly fever Naples	Human	Febrile illness	Sandfly	Europe
Sandfly fever Sicilian	Human	Febrile illness	Sandfly	Europe
Sandfly fever Toscana	Human	Febrile illness, sporadic meningitis or meningo-encephalitis	Sandfly	Europe
Punta Toro	Human	Febrile illness	Sandfly	Central America
Severe fever with thrombocytopenia syndrome	Human	Fever, thrombocytopenia, gastrointestinal symptoms and leukocytopenia	Tick	Asia
Tospovirus				
Tomato spotted wilt virus	>650 plant species	Various symptoms	Thrips	Worldwide

RVFV virion architecture

RVFV virions are spherical, with a diameter of 100 nm. Contrary to initial views that bunyaviruses are pleomorphic, more recent studies using cryoelectron tomography (cryo-ET) of RVFV have revealed highly organized particles (Fig. 3A) [52-54]. The virus displays two glycoproteins, named Gn and Gc, which protrude from a lipid bilayer. The glycoproteins were shown to cluster into 12 pentameric and 110 hexameric capsomers in an unusual T=12 lattice (Fig. 3B). Such a lattice has been shown so far only for Uukuniemi virus (UUKV), also member of the Phlebovirus family [55].

Bunyaviruses lack a matrix protein to link the envelope proteins to the ribonucleoprotein complex (RNP). Instead, direct interaction between glycoprotein C-termini and the RNP has been demonstrated for different bunyaviruses, including RVFV [56-59]. The RNP forms the ribonucleoprotein core, composed of viral RNA encapsidated by nucleoprotein N (Fig. 3C). A few copies of the viral polymerase are associated with the RNP.

Genome organization of RVFV

Like all bunyaviruses, the genome of RVFV is of negative-sense polarity and is divided into three segments, which are named after their size: large (L), medium (M) and small (S) (Fig. 4 A, B).

The L segment encodes the viral RNA-dependent RNA polymerase (RdRp, often named L) [60]. The M segment encodes the surface glycoproteins Gn and Gc, as well as two accessory proteins with molecular weights of 14- and 78 kDa [61]. Gn and Gc are responsible for virus attachment and entry into host cells. The 14-kDa accessory protein is known as NSm. This non-structural protein was reported to have an anti-apoptotic function [62]. The 78-kDa protein was suggested to be a minor structural protein [63-65] and was recently shown to facilitate replication in mosquito vectors [66, 67]. Both accessory proteins are non-essential for virus growth *in vitro* [68, 69]. Viruses lacking these proteins were shown to be attenuated but not avirulent *in vivo* [70].

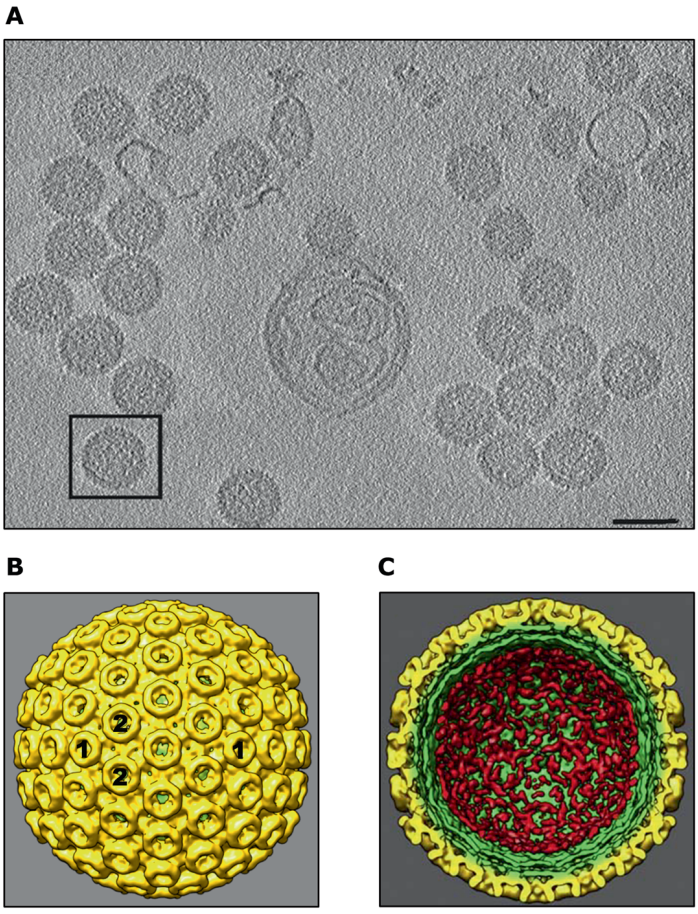


Figure 3. Morphology of RVFV particles (A) Cryo-ET analysis of virus preparations, showing the homogeneity of the virus population. Example of a virus particle is marked with the square [52]. (B) Single-particle cryo-ET map of RVFV, showing glycoprotein protrusions, organized in a T=12 lattice. A pentamer is marked with 1 and hexamers with 2. (C) Virus section revealing the nucleocapsid (red), the lipid envelope (green) and the glycoprotein layer (yellow) [53].

A remarkable feature of the coding sequence of the M segment is the presence of five in-frame start codons (AUG) [71-73] (Fig. 4C), four of which (AUG-1, 2, 4 and 5) are used for translation initiation. The alternative utilization of these AUG codons results in synthesis of different precursor proteins that are post-translationally cleaved. Translation initiation at AUG-1 results in the 78-kDa protein and Gc, at AUG-2 in NSm, Gn and Gc, whereas translation initiation at AUG-4 or 5 results exclusively in Gn and Gc. Interestingly, only the fourth AUG has an optimal translation initiation (Kozak) sequence [74]. This explains why Gn and Gc are the most abundant proteins in infected cells.

The small segment (S) encodes two proteins in an ambisense strategy – the nucleocapsid protein (N) in genomic-sense orientation and a non-structural protein (NSs) in antigenomic-sense orientation. The two genes are separated by an intergenic region (IGR) [60], which contains signals for transcription termination [75-77] (Fig. 4B). The nucleocapsid protein associates with the viral RNA resulting in the formation of ribonucleoprotein (RNP). The N protein interacts with the viral RNA via a deep hydrophobic pocket, thereby completely shielding it from nucleases and other proteins [78]. In addition, packaging of the viral genomic RNA into RNPs is essential for recognition by the viral polymerase in order to serve as a template for transcription and replication [79]. The NSs protein antagonizes host innate immune responses via several different mechanisms, and is therefore considered the major virulence factor [80]. First, NSs is the only RVFV protein that localizes to the nucleus, where it forms filamentous structures [81-83]. The protein sequesters two subunits (p44 and XBP) of the general transcription factor complex TFIIH, thereby suppressing cellular transcription [84], including transcription of the IFN-β gene [85]. Second, NSs recruits the repressor protein SAP30, thereby specifically suppressing the promoter of IFN-β [86]. Third, NSs induces specific degradation of the double-stranded RNA-dependent protein kinase (PKR), thereby preventing phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2) [87, 88]. The latter, when phosphorylated, leads to translational arrest of cellular and viral mRNA.

The coding regions of all three segments are flanked by untranslated regions (UTRs) that contain signals for transcription, replication, packaging and transcription termination [75, 89-92]. The terminal 8 nucleotides of the 3’ and the 5’ UTRs are complementary to each other and are conserved among the viruses of the Phlebovirus genus. These termini form “panhandle” structures that are essential for both replication and transcription [60] (Fig. 4A). Such structures are a common feature of segmented negative-strand RNA viruses [93]

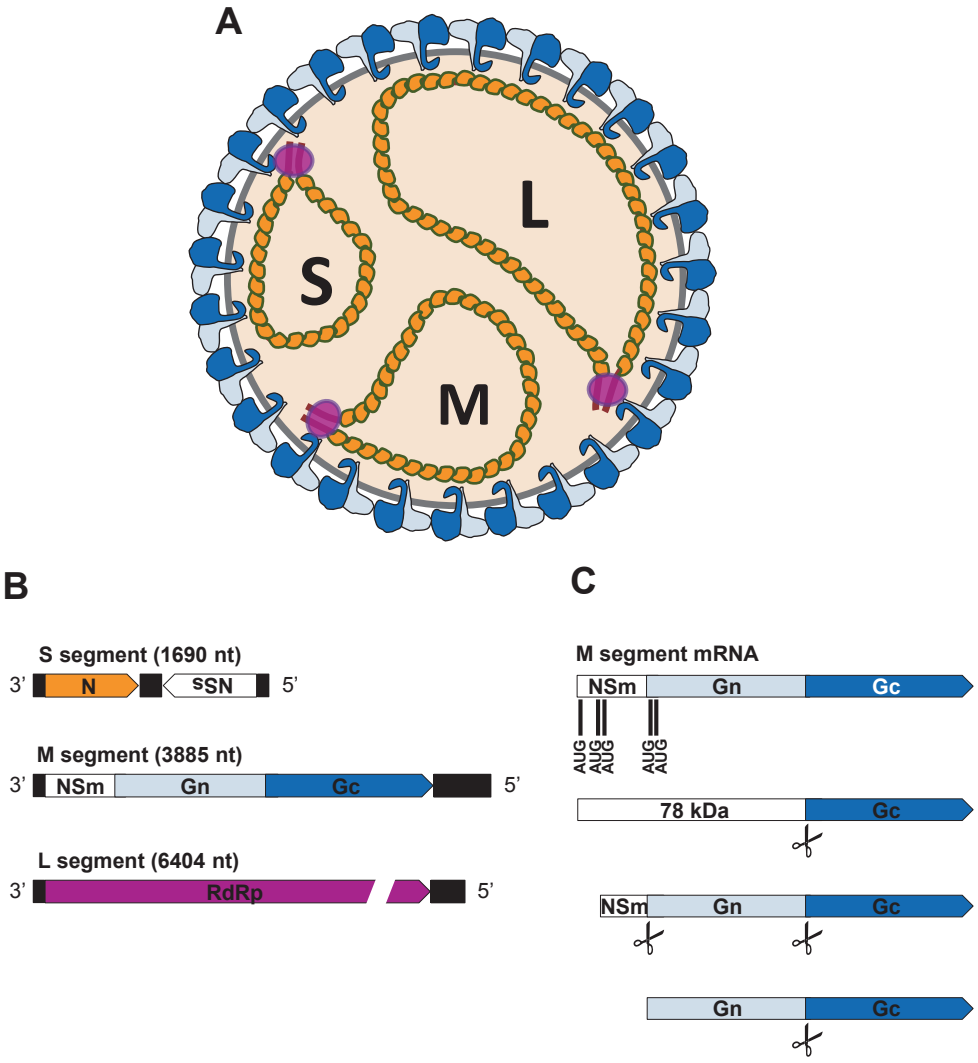


Figure 4. Organization of the RVFV particle and genome. (A) Schematic representation of a RVFV particle. The dark and light blue structures represent Gn and Gc proteins, respectively. The yellow circles represent nucleoprotein packaging the viral RNA, which comprises three segments - L, M and S. All segments form "panhandle" structures, associated with polymerase molecules (purple ovals). (B) Schematic representation of the three genome segments. The arrows indicated the open reading frame of each segment. Black boxes represent untranslated regions. (C) M-segment mRNA encoding the polyprotein precursor with five start codons. The resulting protein products are shown and the cleavage sites are marked with scissors.

Infection cycle

The infection cycle of RVFV (Fig. 5) begins with attachment of the virus particles to the cell surface. Recently, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) was identified as a *bona fide* receptor for RVFV [94]. However, DC-SIGN is expressed only at the surface of dendritic cells and macrophages, while RVFV is known to infect hepatocytes and cells of the central nervous system as well. Additional receptors of RVFV remain to be identified. Interestingly, De Boer *et al.* [95] demonstrated that heparan sulphate, a molecule that is abundantly present on the surface of most mammalian cell types, is required for efficient infection, suggesting an important role of this attachment factor in the pathology of RVFV.

The next phase of the infection cycle is entry, accomplished via clathrin-mediated endocytosis [96]. Virus particles are transported into the cells via endosomes in which gradual acidification takes place. At pH 5.8, corresponding to late endosomal compartments, membrane fusion is triggered [96]. The fusion process is initiated by acid-driven rearrangement of the Gn and Gc proteins. The heterodimers formed by the two glycoproteins dissociate to allow trimerization of the Gc fusion protein. Gc trimers are proposed to trigger the fusion of the endosomal and viral membranes followed by release of the viral genome into the cytosol [96].

Once the viral genome is released into the cytosol, genome replication takes place. The replication starts with primary transcription of the genomic negative-sense RNA into mRNA by RNP-associated viral polymerase. To prime mRNA synthesis, the viral polymerase cleaves cap-containing sequences from host mRNAs with length 12-18 nucleotides and attaches them to the 5’ end of the viral mRNAs, a process known as cap-snatching. The viral mRNAs are truncated at their 3’ terminus and are not polyadenylated [75, 97]. After translation of the viral genes, viral RNA transcription switches to replication mode. Replication involves primer-independent synthesis of full-length, exact copies of the viral segments in antigenomic-sense orientation. These copies in turn serve as templates for synthesis of genomic RNA in genomic-sense orientation. The mechanism of switching between transcription and replication modes is unclear but it has been suggested that the concentration of the N protein plays a role in this process.

RVFV replicates exclusively in the cytoplasm of infected cells and assembles at the Golgi complex. This process is initiated by accumulation of the envelope glycoproteins Gn and Gc in the Golgi, followed by recruitment of newly

synthesized RNPs. This recruitment is presumably mediated by the cytoplasmic tail of Gn [98], which is also involved in Golgi targeting of the Gn/Gc heterodimers [99]. Interestingly, a recent report demonstrated that the short cytoplasmic tail of Gc is also important for virus assembly and release [98]. Newly assembled virus particles bud into the lumen of the Golgi, followed by transport to the cell surface via exocytic vesicles. Virus is released after fusion of the vesicles with the plasma membrane [100]. The only known exception of the described assembly route has been observed in hepatocytes where RVFV was found to bud directly from the plasma membrane as well [101].

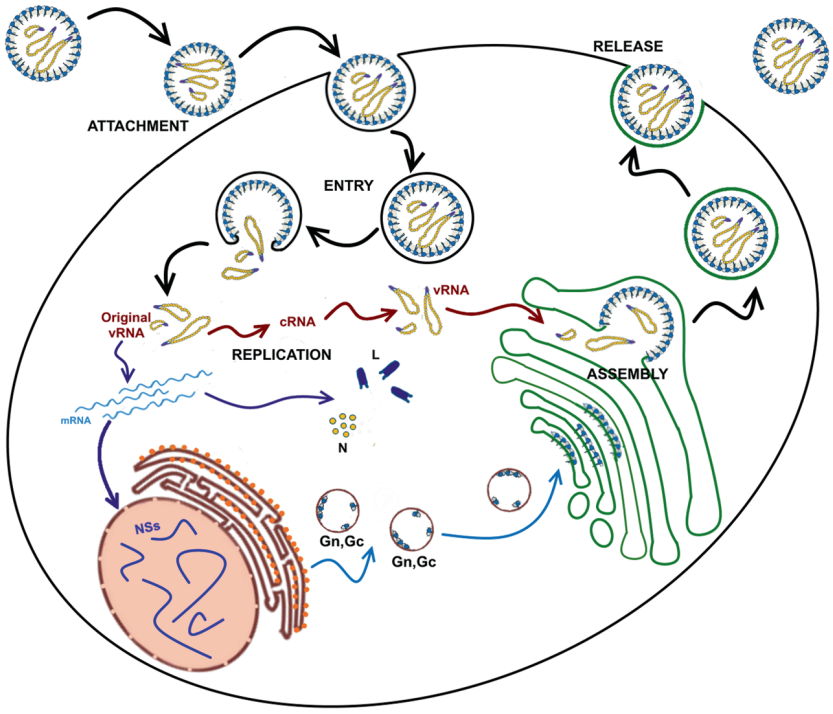


Figure 5. Infection cycle of RVFV. Infection starts with **attachment** of the virus particle to the cell surface, after which **entry** is accomplished via clathrin-mediated endocytosis. **Replication** takes place exclusively in the cytosol. The viral RNA (vRNA) is transcribed into mRNA by viral polymerase, followed by translation of viral proteins by the cellular machinery. The viral genome is replicated by viral polymerase through synthesis of exact copies of each segment, called complementary RNA (cRNA), which on turn serve as template for production of vRNA. The new virus particles are **assembled** at the Golgi complex. Virus particles bud into the lumen of the Golgi and are transported to the cell surface via exocytic vesicles. After fusion of the vesicles with the plasma membrane, virus particles are **released**.

Each virus particle must contain at least one segment of each type (S, M and L) to be able to produce progeny particles in newly infected cells. A model was proposed where specific interactions between the three genome segments regulate co-packaging, in which the M segment plays a pivotal role [102]. However, results from independent research groups have demonstrated that infectious replicon particles of RVFV can be generated that contain only two segments (S and L) and lack the M genome segment [103-105]. Furthermore, two-segmented and three-segmented RVF viruses were created that also lack an M-type genome segment [105, 106]. Finally, earlier studies have shown wild-type virus particles with an RNA content that is not consistent with equimolar amounts of each genome segment [89, 107]. These data favor a model where the three segments are randomly packaged into virions and that the numbers and types of genomes packaged depends on the concentration and, therefore probably, the size of each segment.

Reverse-genetics of negative-strand RNA viruses

Detailed molecular studies of RNA viruses became possible only after the establishment of reverse-genetics technology. In this powerful technology, a cDNA copy of the viral RNA genome is generated and subsequently delivered into permissive cells, where it serves as a template for the production of new viral proteins, viral RNA and ultimately new virus particles. In that manner, viruses are generated entirely from cDNA, a process referred to as "virus rescue". Apart from rescue of viruses, reverse-genetics can be used to introduce various changes in the viral genome and to directly study the effect of these changes on the viral phenotype and biology. Importantly, altering the viral genome with reverse-genetics can generate safe and effective vaccine viruses.

The first significant success with reverse-genetics was achieved by Taniguchi (1978) who managed to rescue the positive-strand RNA bacteriophage Qbeta [108], followed by Racaniello and Baltimore (1981) who first rescued a mammalian positive-strand RNA virus, the poliovirus [109]. Later it was found that *in vitro* transcription of RNA and subsequent transfection into permissive cells is a more efficient method to rescue positive-strand RNA viruses and this technique was successfully applied to many of those viruses [110].

Development of a comparable technology for negative-strand RNA viruses has been considerably more challenging because, unlike the genome of a positive-strand RNA virus, the genome of a negative-strand RNA virus is not infectious. In other words, genomes of positive-strand RNA viruses can be used for

translation of protein directly, whereas protein translation of negative-strand RNA viruses is preceded by primary transcription of viral mRNAs, which requires polymerase and nucleocapsid protein. For that reason, these virus proteins are always packaged into the virions to initiate viral replication.

In early experiments with influenza virus, which is a segmented negative-strand RNA virus, modified RNP complexes containing one novel segment were delivered together with "helper" wild-type influenza virus in cell cultures [111, 112]. This approach was successfully applied to generate viruses containing the novel segment, but only a small percentage of the virus progeny acquired this segment and the use of helper virus required further selection for the virus of interest. A different approach was used for a non-segmented negative-strand RNA virus, the rabies virus, which was rescued in a helper virus-independent manner [113]. In this approach, viral RNA transcripts were delivered into cells in the form of full-length antigenomic cDNA copies. Intracellular viral genome replication was carried out by nucleocapsid and polymerase proteins expressed in the same cells after transfection, resulting in the assembly of functional RNP complexes. A similar method was used by Bridgen and Elliott who were the first to rescue a segmented negative-strand RNA virus, the Bunyamwera orthobunyavirus, entirely from cloned cDNA [114]. Subsequently, several other negative-strand RNA viruses were rescued, including paramyxoviruses, orthomyxoviruses, filoviruses, arenaviruses and bornaviruses [115].

One of the most important requirements for successful rescue of negative-strand RNA viruses is the generation of RNA transcripts that contain termini closely resembling those of the authentic viral genome. Only then, the generated RNA transcripts can function as templates for genome replication by the viral polymerase. To accomplish this requirement, both eukaryotic polymerase I (Pol-I) and phage T7 polymerase have been employed. Pol-I is an endogenous nuclear protein that produces transcripts lacking 5' caps and 3' polyadenylated tails. Viral genome sequences inserted between the promoter and the terminator sequences of Pol-I contain the exact viral genomic termini. T7 polymerase allows cytoplasmic transcription of viral RNAs and is therefore often used for reverse-genetics of viruses that replicate in the cytoplasm, such as the bunyaviruses. The T7 polymerase can be expressed from plasmids, recombinant viruses such as vaccinia or fowlpox viruses or constitutively in cell lines [115]. To create RNAs with 3' termini that exactly correspond to those of the viral genome, the self-cleaving hepatitis delta virus ribozyme sequence can be introduced [116].

Reverse-genetics of bunyaviruses

In bunyavirus research, the T7-based strategy was utilized for the rescue of Bunyamwera virus [114, 117], LaCrosse virus [118] and RVFV [119], while the Pol I system was used for the rescue of Akabane virus [120] and RVFV [121, 122]. The last two studies compared the two systems and concluded that both were equally efficient for the rescue of RVFV. Of note, when the Pol I system is used, the nucleocapsid protein and the viral polymerase protein must be provided in trans to start virus replication. In contrast, successful rescue using the T7 system depends on only the three plasmids encoding the viral cDNAs in antigenomic-sense orientation. This demonstrates that the antigenomic-sense transcripts act not only as replication intermediates, but also as mRNAs [117].

Apart from using reverse-genetics systems to rescue complete bunyaviruses, several groups have pursued to develop minigenome systems [79, 89, 123-129]. In this approach, a gene encoding a reporter protein is placed between the UTRs of a genome segment. As reporters, fluorescent proteins, luciferase or acetyltransferases are most often used. Expression of the reporter protein is only possible if nucleoprotein N and polymerase L are provided in trans, allowing the assembly of RNPs. Both T7 and Pol-I systems have been employed for transcription and replication of minigenomes, with N and L proteins expressed from polymerase II promoters.

Further advancement of minigenome systems was achieved by Habjan *et al.* [130] who demonstrated successful packaging of minigenomes in particles. In that study, a luciferase-encoding minigenome flanked by the UTRs of the M segment of RVFV, was delivered to "donor cells" together with plasmids expressing the N and L proteins. Minigenome expression was confirmed by detection of luciferase activity. By providing the viral glycoproteins Gn and Gc in trans from another expression plasmid, the minigenome was successfully packaged into virus-like particles (VLPs). These particles were shown to resemble the authentic virus and were able to infect new recipient cells, where the minigenome was expressed de novo when the N and L genes were expressed from transfected plasmids. The particles were successfully used by the authors to study the effect of the antiviral MxA protein on virus replication.

Since various members of the family Bunyaviridae, such as RVFV, Crimean-Congo hemorrhagic fever virus (CCHFV) and severe fever with thrombocytopenia syndrome virus (SFTSV) are highly pathogenic and have to be handled in biosafety level-3 or level-4 containment facilities, minigenome systems have

proven invaluable tools for fundamental studies and for the development of vaccines and antiviral therapies.

Vaccines

Classical vaccines

The significant morbidity and mortality caused by RVFV in ruminants, as well as the major socio-economic impact of RVF outbreaks call for effective vaccines to control the disease. Currently three vaccines are commercialized for veterinary use and are produced by Onderstepoort Biological Products (OBP, Onderstepoort, South Africa). The first of these vaccines is based on a live-attenuated virus that was developed by K. C. Smithburn in the 1940's by intracerebral passage of the virus in mice. The resulting "Smithburn" strain is neurotropic and attenuated. The Smithburn virus is inexpensive to produce and can elicit solid immunity in animals after a single application. Due to these properties, the vaccine is still used to protect ruminants in endemic areas, although its residual virulence is well recognized. Consequently, the Smithburn vaccine is not recommended for pregnant and very young animals [131-133]. Another vaccine, based on formalin-inactivated virus was shown to be protective in sheep and cattle [134, 135] and can be safely applied during all physiological stages, but is laborious to produce and requires booster vaccinations. This renders this vaccine less suitable for the control of explosive outbreaks. Recently, a new vaccine was introduced based on an isolate from a human case from the Central African Republic [136]. This isolate, called Clone 13, contains a natural 549-nucleotide deletion in the NSs gene rendering the virus highly attenuated. Clone 13 was shown to be both safe and effective in sheep, cattle and weaner calves under experimental conditions [137, 138]. However, a recent study reported lethal encephalitis in mice inoculated intranasally with RVFV lacking NSs [139]. This finding explains the need for additional studies to firmly establish the safety of Clone 13, both for target animals and vaccine administrators.

The first vaccine for human application was developed by Randall and co-workers [140-142] and was based on formalin-inactivated Entebbe strain, isolated from mosquitoes in Uganda in 1944 [35]. This investigational vaccine was well tolerated in humans and elicited neutralizing antibodies, but required several applications to induce a sufficiently high neutralizing antibody response in all vaccinated individuals. The vaccine was produced by the National Drug Biological Research Company (NDBR) under the commercial name NDBR-103

and was used from 1967 to protect laboratory personnel. In 1979, the Salk Institute, Government Service Division (TSI-GSD), commissioned by the United States army, manufactured an improved version of NDBR-103 under the name TSI-GSD 200, which was also investigational. A recent summary about the use of the vaccine over a 19 year period supported its safety and ability to induce neutralizing antibodies [143]. However, the recommended regimen of a three-dose primary vaccination followed by a booster vaccination clearly calls for more efficient vaccines for application in humans.

A live-attenuated vaccine, originally developed for use in livestock and later evaluated for use in humans, was developed by Caplen and co-workers [144] by serial passages of the virulent strain ZH548 in the presence of the mutagen 5-fluorouracil. The resulting virus, later called MP-12, had accumulated mutations on all three genome segments, resulting in attenuation [145]. The MP-12 vaccine has been extensively tested for efficacy in ruminants and was found efficacious in both sheep and cattle. Although low levels of viremia were detected after vaccination, no untoward effects in lambs, adult sheep or cattle were observed. The vaccine was also found safe for use in ewes and cows during the second trimester of gestation [146-148]. However, when applied in the first trimester of gestation, MP-12 caused teratogenicity and abortions in some of the sheep [149]. In a more recent study performed in well-controlled conditions, the MP-12 vaccine and an authentic recombinant (arMP-12) vaccine were not found to cause untoward effects in vaccinated gestating ewes. However, one of four ewes in both vaccination groups carried a dead fetus at the end of the experiment, although direct association with the vaccines could not be established [150].

Safety of the MP-12 vaccine was studied also in nonhuman primates. In neurovirulence studies with rhesus macaques the vaccine was found not completely innocuous. Residual lesions were, however, classified as mild and of similar severity as those known to occur after administration of the 17D yellow fever vaccine (Morril, 2003). Subsequently, the MP-12 vaccine was evaluated in trials including a total of 63 healthy volunteers. Although the results of these studies still await publication, it was reported that no serious adverse effects and no reversion to virulence were observed [151]. The safety record of MP-12 was found sufficient to exclude the virus from the Select Agent list and the vaccine virus may be handled in BSL-2 facilities in the USA.

Next-generation vaccines

Live-attenuated vaccines created by reverse-genetics

Due to the remaining concerns on the safety of the Clone 13 and MP-12 vaccines, efforts are continued to develop vaccines of enhanced safety without compromising efficacy. To this end, reverse-genetics has been employed to introduce attenuating modifications in the RVFV genome. Attenuation is preferably achieved by deleting genes or parts thereof, so that the chance of reversion to virulence is minimized. Deletions of the NSs and NSm coding regions, which are non-essential for efficient replication in tissue culture, were shown to be highly attenuating and are therefore preferred targets for deletion. Deletion of the NSm-coding region from the MP-12 genome resulted in a vaccine candidate that has an efficacy comparable to the parental strain and was shown to be of improved safety [150]. A reassortant virus combining the M and L segments of the MP-12 virus and the S segment of Clone 13 was also created, named R566 [152]. The efficacy of this vaccine is described in Chapter 4 of this thesis. In another attempt to develop a live-attenuated vaccine by reverse-genetics, both the NSm-coding region and the NSs gene were deleted from the human isolate ZH501, giving rise to the Δ/Δ vaccine strain, recently renamed to DDvax. This vaccine was shown to be safe for rats and gestating sheep and provided solid protection in both species [153, 154]. The demonstrated safety of the DDvax vaccine was found sufficient to remove it from the Select Agent list and to downscale the strain to BSL-2 by the US Centers for Disease Control and Prevention and the National Institutes of Health. It is regarded as a promising live-attenuated candidate vaccine for use in livestock.

Very recently, a live vaccine based on a 4-segmented RVFV was created in our laboratory. In this vaccine, designated 4s-ΔNSs, the M segment is split into two parts encoding either Gn or Gc. Although this modification alone resulted in a virus that was completely innocuous in mice, the NSs gene was omitted from the genome to optimize its safety profile. This vaccine protected mice from a lethal RVFV challenge and induced sterile immunity in sheep after a single vaccination [106, 155]. Due to the demonstrated safety profile of 4s-ΔNSs, the vaccine was recently downscaled to BSL-2 in The Netherlands.

Subunit vaccines

The surface glycoprotein Gn of RVFV is highly immunogenic and the dominant target for neutralizing antibodies [156]. This renders the Gn protein a preferred candidate for subunit vaccine development. After pioneering work of Collett and

Schmalljohn in the late 1980's, who evaluated the immunogenicity of vaccinia- or baculovirus-produced RVFV glycoproteins [157], De Boer *et al.* produced a soluble form of the Gn ectodomain in Drosophila insect cells [158]. This protein, when used in a vaccine formulation with Stimune water-in-oil adjuvant, elicited neutralizing antibodies in both mice and lambs. Vaccinated mice were protected from lethal challenge and lambs had significantly lower viremia after challenge, as compared to unvaccinated controls [158, 159].

A different approach for subunit vaccine development using both surface glycoproteins Gn and Gc is the generation of virus-like particles (VLPs). Expression of Gn and Gc was sufficient to produce VLPs [158, 160], but co-expression of the nucleoprotein N was shown to increase the yields and was shown to improve particle stability [161, 162]. VLPs resemble strongly the authentic virus by structure and present Gn and Gc in a native conformation. Consequently, VLPs are generally highly immunogenic and can be efficacious even in the absence of adjuvant. VLP-based RVF vaccines were shown to induce neutralizing antibodies in mice and rats and to protect both species from a lethal dose of RVFV [158, 160, 161].

Subunit vaccines are completely safe to use since neither the vaccine composition, nor its production involves the use of a replicating virus. Drawbacks of these vaccines include production limitations, the need for multiple applications to achieve optimal efficacy and inflammation reactions that can develop as a consequence of co-administered adjuvants.

DNA vaccines

DNA vaccines offer an alternative strategy for the development of vaccines of optimal safety. Several studies have described the use of such vaccines against RVFV [163-166]. Plasmids encoding either the glycoproteins or the nucleocapsid protein were consistently shown to induce only partial protection in mice, even when boosted with a poxvirus vector expressing the same proteins [163]. Interestingly, the efficacy of a DNA vaccine was greatly improved when three copies of the complement protein C3d were fused to Gn. This vaccine provided full protection from morbidity and mortality in mice and induced total IgG levels comparable to those of mice vaccinated with a Venezuelan encephalitis virus-based replicon vaccine, expressing Gn (Rep-Gn). However, the isotype specificities differed, as Rep-Gn vaccination elicited a balanced IgG1 – IgG2 antibody response, while DNA vaccination induced predominantly an IgG1 response, even when boosted with Rep-Gn. Furthermore, no IFN-γ secreting spleen cells were detected in mice vaccinated with the DNA construct, whereas

these cells were detected in mice that received the Rep-Gn vaccine [165]. In another study, fusion of a ubiquitin moiety to the nucleocapsid protein seemed to improve vaccine efficacy in an IFNAR-/- mouse model [166]. Altogether these studies reveal that DNA vaccines, although safe, need serious improvement in efficacy before they can be employed for protection of animals or humans against RVFV.

Vaccines based on viral vectors

Various vector vaccines for the control of animal diseases have been licensed for use in animals [167, 168] and more recently also for the first time for use in humans [169-171] . Viral vector vaccines deliver a gene of interest into specific target cells of a host. Apart from high-level *in vivo* expression of an immunogenic protein of choice, replication of the vaccine virus triggers innate responses that can function as a highly effective adjuvant. Vectors can be advantageous over attenuated vaccines when safety concerns prohibit the use of live-attenuated vaccines, and trigger a broader immune response compared to inactivated- or subunit vaccines [172]. A large variety of vector platforms have been created and evaluated, including those based on DNA viruses (poxviruses and adenoviruses), positive-strand RNA viruses (togaviruses and flaviviruses) and negative-strand RNA viruses (rhabdoviruses and paramyxoviruses).

The idea for a vector-based vaccine against RVFV was first exploited by Wallace and co-workers, who used the poxvirus lumpy skin disease virus (LSDV), which mostly affects cattle [163, 173]. Poxviruses are widely explored as viral vectors because of their stability and capability to accommodate large and multiple foreign genes. LSDV was chosen as a vaccine vector with the intention to create a multivalent vaccine that can be used to protect cattle, sheep and goats against both endemic poxviruses and RVFV [173]. A major goal of this approach is to overcome the general reluctance of farmers to vaccinate ruminants against RVFV during interepidemic periods.

The attenuated South African Onderstepoort LSDV vaccine strain was used to express both the Gn and Gc proteins of RVFV, resulting in the rLSDV-RVFV vaccine. A prime-boost vaccination regimen with rLSDV-RVFV was comparably effective as the OBP Smithburn vaccine in the same study, and elicited similar antibody titers against RVFV in mice and sheep [163]. Sheep also seroconverted for LSDV-specific antibodies. Interestingly, LSDV expressing only the RVFV nucleoprotein conferred partial protection in mice, although no antibodies against N were detected. The relatively mild RVFV challenge, however, precludes comparison of the results with those from other studies.

In related studies, Soi *et al.* used the KS-1 strain of LSDV as a vector, expressing the NSmGn-coding region of RVFV [174]. This vaccine elicited neutralizing antibodies against both RVFV and LSDV after prime-boost vaccination of sheep. The vaccinated animals displayed reduced fever and viremia upon RVFV challenge. Studies in mice revealed that protection from lethal RVFV challenge was dose- and route dependent and protected at best 70% of the mice after a single intraperitoneal administration of the recombinant KS-1 vaccine. More recently, the KS-1 strain was used to validate inbred MBT/pas mice as a potential small animal model for evaluation of vaccines against RVFV. The KS-1 candidate induced neutralizing antibodies, stimulated lymphocyte proliferation, and provided protection from virulent RVFV challenge in 75% of the mice after a prime-boost vaccination applied via combination of three different routes, intramuscular, intraperitoneal and subcutaneous [175].

Vaccinia virus recombinants were also used to develop vector vaccines against RVFV. Modified vaccinia virus Ankara expressing Gn and Gc of RVFV was fully protective in mice. The vaccinated animals developed neutralizing antibodies and glycoprotein-specific T-cell responses [176]. MVA expressing the RVFV nucleoprotein was partially protective, consistent with previous reports on using N as a vaccine antigen [163, 164, 177]. Recently an MVA vector expressing Gn and Gc of RVFV was tested in lambs [178]. This study revealed only moderate reduction of clinical signs in vaccinated animals after challenge. Comparable amounts of RNA in the blood of vaccinated and control animals were detected. These results led the authors to conclude that the vaccination protocol requires further optimization. Another variant of vaccinia virus, the attenuated replication-competent Vaccinia virus (rVACV), expressing Gn and Gc, provided 50% and 90% protection in mice after one or two vaccinations, respectively. Nevertheless, this vaccine elicited high titers of neutralizing antibodies in baboons, following a prime-boost regimen and caused only minor skin lesions. This finding holds promise for a possible human application [179].

The generally suboptimal performance of poxvirus-based vectors against RVFV explains the continuous search for more potent vector platforms. An adenovirus system, which is being extensively evaluated for diverse applications, was evaluated by Holman *et al.* [180]. The authors utilized an adenovirus vector system expressing both glycoproteins of RVFV (CAdVax-RVF) and demonstrated that a single vaccination protected all mice from an otherwise lethal challenge dose. In the same study, the influence of pre-existing immunity was evaluated. The authors reported reduced survival of mice after administrating a lethal challenge dose, when these mice were previously exposed to a homologous

adenovirus vector. This result, when extrapolated to the human population, reveals that pre-existing immunity can interfere with the efficacy of adenovirus vectors. To circumvent this problem, Warimwe *et al.* [181] employed a chimpanzee adenovirus vector that seems to have low seroprevalence in the human population and is serologically distinct from the human adenoviruses [182]. Analogous to the CAdVax-RVF vector, the chimpanzee adenovirus vector was constructed to express the Gn and Gc glycoproteins of RVFV (ChAdOx1-GnGc) and the efficacies of the two vectors were compared in mice. A single vaccination with both vectors provided 100% protection from an otherwise lethal challenge with RVFV. However, the neutralizing antibodies and IFN-γ responses in ChAdOx1-GnGc vaccinated mice were lower than in the CAdVax-RVF vaccinated mice in unadjuvanted vaccine preparations. Two commercially available adjuvants, Adda-Vax™ and Matrix-M™ were found beneficial for improving neutralizing antibody responses in ChAdOx1-GnGc vaccinated animals, and Adda-Vax™ vastly improved cellular immune responses in CAdVax-RVF vaccinated animals. Further studies in natural target species of RVFV are needed to establish the potential differences in efficacy of the two adenovirus vectors.

The vector vaccine furthest in development was created by Kortekaas and co-workers and is based on the Paramyxovirus Newcastle disease virus (NDV) [183, 184]. NDV has several properties that render it a promising vaccine vector. First, this virus is restricted to avian species. Consequently, mammals do not have pre-existing immunity that could otherwise compromise vaccine efficacy. Second, the virus can be grown in embryonated chicken eggs to very high titers, presenting means of production that are low-tech and cost-effective. Third, as this vaccine does not encode the nucleoprotein of RVFV, it can be used to differentiate infected from vaccinated animals (DIVA). The vaccine, based on avirulent NDV strain La Sota expressing Gn and Gc of RVFV, completely protected sheep from viremia and morbidity after a single vaccination. This vaccine is currently being further developed by Deltamune in South Africa.

Vaccines based on replicon vectors

Virus replicons are able to perform genome replication in infected cells, but are incapable of generating progeny virus particles since they are deprived of one or more genes encoding surface glycoproteins. Thereby, they do not spread from the site of initial infection. Virus replicon particles are developed with the aim to optimally combine the efficacy of live vaccines with the safety of inactivated vaccines.

First attempts to use replicon particles to develop RVF vaccines were based on two alphaviruses – Venezuelan equine encephalitis virus (VEEV) and Sindbis virus (SINV) [185]. The alphavirus replicons were designed to express either both surface glycoproteins or only the Gn protein of RVFV. A single vaccination with VEEV-Gn protected all mice from lethal challenge, while SINV did not protect any of the mice. The authors explained this contrasting difference in vector efficacy with the higher level of RNA replication of VEEV and its greater resistance to type-I interferons. Unfortunately, a strong interference of Gn expression with VEEV replicon packaging was observed, making the production of these replicons technically challenging. In another study, Heise and co-workers described the successful use of two different SINV replicons, encoding the complete open reading frame of the M segment. Prime-boost vaccination with either construct protected all mice from lethal challenge and elicited neutralizing antibodies in both mice and sheep [186]. The ability of VEEV replicons expressing Gn to protect mice from a lethal challenge dose was confirmed by Bhardwaj *et al.* [165]. Efficacy of alphavirus replicon vectors in the natural target animals against RVFV challenge infection is not yet reported.

RVF vaccines based on replicon particles

Using reverse genetics, our group developed bunyavirus replicon particles, referred to as nonspreading RVFV (NSR). These particles are briefly introduced below and are described in detail in Chapter 2 of this thesis. NSR was tested in animal experiments and proved to be highly efficacious in mice and sheep (Chapter 2 and [159]). Similar replicons (VRP_{RVF}) were described by Dodd *et al.* and were shown to fully protect mice from RVFV challenge [104]. We subsequently improved the efficacy of our NSR replicon particles by introducing the Gn gene in the NSR genome, resulting in NSR-Gn (Chapter 3). A single vaccination of lambs was shown to induce sterilizing immunity (Chapter 3 and 4). Very recently, Murakami *et al.* described the production of MP-12-based replicons. These replicons were tested in mice and protected 55% of the animals from an otherwise lethal RVFV challenge [187]. In the same study, the development of a single-cycle replicable RVF vaccine based on the MP-12 strain (scMP-12) is described. scMP-12 encodes a membrane fusion-defective mutant of Gc protein, rendering it capable of only a single propagation cycle. In an efficacy study, scMP-12 was shown to protect 26 from 29 of mice, challenged with a lethal dose of RVFV, while an identical dose of the parental MP-12 vaccine provided 100% protection.

Thesis aim and outline

RVFV is a serious pathogen for both ruminants and humans and is recognized as a notifiable disease by the World Organization for Animal Health (Office International des Epizooties) and as a Category A overlap Select Agent by the Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA). Outbreaks of RVF have a major impact on the agricultural sector in affected areas with severe socio-economic consequences. Moreover, the virus has shown an impressive capability of crossing large geographic areas. Globalization, climate change and the global distribution of potential vectors constitute a significant risk of further spread of the virus to new areas. All these features explain the need for safe, effective and affordable vaccines for emergency vaccination in both endemic and yet unaffected areas.

The aim of the work described in this thesis was to develop a RVF vaccine that optimally combines the safety of inactivated vaccines with the efficacy of live-attenuated vaccines. The research was initiated by the creation of a nonspreading RVFV, abbreviated as NSR. NSR particles are incapable of autonomous spread and are therefore inherently safe. The NSR vaccine proved to be highly efficacious as a RVF vaccine in target species. The remarkable efficacy of NSR vaccination prompted the development of an NSR-based vector vaccine for the prevention of influenza and the use of NSR particles for prophylactic or therapeutic vaccination against cancer. To gain insight into the molecular basis of NSR efficacy, the interaction of NSR particles with dendritic cells was studied.

Chapter 2

In Chapter 2, we report the first successful creation of RVFV replicon particles. We describe a cell line that stably maintains replicating RVFV genome segments L and S. The S segment was modified to encode the enhanced green fluorescent protein (eGFP), enabling easy detection of genome replication. The two segments are efficiently packaged into particles following a simple transfection step with one expression plasmid that encodes the viral glycoproteins. The resulting particles are able to infect new cells, resulting in autonomous genome replication and expression of the eGFP reporter. However, due to the absence of the M segment, no progeny particles are produced. The latter feature enables the safe application of NSR outside biosafety level-3 facilities, greatly facilitating both fundamental and applied scientific studies on RVFV. We demonstrate that a

single vaccination provides full protection in a mouse model, underscoring the vaccine potential of NSR.

Chapter 3

The promising results obtained with NSR vaccination of mice prompted further efficacy studies in lambs. In a first study, that is not included in this thesis, we demonstrated that NSR vaccination prevents clinical disease and reduces viremia to levels undetectable by virus isolation [159]. However, low levels of viral RNA were detected in vaccinated lambs following challenge infection, suggesting that protection was not sterile. In Chapter 3, we report the improvement of the vaccine by introducing the Gn gene, which is the major target for neutralizing antibodies, in the NSR genome. To compare immune responses elicited by NSR and NSR-Gn, mice were vaccinated with either NSR or NSR-Gn and both humoral and cellular immune responses were analyzed. This study demonstrated that NSR-Gn elicits superior CD8 and CD4-restricted memory responses and higher titers of neutralizing antibodies. Further evaluation of NSR-Gn in lambs demonstrated that a single vaccination can completely prevent viremia as determined by our most sensitive PCR. Furthermore, no boosts in neutralizing antibodies were elicited by challenge infection, underscoring the notion that sterile immunity was obtained.

Chapter 4

In the work described in Chapter 4, the efficacy of NSR-Gn was compared with R566 and Clone 13 in lambs. Not all animals were protected from viremia and pyrexia after R566 vaccination, while sterile protection was achieved after both NSR-Gn or Clone 13 vaccination.

Chapter 5

The work described in Chapter 5 was performed to evaluate the possible application of NSR as a vector vaccine, using a mouse model of lethal influenza. NSR particles were developed that encode either the full-length, membrane-anchored hemagglutinin (HA) or the soluble ectodomain (sHA), fused to an artificial multimerization domain, resulting in NSR-HA and NSR-sHA respectively. Mice that received the NSR-sHA vaccine, either via intramuscular or intranasal route, were not protected. In contrast, a single intramuscular vaccination with

NSR-HA protected all mice from mortality, although mild weight loss was not prevented. Interestingly, mice vaccinated via the intranasal route were completely unaffected by the influenza virus challenge. Analysis of cytokine responses suggested that NSR vaccination induces a polarized Th1 response.

Chapter 6

The work in chapter 6 describes a study on the effect of NSR infection on human monocyte-derived DCs. We observed that NSR infection resulted in upregulation of the surface molecules CD40, CD80, MHCI and MHCII, indicative of DC maturation. However, a downregulation in the surface expression of CD83 was detected. Considering that CD83 is the most prominent marker of DC maturation, our finding suggests that NSR-infected DCs did not fully mature. The results from experiments aiming to elucidate the molecular basis of the observed CD83 downregulation indicate that it occurs at the translational level. In contrast to infected DCs, bystander DCs displayed a fully mature phenotype, suggesting that these cells play an important role in NSR-mediated immunity.

Chapter 7

In Chapter 7, we report the evaluation of NSR as a vector vaccine for cancer immunotherapy. A well-established ovoalbumin (OVA) mouse tumor model was used to evaluate the efficacy of a prophylactic or therapeutic vaccination regimen. While all control mice developed solid tumors and reached the humane end point within 26 days after tumor cell inoculation, significant delays in tumor growth were observed in vaccinated mice. Two out of 10 therapeutically-vaccinated mice and 6 out of 10 mice that received prophylactic treatment cleared the tumors completely. Analysis of tumor cells collected from mice with relapse demonstrated that these cells had lost OVA expression, indicating that tumor cells expressing OVA were efficiently cleared by vaccination.

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Creation of a nonspreading Rift Valley fever virus

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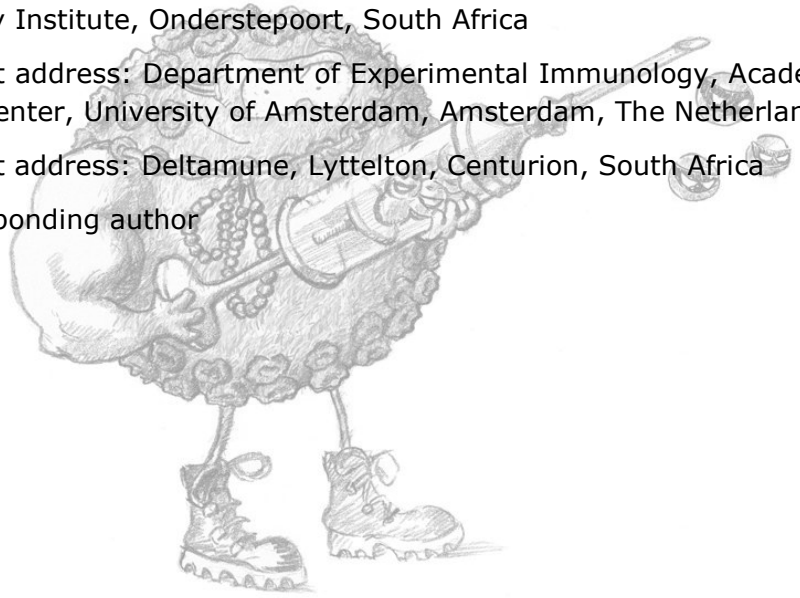

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Abstract

Rift Valley fever virus (RVFV) is a mosquito-borne zoonotic bunyavirus of the genus *Phlebovirus* and a serious human and veterinary pathogen. RVFV contains a three-segmented RNA genome, which is comprised of the large (L), medium (M) and small (S) segment. The proteins that are essential for genome replication are encoded by the L and S segments, whereas the structural glycoproteins are encoded by the M segment. We have produced BHK replicon cell lines (BHK-Rep) that maintain replicating L and S genome segments. Transfection of BHK-Rep cells with a plasmid encoding the structural glycoproteins results in the efficient production of RVFV replicon particles (RRPs). To facilitate monitoring of infection, the NSs gene was replaced by the eGFP gene. RRPs are infectious for both mammalian and insect cells but are incapable of autonomous spread, rendering their application outside biosafety containment completely safe. We demonstrate that a single intramuscular vaccination with RRPs protects mice from a lethal dose of RVFV and show that RRPs can be used for rapid virus-neutralization tests that do not require biocontainment facilities. The methods reported here will greatly facilitate vaccine and drug development as well as fundamental studies on RVFV biology. Moreover, similar systems can possibly be developed for other members of the bunyavirus family as well.

Introduction

The family *Bunyaviridae* is divided into five genera, of which four (*Orthobunyavirus, Nairovirus*, *Phlebovirus* and *Hantavirus*) include numerous virus species capable of causing severe disease in both animals and humans. Well known examples are hantaanvirus (HTNV; genus *Hantavirus*), Crimean-Congo hemorrhagic fever virus (CCHFV; genus *Nairovirus*) and Rift Valley fever virus (RVFV; genus *Phlebovirus*). Although RVFV, HTNV and CCHFV cause severe disease with high case fatality, no vaccines are available for the prevention of these diseases in humans and no antiviral agents are registered for postexposure treatment. The development of such control tools is complicated by the fact that these viruses must be handled under high biosafety containment.

In the veterinary field, RVFV is the most feared bunyavirus. The mortality rate in adult ruminants can be up to 20%, whereas fatality rates in unborn and young animals can be even more dramatic, approaching 100% [1, 2]. The human case-fatality rate is historically estimated to be below 1%, although considerably

higher mortality rates are also reported [3-5]. RVFV is currently largely confined to the African continent and the Arabian Peninsula, but mosquitoes capable of transmitting RVFV are not restricted to these areas [6-8]. This explains the growing concern for RVFV incursions into previously unaffected areas including Europe, Australasia and the Americas [9-13].

Like other bunyavirus family members, RVFV contains a three-segmented RNA genome, comprising a large (L), medium (M) and small (S) segment [14]. The L segment encodes the viral RNA polymerase. The M segment contains five in-frame start codons which give rise to the structural glycoproteins Gn and Gc and two major non-structural proteins, referred to as NSm1 and NSm2 (Fig. 1). The S segment encodes the non-structural NSs protein and the nucleocapsid (N) protein. The NSs protein suppresses host innate immune responses and was shown to be the primary virulence factor of the virus [15-18].

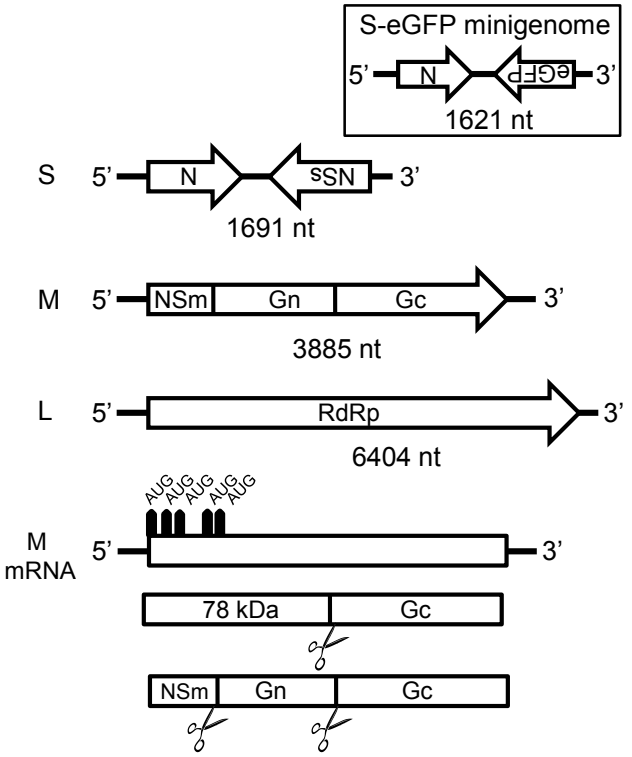

Figure 1. RVFV genome organization and expression strategy of the M genome segment. (A) Schematic representation of the RVFV small (S), medium (M) and large (L) genome segment in antigenomic orientation. Inset: The S-based minigenome containing the eGFP gene used in the current work. (B) Coding strategy of the M genome segment. The mRNA encoded by the M segment is translated into a polyprotein that is processed into the NSm1, NSm2, Gn and Gc proteins.

The recent establishment of a reverse-genetics system for RVFV has provided important new insights into its biology [11, 19-21]. A few years after the first successful rescue of RVFV from cloned cDNA, the packaging of a reporter minigenome into virus-like particles (VLPs) was reported [22]. The VLPs were produced by transient expression of the NSm, Gn, Gc, N and L proteins in the presence of the reporter minigenome. In this system, the N and L proteins produced from protein expression plasmids facilitate replication of the minigenome. The minigenome is subsequently packaged by the structural glycoproteins into so called 'infectious VLPs' (iVLPs), which are able to transport the RNA to receiving cells. Whereas primary transcription in these cells occurs, replication of the minigenome and high-level reporter gene expression depends on *de novo* production of N and L proteins from transfected plasmids [22].

A recent study reported the co-packaging of the M and S genome segments into VLPs [23]. Packaging of the L genome segment into VLPs was not accomplished in these studies and it was proposed that the M genome segment either alone or in a coordinated action with the S segment, is essential for packaging of the L segment. We here describe efficient methods to produce large amounts of virus particles that contain both S and L genome segments and thereby demonstrate that the M segment is not essential for this process. By virtue of the L and S genome segments, the particles are capable of autonomous genome replication and high-level gene expression. However, since the M genome segment is absent, the particles are incapable of autonomous spread. The so-called RVFV replicon particles (RRPs) were produced to titers exceeding 10^7 infectious particles/ml.

We propose that RVFV RRPs optimally combine the efficacy of live vaccines with the safety of inactivated vaccines and support this notion by demonstrating that a single intramuscular vaccination with 10^6 RRPs completely protects mice from a lethal dose of RVFV. We also show that RRPs can be used for rapid virus-neutralization tests that do not require biosafety containment facilities.

The methods described here will greatly facilitate both fundamental and applied research on RVFV and can potentially be established for other members of the bunyavirus family as well.

Materials and methods

Cells and growth conditions

BSR-T7/5 were kindly provided by Prof. Dr. K. Conzelmann (Max von Pettenkofer-Institut, München, Germany). BSR-T7/5 cells, BHK cells and derivatives were grown in Glasgow Minimum Essential Medium (GMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 4% tryptose phosphate broth (Invitrogen), 1% minimum essential medium non-essential amino acids (MEM NEAA, Invitrogen) and 5-10% Fetal Bovine Serum (FBS; Bodinco, Alkmaar, The Netherlands). For maintenance of stable cell lines, geneticin (G-418; Promega, Madison, WI, USA) was used at a concentration of 1 mg/ml. For the production of RRPs for the vaccination-challenge trial, cells were grown in Optimem (Invitrogen) supplemented with 2% FBS. Cells were grown at 37^{o}C and 5% CO2. *Drosophila* (S2) cells were grown in Schneider's medium (Invitrogen) at 28^{o}C. *Aedes albopictus* C6/36 cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FBS at 28^{o}C and 5% CO_2.

Plasmids and viruses

Plasmid pCIneo-GnGc contains the open reading frame of the M segment of RVFV strain 35/74, starting at the fourth methionine codon. The GnGc-coding sequence was codon-optimized for optimal expression in mammalian cells and synthesized by the GenScript Corporation (Piscataway, NJ, USA). Plasmids pCIneo-M and pCAGGS-M contain cDNA of the authentic RNA sequence of the M segment starting at the first methionine codon. Expression of genes from pCIneo is controlled by a cytomegalovirus (CMV) immediate-early enhancer/promoter, whereas the expression of genes from the pCAGGS plasmid is controlled by a CMV immediate enhancer/ß-actin (CAG) promoter [24].

RVFV strain 35/74 was isolated from a liver of a sheep that died during a RVFV outbreak in the Free State province of South Africa in 1974 [25]. The virus was passaged four times in mouse brain and three times in BHK cells. Amplification of the genome was performed by a one-step RT-PCR using primers previously described by Bird *et al*. [26]. PCR products were purified from agarose gels and used for GS FLX sequencing at Inqaba Biotec (Pretoria, South Africa) essentially as described [27]. The consensus sequences corresponding to each genome segment were synthesized and cloned in pUC57, a standard cloning vector of the GenScript Corporation (Piscataway, NJ, USA). pUC57-L, pUC57-M and pUC57-S encode the RVFV L, M and S genome segment in antigenomic orientation, respectively. The complete sequence of the L, M and S genome sequences can

be found on GenBank under the accession numbers JF784386, JF784387 and JF784388, respectively. Of note, the cDNA of the M segment present in plasmid pUC57-M contains a silent A to G mutation at position 182. The transcription plasmids each contain a complete copy of the viral RNA segments and are flanked by a minimal T7 promoter and a hepatitis delta virus ribozyme sequence. In pUC57-S-eGFP, the NSs gene is replaced by the gene encoding eGFP (Fig. 1, inset).

Rescue of recombinant

RVFV strain 35/74. BSR-T7/5 cells were seeded in 6-well plates and were co-transfected with 1 µg of plasmids pUC57-L, pUC57-M and pUC57-S using jetPEI transfection reagent according to the instructions of the manufacturers (Polyplus-transfection SA, Illkirch, France). After 6 days of incubation, the medium was collected and used for virus titration on BHK cells.

Alternatively, BHK cells were infected with a recombinant fowlpox virus (FPV) that produces T7 polymerase [28, 29]. This virus, named fpEFLT7pol (here referred to as FP-T7), was previously kindly provided by the Institute for Animal Health (IAH, Compton, UK). After incubation with FP-T7 for 1 h and recovery for another hour, the cells were treated in a similar way as described for BSR-T7/5 cells. Virus titers were determined as 50% tissue culture infective dose ($TCID_{50}$) using BHK cells and were calculated using the Spearman-Kärber method [30, 31].

Production of RRPs.

For the production of RRPs using the three-plasmid system, BHK or BHK-GnGc cells were seeded in 6-well plates and incubated with FP-T7 for 1.5-2 h at 37^{o}C. Medium was refreshed and cells were allowed to recover for 1 h. Cells were subsequently transfected with 600 ng each of plasmid pUC57-L, pUC57-S-eGFP and pCAGGS-M. The medium was refreshed the next day. Supernatants were harvested after 72 hrs, pre-cleared by slow-speed centrifugation at RT and stored at 4^{o}C until further use.

For the production of RRPs using the one-plasmid system, HEK293T cells were seeded in 6-well plates and infected with RRPs at a multiplicity of infection (m.o.i). of 3. Three days later, the cells were passaged to wells of a 6-well plate and transfected with 1 µg of pCAGGS-M. RRPs were collected from the culture medium at 72 hrs post transfection. Alternatively, BHK-Rep cells were transfected with pCAGGS-M and RRPs were collected at different time points.

The titers of RRPs were determined by $TCID_{50}$ assays using BHK cells and were calculated using the Spearman-Kärber method [30, 31]. Infectivity was detected by monitoring eGFP expression using a Zeiss fluorescence microscope.

For the production of RRPs for the vaccination of mice, BHK-Rep cells were grown in Optimem supplemented with 2% FBS. The cells were transfected with pCAGGS-M and after 24 hrs the medium was collected. The RRPs in the collected medium were concentrated using Amicon filters (Millipore, Billerica, MA, USA) and subsequently diluted in PBS to a titer of $10^{7.3}$ $TCID_{50}$/ml.

Northern blotting.

RNA probes were prepared by T7-based *in vitro* transcription in the presence of digoxigenin-11-UTP according to the instructions of the DIG Northern Starter Kit (Roche, Woerden, The Netherlands). Templates for *in vitro* transcription were produced by PCR using cDNA of the S, M and L segments as template and dedicated [29]378 of the antigenomic-sense S segment, the M probe 3545 to 3885 (antigenomic-sense orientation) and the L probe nucleotides 1 to 320 (antigenomic-sense orientation).

Viral RNA of recombinant RVFV strain 35/74 (r35/74) was extracted using the High Pure Viral RNA Kit (Roche) according to the instructions of the manufacturers. RVFV RNA from the BHK-Rep and BHK-Rep2 cells was extracted using the RNeasy kit according to the instruction of the manufacturers (Qiagen, Hilden, Germany). RNA was separated by electrophoresis using the glyoxal/dimethyl sulfoxide system provided by the Northern-Max®-Gly kit (Ambion, Austin, TX, USA). Size-fractionated RNA was transferred by blotting onto positively-charged nylon membranes (Roche) using the transfer buffer of the Northern-Max®-Gly kit. RNA was fixed on the membrane by baking at 80°C for 30 min. Hybridization of DIG-labeled RNA probes was performed using UltraHyb hybridization buffer and wash solutions according to the recommendations of the manufacturer (Ambion).

Detection of hybridized probes was performed using the DIG Northern Starter Kit (Roche) according to the manufacturer's protocol. Briefly, blots were incubated in blocking solution for 30 min and subsequently incubated with alkaline phosphatase-conjugated anti-DIG antibody for 30 min, followed by washing twice in washing buffer. After equilibration in detection buffer, blots were incubated with the chemiluminescent substrate supplied by the DIG kit (CDP-Star) and exposed to X-ray films (Amersham Hyperfilm).

Polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Proteins were separated in Bis-Tris gradient gels (Invitrogen) and analyzed by Western blotting as described [32]. For Western blot analysis of Gn and Gc, rabbit peptide antisera were used [33]. Monoclonal antibody (mAb) F1D11 (kindly provided by Dr. Alejandro Brun, CISA-INIA, Madrid, Spain) was used for the detection of the N protein.

Immunoperoxidase monolayer assays (IPMAs).

IPMAs were performed as described previously [33]. For the detection of the Gn and Gc proteins, a polyclonal sheep antiserum was used [32].

Flow cytometry.

Flow cytometry was performed using a CyAn ADP flow cytometer (Beckman Coulter), equipped with a 488 nm laser. For the analysis of the data, the Summit v4.3 software was used.

Transmission electron microscopy (TEM).

RRPs were applied to copper Formvar-carbon coated grids (Stork Veco BV, Eerbeek, The Netherlands). The grids were stained with 1% phosphotungstate (PTA) at pH 7 (Merck, Darmstadt, Germany). Images were recorded at a calibrated magnification of 60,000 x using a FEI Tecnai 12 electron microscope.

Virus neutralization test (VNT) using RRPs.

Classical and RRP VNTs were performed with sera from lambs that were previously experimentally infected with the 35/74 virus. To confirm the presence of RVFV-specific antibodies, the sera were analyzed by the recN RVFV ELISA (BDSL, Ayrshire Scotland, UK) prior to analysis by VNT. The classical VNT was performed as described previously [33]. For the RRP VNT, serum dilutions were prepared in 96-well plates in 50µl GMEM supplemented with 5% FBS, 4% TPB, 1% MEM NEAA, 1% pen/strep. Culture medium containing ~200 RRPs in a 50µl volume was added to the serum dilutions and incubated for 1.5 h at room temperature. Next, 50µl of growth medium containing 40 000 BHK cells was added to each well. Plates were incubated at 37oC and 5% CO2. After 36-48 hrs the neutralization titer was calculated using the Spearman-Kärber method [30, 31].

Vaccination and challenge of mice.

Female BALB/c mice (Charles River laboratories, Maastricht, The Netherlands) were housed in groups of five animals and kept under biosafety level-3 containment. Groups of 10 mice were vaccinated via the intramuscular or subcutaneous route either once on day 21 or two times on days 0 and 21 with 10^6 $TCID_{50}$ of RRPs in 50 µl PBS. One group of nine mice was left untreated (Mock). The body weights of the mice were monitored weekly. On day 42, all mice were challenged via the intraperitoneal route with $10^{2.7}$ $TCID_{50}$ of RVFV strain 35/74 in 0.5 ml culture medium. Challenged mice were monitored daily for visual signs of illness and mortality.

This experiment was approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen University and Research Centre.

Results

Establishment of a three-plasmid system for the production of RRPs.

RVFV strain 35/74 was readily rescued from cDNA by transfection of BSR-T7/5 cells with plasmids pUC57-S, pUC57-M and pUC57-L encoding the viral RNA segments in antigenomic orientation. Our next aim was to produce only the L and S genome segments and to package these segments into RRPs by providing the structural glycoproteins Gn and Gc in trans from a protein-expression plasmid. To facilitate monitoring of genome replication, a reporter minigenome was produced in which the non-essential NSs gene of the S genome segment is replaced by the enhanced green-fluorescent protein (eGFP) gene (Fig. 1, inset). The resulting plasmid was named pUC57-S-eGFP. Co-transfection of pUC57-L and pUC57-S-eGFP into BSR-T7/5 cells resulted in only very few eGFP-positive cells and co-transfection with a plasmid providing the NSm, Gn and Gc proteins (i.e. pCAGGS-M) did not result in the production of RRPs.

To improve the system, we evaluated the use of a recombinant fowlpox virus as a source of T7 polymerase [28, 29]. The pUC57-S plasmid was transfected on its own into BSR-T7/5 cells and into BHK cells that were infected with FP-T7 prior to transfection. Whereas the N protein was not detected in BSR-T7/5 cells transfected with pUC57-S, FP-T7-infected BHK cells that were transfected with this plasmid stained intensely with a monoclonal antibody (mAb) specific for the N protein (Fig. 2).

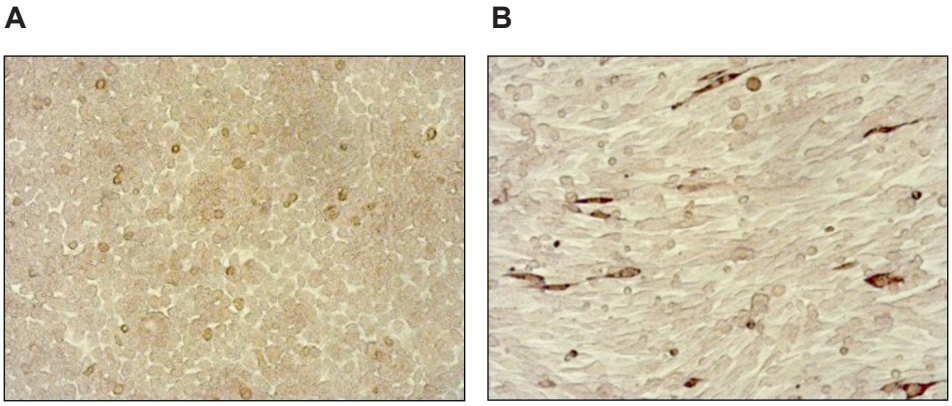

Figure 2. Expression of the N protein from the antigenomic-sense S segment. BSR-T7/5 cells (A) or FP-T7-infected BHK-21 cells (B) were transfected with plasmid pUC57-S, encoding the RVFV S genome segment in antigenomic-sense orientation. Expression of the RVFV N protein was detected using an N protein-specific mAb and HRP-conjugated anti-mouse IgG antibodies.

BHK cells were infected with the fowlpox virus (FP-T7) and subsequently transfected with pUC57-S-eGFP only, co-transfected with pUC57-S-eGFP and pUC57-L or co-transfected with pUC57-S-eGFP, pUC57-L and pCAGGS-M. Transfection with only pUC57-S-eGFP did not result in eGFP expression (Fig. 3A). After 72 hrs, eGFP expression was observed in a small percentage (0.33%) of cells that were co-transfected with pUC57-S-eGFP and pUC57-L. However, when pCAGGS-M was added to the transfection mixture, about one quarter of the cells expressed eGFP (Fig. 3A). This finding suggested that infectious particles were formed upon introduction of the pCAGGS-M plasmid, resulting in an increase in the number of eGFP-expressing cells. Collected supernatant was added to BHK cell monolayers and after 36 hrs, infection was monitored by fluorescence microscopy. Only cells incubated with the supernatant obtained from cells transfected with all three plasmids revealed eGFP-expression. This confirmed that we were successful in producing RVFV replicon particles (RRPs). Three independently performed experiments yielded an average RRP titer of $10^{4.8}$ $TCID_{50}$/ml. The three-plasmid system for the production of RRPs is schematically depicted in Fig. 3B.

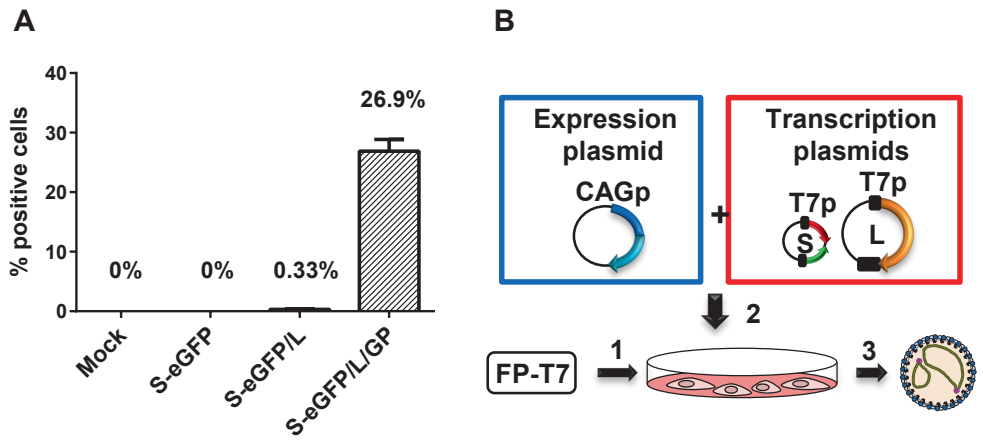

Figure 3. Production of RRPs by the three-plasmid system. (A) BHK cells were infected with FP-T7 and subsequently remained untreated (mock) or were transfected with plasmid pUC57-S-eGFP (S-eGFP) only, in combination with plasmid pUC57-L encoding the RVFV L genome segment (S-eGFP/L), or co-transfected with the aforementioned plasmids and pCAGGS-M (GP), encoding the structural glycoproteins (S-eGFP/L/GP). The percentage of eGFP-positive cells was determined by flow cytometry (N=3 ± SD). (B) Schematic representation of the three-plasmid system. BHK cells are first infected with FP-T7 (step 1) and subsequently transfected with transcription plasmids pUC57-L (L), pUC57-S-eGFP (S) and expression plasmid pCAGGS-M (step 2). After 24 hrs, the culture medium containing the RRPs can be collected (step 3). Transcription from the expression plasmid is controlled by a CAG promoter (CAGp), transcription from the transcription plasmids is controlled by a T7 promoter (T7p). Untranslated regions are depicted as black boxes.

Establishment of a one-plasmid system for the production of RRPs.

The RRP titers obtained with the three-plasmid system never exceeded 10^5 $TCID_{50}$/ml. To develop a system for the continuous production of high-titer RRPs, we created stable BHK cell lines that constitutively produce the Gn and Gc proteins. BHK cells were transfected with pCIneo-GnGc and clones with integrated plasmids were grown in the presence of G-418. Whereas an anti-Gn/Gc serum revealed clear glycoprotein expression 1-2 days after transfection, after cloning the cells, only very few selected clones revealed Gn/Gc expression by IPMA and in all cases expression seemed very low. One clone that revealed the most intense staining in IPMAs (Fig. 4) was selected and named BHK-GnGc.

It was previously demonstrated that expression of the Gn and Gc proteins in both mammalian [34] and insect cells [33] results in the production of VLPs. To determine if VLPs were produced by BHK-GnGc cells, supernatants were ultracentrifuged (100 000 x *g*, 2 h) and the proteins present in the collected pellets were analyzed by Western blotting. Neither the Gn nor the Gc protein was detected in the pellet fractions (data not shown), suggesting that either no

VLPs were produced or, more likely, that glycoprotein production was too low to allow detection.

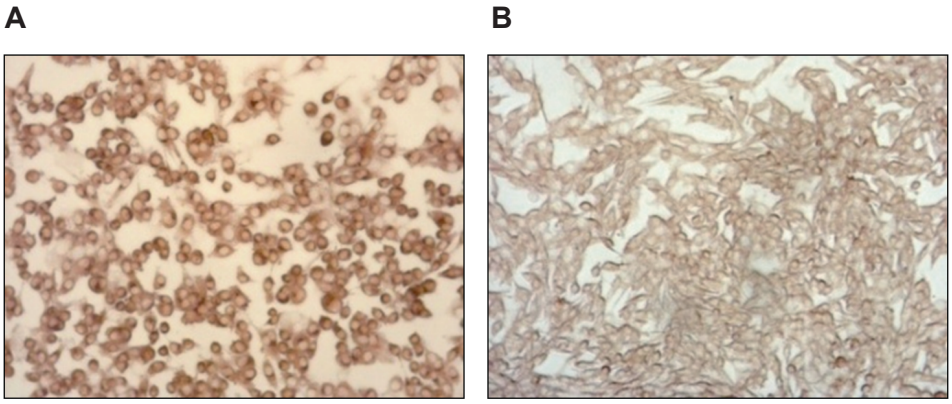

Figure 4. RVFV glycoprotein expression in BHK-GnGc cells. BHK cells were transfected with plasmid pCIneo-GnGc, encoding the RVFV structural glycoproteins Gn and Gc. The BHK-GnGc cells were cloned by limiting dilution. Expression of Gn and Gc was detected by staining of BHK-GnGc cells with polyclonal antibodies specific for the Gn and Gc proteins (A). Panel (B) shows the similarly treated BHK parent cells.

Although the BHK-GnGc cells apparently produced only very limited amounts of Gn and Gc, we hypothesized that these cells might tolerate Gn/Gc expression from plasmid to higher extend than normal BHK cells and thereby be more suitable for RRP production. To substantiate this hypothesis, BHK-GnGc cells were infected with FP-T7 and subsequently co-transfected with the three plasmids. In an attempt to increase the number of eGFP-expressing cells, the cells were next transfected with pCAGGS-M only. This whole procedure was then repeated, followed by two cell passages and introductions of the pCAGGS-M plasmid (Fig. 5A). At this point, flow cytometry demonstrated that >90% of the BHK-GnGc cells were positive for eGFP expression. The resulting cell line, which maintains the L genome segment and the S-eGFP minigenome, was designated BHK-Rep. Transfection of BHK-Rep cells with pCAGGS-M after cell passage 83, 103 and 105 yielded an average RRP titer of $10^{6.7}$ $TCID_{50}$/ml. It is important to note that we also used a similar protocol starting with wildtype BHK cells instead of BHK-GnGc cells. In these experiments, reporter gene expression did not continue to increase and we were therefore unable to produce a cell line with >90% reporter gene expression using this procedure.

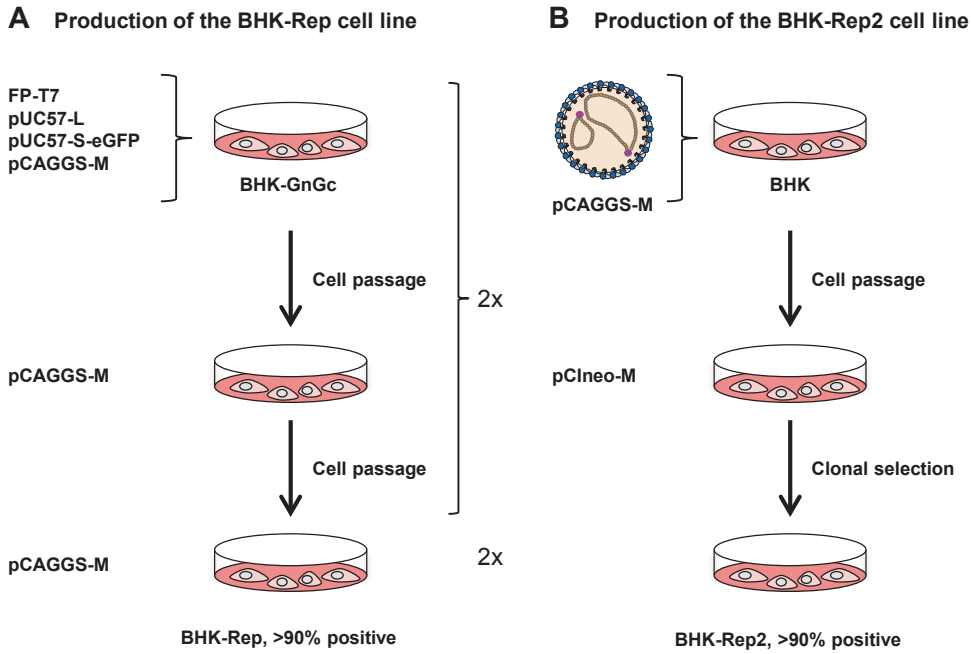

Figure 5. Cartoon representing the construction of the BHK-Rep (A) and BHK-Rep2 (B) cell lines. Construction of the BHK-Rep cells started with a BHK cell line constitutively expressing low amounts of the Gn and Gc proteins (BHK-GnGc), whereas wild-type BHK cells were used for the production of the BHK-Rep2 cell line. Stable glycoprotein expression was achieved in these cells by introducing the pCIneo-M plasmid. The S-eGFP and L genome segments were introduced in the BHK-Rep cell line by FP-T7-driven transcription from plasmids, whereas these genome segments were introduced in the BHK-Rep2 cells by infection with RRPs. Transfection with pCAGGS-M was used to produce RRPs that assist in spread of the genome segments among cells.

The NSm protein was previously reported to suppress virus-induced apoptosis [35], we reasoned that a cell line constitutively expressing not only Gn and Gc but also the NSm proteins could be more efficient in the constitutive production of RRPs by suppressing apoptosis. To this end, we aimed to produce a BHK-Rep cell line with a stably integrated plasmid encoding the NSm, Gn and Gc proteins (i.e. pCIneo-M). For convenience, the L and S-eGFP genome segments were not introduced into BHK cells by transfection of plasmids, but instead by infection of the cells with RRPs combined with a transfection with pCAGGS-M. Cells were passaged and subsequently transfected with the pCIneo-M plasmid and grown in the presence of G-418. By this way, BHK-Rep cells were produced without introducing FP-T7 and the transcription plasmids. The cells were used for cloning by end-point dilution and a selected clone was named BHK-Rep2. The steps followed to produce the BHK-Rep2 cell line are depicted in Fig. 5B. Although also

this cell line did not constitutively produce RRPs, transfection of this cell line with the pCAGGS-M plasmid yielded an average RRP titer of $10^{7.2}$ $TCID_{50}$/ml (N=6).

To determine if other mammalian and insect cells can be infected with RRPs, human embryonic kidney 293 cells (HEK293T), *Drosophila* S2 cells and *Aedes albopictus* C6/36 cells were infected with RRPs at an m.o.i. of 1 (calculated using the titer determined on BHK cells). This experiment demonstrated that both mammalian and insect cells can be readily infected with RRPs (Fig. 6). Expression of eGFP in mammalian cells and insect cells was optimal at 42 or 72 hours post infection, respectively.

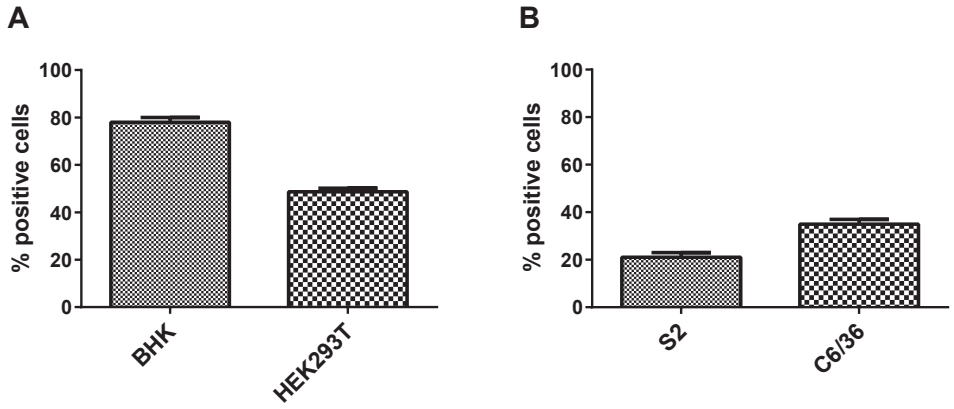

Figure 6. RRP infection of mammalian (A) and insect cells (B). BHK, human embryonic kidney 293T cells (HEK293T), *Drosophila* S2 cells and *Aedes albopictus* C6/36 cells were infected with RRPs at an m.o.i. of 1. The number of positive cells was determined by flow-cytometry at 42 (BHK and HEK293T) or 72 hours post infection (S2 and C6/36). Histograms show averaged results of three independent measurements with SD.

It is important to note that the one-plasmid system is not restricted to BHK-Rep cells. Transfection of RRP-infected HEK293T cells with the pCAGGS-M plasmid yielded an RRP titer of $10^{6.5}$ $TCID_{50}$/ml. This demonstrates that alternative cell types can be used for the production of RRPs by combining an RRP infection with a transfection of the pCAGGS-M plasmid (Fig. 7).

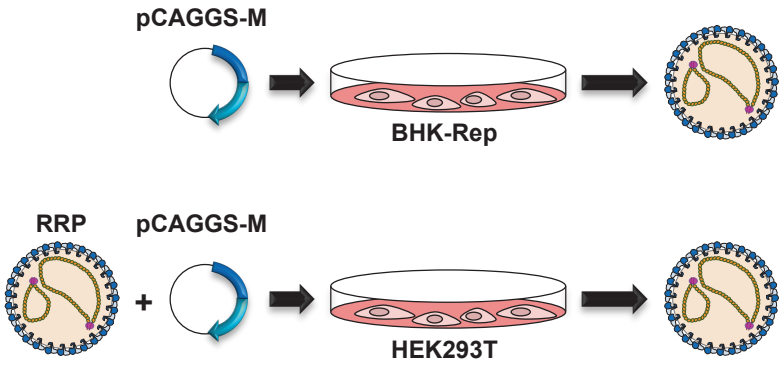

Figure 7. Schematic representation of the one-plasmid systems. Transfection of BHK-Rep cells with pCAGGS-M results in the production of RRPs. Alternatively, RRPs can be produced by infection of potentially any type of mammalian cell, such as HEK293T cells, by infection with RRPs followed by transfection with pCAGGS-M.

Characterization of BHK-Rep cells.

Persistence of RVFV RNA in cells was previously reported by Billecocq *et al*. [36]. These authors detected defective interfering (DI) RNAs with large internal deletions and suggested that these DI RNAs could be responsible for the persistent infection. Considering this, we studied if abnormal viral RNA segments were present in the BHK-Rep and BHK-Rep2 cells. Northern blotting was performed with total RNA extracted from BHK-Rep and BHK-Rep2 cells at cell passage 13 and 45, respectively. S, M and L-specific probes revealed only the S and L segments, confirming that no M segment is present in the BHK-Rep cells (Fig. 8). The S-eGFP and L segments were detected at the same positions as the S and L segment of the recombinant virus, suggesting that no significant levels of DI RNAs were present.

To determine if mutations were introduced upon replication of the L and S-eGFP genome segments in BHK-Rep cells, cDNA was produced and sequenced, with the exception of the primer binding regions on the extreme 3' and 5' ends, using standard techniques. Only a single mutation (G1181→A) was detected in the L genome segment present in the BHK-Rep cells, resulting in an amino acid substitution from glycine to glutamic acid at position 388 of the L protein. This mutation was also detected in the BHK-Rep2 cells, together with a silent mutation in the L gene (A1602→C) and two mutations in the 5' untranslated region (T6325→C and T6367→C). In the S-eGFP segments, only a single silent mutation was detected in the BHK-Rep2 cells (C65→T). The number of the

mutations corresponds to their position in the antigenomic-sense RNA. We did not further study the effect of these mutations.

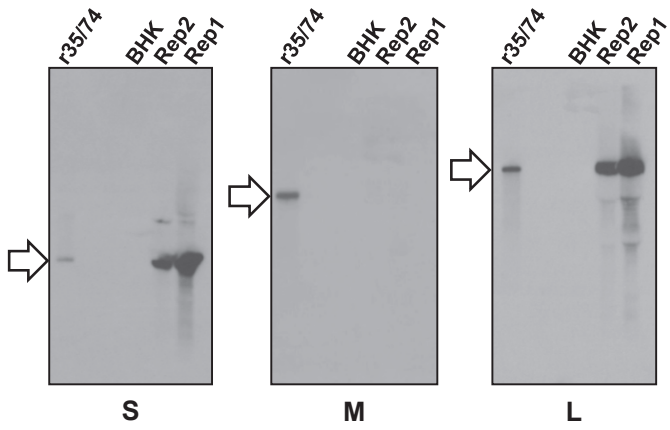

Figure 8. Northern blot analysis of the viral RNA segments present in BHK-Rep cells. Total RNA of BHK-Rep and BHK-Rep2 cells was extracted, separated by electrophoresis using the glyoxal/dimethyl sulfoxide system and transferred to positively-charged nylon membranes. Hybridization was performed with DIG-labeled S, M and L probes and hybridization was detected by phosphatase-conjugated anti-DIG antibodies. As a positive control and a reference for size, RNA extracted from recombinant RVFV 35/74 was used. Wildtype BHK cells were used as a negative control. The specificities of the probes used for hybridization of each blot is indicated below each panel. The positions of the S, M and L segments are indicated by arrows.

The viral RNA segments were introduced in BHK-Rep cells by plasmids that were transcribed by the FP-T7 virus. Although it is well established that FPV does not replicate in mammalian cells [28], the BHK-Rep and BHK-Rep2 cells were tested for the presence of FP-T7 by PCR. No FPV DNA was detected in these assays (data not shown).

Characterization of RRPs.

To establish the kinetics of RRP production, BHK-rep cells were transfected with pCAGGS-M and the culture medium was collected at different time points post transfection. This experiment demonstrated that a titer close to 10^6 $TCID_{50}$/ml was obtained already after 22 h (Fig. 9A).

To demonstrate that RRPs are incapable of autonomous spread, BHK cells were infected with RRPs at an m.o.i. of 1. After two days, eGFP expression was observed by fluorescence microscopy (Fig. 9B, left panel). BHK cells were incubated with the collected pre-cleared supernatant and after three days, cells

were monitored for eGFP expression. No eGFP expression was observed, demonstrating that no progeny infectious particles were produced by the RRP-infected BHK cells (Fig. 9B, right panel).

To visualize RRPs by transmission electron microscopy (TEM), BHK-Rep2 cells were transfected with pCAGGS-M. After 30 h, the RRPs present in pre-cleared culture supernatant were concentrated using Amicon filters and subsequently coated on Formvar carbon-coated grids. Grids were stained with 1% PTA and analyzed by TEM. Representative particles of 80 ± 2 nm are depicted in Fig. 9C. The average particle size observed varied from 80-100 nm.

To visualize RRP proteins, RRPs were pelleted by ultracentrifugation. The proteins were separated in polyacrylamide gels, transferred to nitrocellulose membranes and detected using peptide antisera specific for the Gn and Gc proteins or a mAb specific for the N protein. Analysis of the supernatant obtained from non-transfected BHK-Rep cells revealed only the N protein (Fig. 9D). This result suggests that the RVFV N protein is released from cells, resembling results previously described from studies on CCHFV [37]. Analysis of supernatant from BHK-Rep cells transfected with pCAGGS-M revealed the NSm1 protein, the Gn and Gc proteins and the N protein (Fig. 9D).

Establishment of a novel virus-neutralization test (VNT).

To determine if RRPs can be used in VNTs, sera obtained from experimentally infected lambs were used in the classical VNT as described previously [33] and used in a novel VNT that uses RRPs instead of live virus. The sera were also tested by ELISA. Similarly as is performed with the complete virus in the classical VNT, serum dilutions were pre-incubated with RRPs and the mixtures were subsequently incubated with BHK cells. Whereas in the classical VNT the lack of neutralization is detected by cytopathic effect, in the RRP VNT, eGFP expression demonstrates lack of neutralization. Titers are determined in both assays using the Spearman-Kärber method [30, 31].

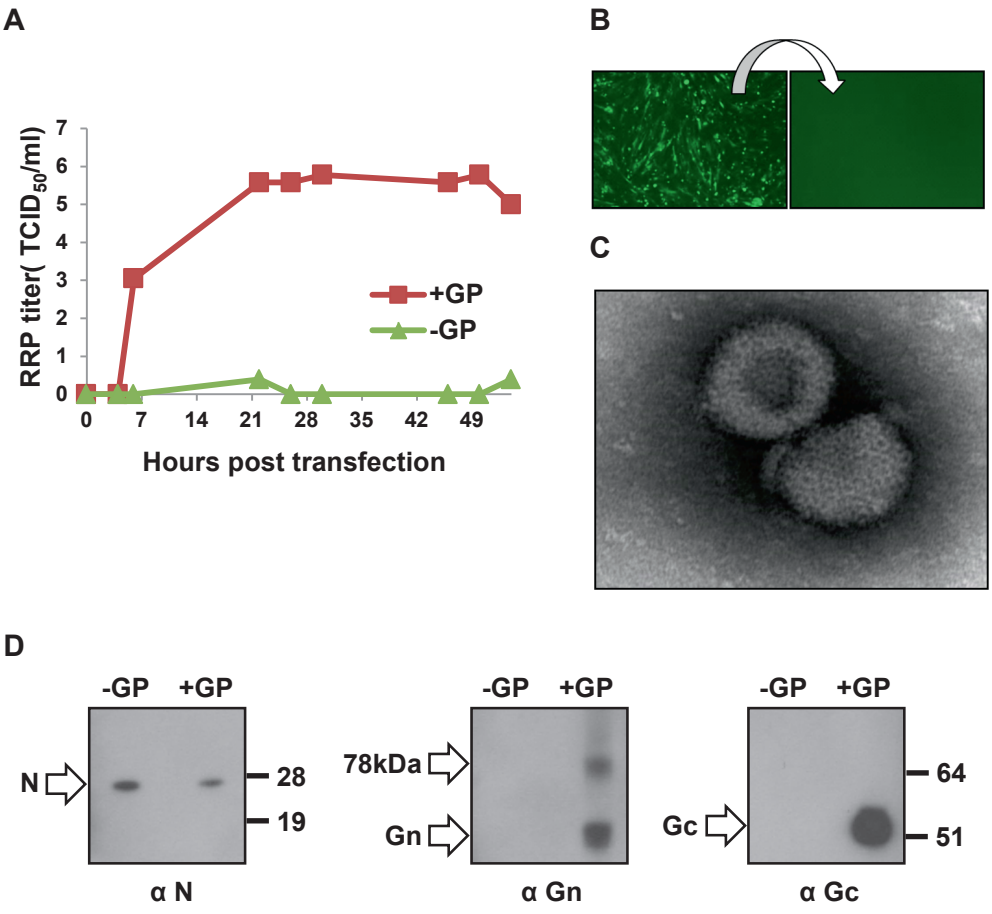

Figure 9. Characterization of RRPs. (A) BHK-Rep cells were either left untreated (-GP) or transfected with pCAGGS-M (+GP) and RRP titers in the collected supernatant were determined at different time points post transfection. (B) To demonstrate that RRPs are non-spreading, BHK cells were infected with RRPs and after two days, eGFP expression was observed in infected cells (left panel). Fresh BHK cells were incubated with the collected supernatant and monitored for eGFP expression after three days (right panel). (C) Electron micrograph of RRPs. Concentrated RRPs were stained with 1% PTA and analyzed by TEM. Scale bar represents 50 nm. (D) To visualize RRP proteins, culture medium of BHK-Rep cells (-GP) or of BHK-Rep cells transfected with pCAGGS-M (+GP) was ultracentrifuged at 100 000 x *g* for 2 h. The proteins present in the pellets were separated in 4-12% Bis-Tris gels and subsequently transferred to nitrocellulose blots. Specific proteins were detected by an anti-Gn (αGn) or anti-Gc (αGc) peptide antiserum or a mAb specific for the N protein (αN). The positions of the NSm, Gn, Gc and N protein are indicated by arrows. Molecular weight standard proteins are indicated to the right in kilodaltons.

The experiment revealed that the so-called RRP VNT has an optimal readout between 36 and 48 hrs and is of equal, if not higher sensitivity than the classical VNT (Table 1).

Table 1. Comparison of the Classical VNT and RRP VNT

Serum sample	Neutralization titer in VNT[a]		ELISA result[b]
	Classical	RRP	
4308	3.56	3.94	POS
4309	4.09	4.16	POS
4310	0	0	NEG
4311	4.01	4.24	POS
4312	3.71	4.76	POS
4314	3.56	4.46	POS
4315	3.71	4.39	POS
4318	4.16	4.39	POS
4321	0	0	NEG
4324	4.24	4.31	POS
4328	4.01	4.69	POS

[a] Reported as $\log_{10}$ 50% end-point titres.

[b] Sera were analysed by the recN ELISA (BDSL).

Vaccination and challenge of mice.

To study the vaccine efficacy of RRPs, groups of 10 mice were immunized with 50 µl of an inoculum containing 10^6 $TCID_{50}$ RRPs, via either the subcutaneous or intramuscular route, either once or twice, with a three week interval. One group of 9 non-vaccinated mice was added as a control group. The mice were challenged on day 42 with a known lethal dose of RVFV strain 35/74. All non-vaccinated mice displayed overt clinical signs and weight loss and eight of a total of nine non-vaccinated mice succumbed to the infection within four days after challenge. One mouse survived for twelve days, but eventually died. The percentage of survival in the groups of mice vaccinated either once or twice via the subcutaneous route was 60%. In contrast, 100% of the mice vaccinated via the intramuscular route, either once or twice, survived the challenge (Fig. 10). These mice did not show any clinical signs or weight loss throughout the experiment. This demonstrates that a single intramuscular vaccination with 10^6 RRPs can protect mice from a lethal dose of RVFV.

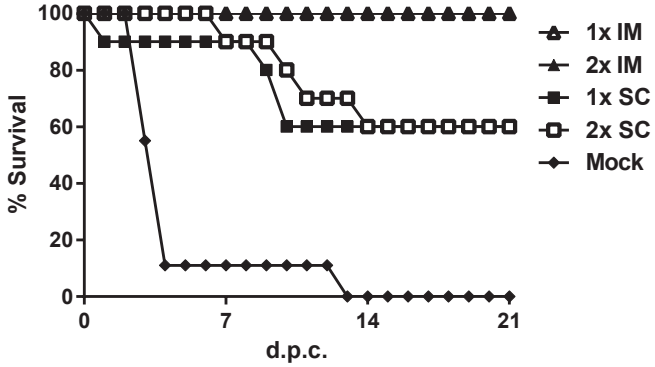

Figure 10. Vaccine efficacy of RRPs. Mice were either non-vaccinated (N=9; Mock) or vaccinated (N=10) either once (1x) or twice (2x) via the intramuscular route (IM) or subcutaneous route (SC) with 10^6 $TCID_{50}$ of RRPs. Mice were challenged with a known lethal dose of RVFV strain 35/74 via the intraperitoneal route. The mortality rates were determined until 21 days post challenge (d.p.c.).

Discussion

Here, we report the creation of a non-spreading bunyavirus. Critical to the production of the RVFV replicon particles (RRPs), was the use of FPV as a source of T7 polymerase. Despite much effort, production of RRPs using BSR-T7/5 cells, which are routinely used for bunyavirus reverse-genetics, never succeeded.

Rescue of a bunyavirus requires only three T7-transcripts representing the viral RNA molecules in antigenomic-sense orientation. This suggests that the antigenomes not only act as replication intermediates, but also as messenger RNAs [38]. Although in our experiments expression of the N protein from antigenomic-sense S RNA was not detectable in BSR-T7/5 cells, expression was readily detected in FP-T7-infected cells. T7-based transcription in BSR-T7/5 cells results in uncapped RNAs, whereas the FP-T7 virus provides its own capping enzyme [28]. We propose that this explains the improved gene expression from antigenomic viral RNAs and thereby the superiority of FP-T7-infected cells over BSR-T7/5 cells to drive minigenome expression.

In is interesting to note that the first rescue of a bunyavirus (i.e. Bunyamwera virus (BUNV)) from cDNA was performed using another poxvirus, namely vaccinia virus (VV) as a source of T7 polymerase (i.e. vTF7-3) [39]. The protocol for BUNV rescue was later adapted to using BSR-T7/5 cells, which circumvented the need to remove the VV from the culture medium after bunyavirus rescue [38]. In our studies, we preferred FPV over VV to provide T7 polymerase since the former is not only much less cytopathic, but also does not replicate in

mammalian cells [28]. There is thus no FPV to be removed from the culture medium after rescue of a bunyavirus.

Although production of RRPs using the initially established three-plasmid system is highly reproducible, yields of RRPs using this system never exceeded 10^5 $TCID_{50}$/ml. We therefore aimed to develop a system that allows for the constitutive production of higher titers of RRPs. Stable cell lines were created that constitutively produce the structural glycoproteins Gn and Gc, but the levels of expression were too low for efficient trans-complementation. However, it was remarkable to find that repeated passage of these cells when containing replicating L and S-eGFP genome segments did not result in cytopathic effect or loss of reporter gene expression. Reporter gene expression in the resulting BHK-Rep cells was maintained for at least 100 cell passages. Importantly, when the RVFV genome segments were introduced in wildtype BHK cells, expression of the reporter was quickly lost upon passage of the cells. Analysis of the culture medium of BHK-Rep cells was occasionally found to contain low titers of RRPs, varying from undetectable levels to 100 $TCID_{50}$/ml. From this, we first hypothesized that the low amounts of Gn and Gc endogenously produced by the BHK-Rep cells ensures that cells that lose the genome segments are re-infected with RRPs, thereby re-introducing these segments. This hypothesis was however rejected, since passage of the BHK-Rep cells in the presence of a neutralizing serum did not result in a decrease in the number of eGFP-positive cells (data not shown). It therefore remains unclear at this point how BHK-Rep cells efficiently maintain the RVFV genome segments.

By establishing the BHK-Rep cells, a one-plasmid system for the production of RRPs was provided that can reproducibly yield titers of up to 10^7 $TCID_{50}$/ml even after 100 cell passages. We also demonstrate that the one-plasmid system is not restricted to using BHK-Rep cells. When combining an infection with RRPs with a transfection of the plasmid encoding the structural glycoproteins, any mammalian or insect cell can potentially be used for the production of RRPs and the use of FP-T7 and the transcription plasmids is no longer required. To underscore this notion, we show that HEK293T cells can be used for the efficient production of RRPs.

The VNT is the gold-standard diagnostic assay for the serologic confirmation of RVFV infection and the prescribed test for animal trade [40]. The classical VNT requires handling of live RVFV and must therefore be performed in appropriate biosafety containment facilities. Another drawback of the classical VNT is that the assay requires 5-7 days for completion. We show that eGFP-expressing RRPs can be used for VNTs that do not require biocontainment facilities and that this

so-called RRP-VNT requires only 24-48 hrs for completion. Apart from the RRPs developed in the current work, attenuated viruses containing the eGFP gene can potentially be similarly applied.

Finally, we demonstrate that RRPs can be used as a highly effective vaccine. We and others have previously reported efficient VLP production systems and VLPs comprising only the Gn and Gc proteins are highly effective vaccine candidates [33, 34, 41-44]. Mandell *et al*. demonstrated that including the N protein in VLPs improves vaccine efficacy [34], which could be due to a stabilization of the particle, and/or by partial protective efficacy mediated by the N protein alone [45]. It was subsequently reported that so-called infectious VLPs capable of primary transcription of the N gene are remarkably effective, providing complete protection in mice after a single vaccination [44]. The RRPs produced in the current work could be even more efficacious, since the particles are capable of autonomous genome replication and high-level gene expression. Replication of the RRP genome *in vivo* could induce interferon and other innate immune responses and *in vivo* production of the L and N proteins could also induce adaptive cellular immune responses. Although additional studies are required to characterize in detail the innate and adaptive immune responses elicited by RRPs, we already demonstrate here that a single intramuscular vaccination with RRPs protects mice against a lethal challenge dose. Experiments are planned to study the immune response elicited by RRP vaccination in detail and to study the vaccine efficacy of RRPs in sheep.

Apart from applied research, our methods will also facilitate studies on RVFV genome replication and packaging. In this respect, it is interesting to note that the current work already demonstrates that the M genome segment is not required for packaging of the L segment, contrasting a recent suggestion [23]. Our system is also particularly useful for functional studies on the structural glycoproteins Gn and Gc. Mutations can be easily introduced in the pCAGGS-M plasmid and their effects on host cell attachment, entry and fusion can be studied independently. Since the system does not depend on live virus, even the nature of lethal mutations can be studied. An alternative system that can be used for such studies is the previously described system to produce iVLPs [22, 44, 46]. Reporter gene expression in iVLP-infected cells however requires co-transfection with helper plasmids, which likely renders this system more prone to experimental variation than the one-plasmid system described here.

In summary, the methods reported here allow both fundamental and applied research on RVFV to be conducted outside biocontainment facilities and will thereby greatly facilitate the development of novel therapeutics and vaccines for

the control of RVFV. A newly identified member of the phlebovirus genus causing severe fever with thrombocytopenia syndrome (SFTS) in humans recently emerged in China [47] and also members of other genera of the bunyavirus family such as CCHFV and HTNV continue to be of serious public health concern. The methods described here could facilitate the future control of these highly pathogenic bunyavirus as well.

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3

A single vaccination with an improved nonspreading Rift Valley fever virus vaccine provides sterile immunity in lambs

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Abstract

Rift Valley fever virus (RVFV) is an important pathogen that affects ruminants and humans. Recently we developed a vaccine based on nonspreading RVFV (NSR) and showed that a single vaccination with this vaccine protects lambs from viremia and clinical signs. However, low levels of viral RNA were detected in the blood of lambs shortly after experimental infection. These low levels of virus, when present in a pregnant ewe, could potentially infect the highly susceptible fetus. We therefore aimed to further improve the efficacy of the NSR vaccine. Here we report the expression of Gn, the major immunogenic protein of the virus, from the NSR genome. The resulting NSR-Gn vaccine was shown to elicit superior CD8 and CD4-restricted memory responses and improved virus neutralization titers in mice. A dose titration study in lambs revealed that the highest vaccination dose of $10^{6.3}$ $TCID_{50}$/ml protected all lambs from clinical signs and viremia. The lambs developed neutralizing antibodies within three weeks after vaccination and no anamnestic responses were observed following challenge. The combined results suggest that sterile immunity was achieved by a single vaccination with the NSR-Gn vaccine.

Introduction

Rift Valley fever virus (RVFV) is a mosquito-transmitted pathogen that infects domesticated ruminants as well as humans. The virus circulates on the African continent and has made several incursions in countries outside the African mainland [1-5]. In ruminants, RVFV causes massive abortion storms and high mortality among young animals [6-8]. In humans the infection manifests mainly as a self-limiting febrile disease with abrupt onset and symptoms, including headache, nausea, malaise, myalgia, arthralgia as well as gastro-intestinal disorders. In some cases complications are developed, which include retinal damage, jaundice, neurological disease and hemorrhagic fever [9, 10]. The case fatality rate in humans is historically reported to be between 0.5 and 2% [11].

RVFV belongs to the *Phlebovirus* genus of the family *Bunyaviridae*. Typical for a bunyavirus family member, RVFV contains a three-segmented negative-strand RNA genome, comprising a large (L), medium (M) and small (S) segment [12]. The L segment encodes the viral RNA-dependent RNA-polymerase. The S segment encodes the nucleocapsid (N) protein and a non-structural protein named NSs. The polymerase and the nucleocapsid protein together form the replication machinery of the virus. NSs was identified as the major virulence factor and functions as an antagonist of host innate immunity [13-17]. The M

segment encodes the two structural glycoproteins Gn and Gc as well as two accessory proteins [18]. The first is a 14-kDa nonstructural protein known as NSm, which was shown to have an anti-apoptotic function [19]. The second is a protein of 78-kD of which the function is yet unresolved.

RVFV can be transmitted by an impressive variety of mosquito species, which are globally prevalent. This explains the fear for future RVF outbreaks in currently unaffected areas, such as Europe and the USA, where both large populations of susceptible livestock and vector species are present. The risks of introduction and subsequent spread of the virus are furthermore increasing by continued globalization and climate change, respectively [20-24]. Only recently, a new virus of the *Bunyaviridae* family, the Schmallenberg virus (SBV, genus *Orthobunyavirus*), emerged and spread within a few months accross Europe [25]. SBV was able to spread so rapidly due to the abundance of competent *Culicoïdes* midges and large numbers of immunologically naïve target animals. This incursion, as well as that of the bluetongue virus in Northern Europe in 2006, exemplifies how quickly exotic arboviruses can adapt to new environments. The high impact of RVFV on both animal and human health underlines the need for a safe and effective vaccine that can be used to control RVFV not only in areas of endemicity, but also in currently unaffected areas.

Several vaccines are available for veterinary use in South Africa and some other countries on the African continent. A live-attenuated vaccine, the so-called Smithburn vaccine, elicits solid immunity but is not safe for pregnant animals [26, 27]. An inactivated whole-virus vaccine, on the other hand, is safe to use during all physiological stages, but is expensive to produce and requires booster administrations for optimal protection [28]. Recently, a third vaccine was registered for use in South Africa, named Clone 13. This vaccine was shown to be efficacious and safe in animal studies involving sheep [29] and cattle [30]. However, data on its safety and efficacy in the field have yet to be reported.

An alternative strategy to develop a live-attenuated vaccine resulted in the mutagen-attenuated MP-12 strain [31, 32]. Although effective in livestock [33-36], concerns remain about its residual virulence [37, 38]. To attenuate the MP-12 vaccine virus further, derivatives were created that contain deletions in the NSm-coding region and/or the NSs gene. The MP-12ΔNSs was found to be poorly immunogenic, whereas the MP-12ΔNSm vaccine showed promise in sheep [38] and cattle [39]. A similar approach was used to develop a ΔNSs/ΔNSm vaccine virus based on the virulent ZH501 human isolate, which appears to be safe and protective in pregnant ewes [40].

Alternative vaccine development strategies that focus on optimal safety have resulted in the development of experimental subunit vaccines, DNA vaccines and vector vaccines, most of which await further evaluation in the target species. These candidate vaccines are described in several comprehensive reviews [11, 41-44].

To create a vaccine that optimally combines the efficacy of live vaccines with the safety of inactivated vaccines, we and others previously reported the creation of RVFV replicon particles. These particles contain two of the three viral genome segments, L and S, where the NSs gene of the S segment is exchanged for the gene encoding enhanced green fluorescent protein (eGFP) [45, 46]. A single vaccination with the resulting nonspreading RVFV (NSR) vaccine was shown to induce neutralizing antibodies and to protect lambs from viremia and clinical signs [47]. However, minor amounts of viral RNA were detected in the blood of the lambs shortly after experimental infection. We recently found that RVFV can be transmitted vertically in ewes without detection of maternal viremia by our most sensitive qRT-PCR [48]. Although viral RNA was not detected in the blood of these ewes, it was detected in different organs of both ewes and fetuses. This finding suggests that sterile immunity is needed to prevent vertical transmission of the virus. To develop a vaccine that induces sterile immunity after a single vaccination, we decided to improve our NSR vaccine by expressing the major immunogenic protein, Gn, from the NSR small genome segment, resulting in NSR-Gn. In the current study, we demonstrate that expression of Gn from the NSR genome improves Gn-specific humoral and cellular immune responses in mice. A subsequent dose-titration experiment in lambs demonstrated that sterile immunity can be achieved by a single vaccination with the NSR-Gn vaccine.

Materials and methods

Ethics statement

All animal experiments were conducted in accordance with the Dutch Law on Animal Experiments (Wod, ID number BWBR0003081) and approved by the Animal Ethics Committee of the Central Veterinary Institute of the Wageningen UR (Permit Numbers: 2012148, 2012108). All manipulations with the animals were limited to the possible minimum. For the challenged lambs, a human end point (HEP) was defined as the state when animals are not able to get up on their own, even after slight stimulation. If lambs reached HEP, they were humanly euthanized.

Preparation of the challenge virus

A recombinant RVFV was used as challenge virus. The virus was produced from cDNA as described previously [45] with sequences derived from strain 35/74 [49], thereby generating rec35/74. The titer was determined as 50% tissue culture infective dose ($TCID_{50}$) on baby hamster kidney (BHK) cells, using the Spearman-Kärber algorithm. The virus was handled under biosafety level-3 laboratory conditions in class-III biosafety cabinets.

Cells and growth conditions

BHK cells were grown in Glasgow minimal essential medium (GMEM; Invitrogen, CA, USA), supplemented with 4% tryptose phosphate broth (Invitrogen), 1% minimum essential medium nonessential amino acids (MEM NEAA, Invitrogen), 1% Penicillin-Streptomycin (Invitrogen) and 5% fetal bovine serum (FCS; Bodinco, The Netherlands). BHK-GnGc cells [45] and derivatives thereof were grown in the above described medium supplemented with 10% FCS and 1 mg/ml Geneticin (G-418; Promega, USA). For clarity, complete medium supplemented with either 5 or 10% FCS is here referred to as GMEM(5) and GMEM(10), respectively. Transfections were performed in Opti-MEM® (GlutaMAX™; Invitrogen), supplemented with 0.2% FCS.

Plasmids

The plasmid encoding the complete L genome segment and the plasmid encoding the S genome segment in which the NSs gene is replaced by the eGFP gene (pUC57-L and pUC57-S-eGFP, respectively) were described previously [45]. In the current work, the eGFP gene of the pUC57-S-eGFP plasmid was replaced for the Gn gene, starting at the fourth methionine of the M segment open reading frame and ending at the sequence "PIPRHAPIPR". The nucleotide sequence of the Gn gene was codon optimized for expression in human cells. Plasmid pCAGGS-M encodes the complete glycoprotein precursor of RVFV under control of the CMV immediate enhancer/β-actin (CAG) promoter [50].

Flow cytometry

Cells were washed with PBS and permeabilized and fixed with Cytofix/Cytoperm solution (BD biosciences, NJ, USA). A monoclonal antibody (mAb) specific for the N protein was used as primary antibody and FITC-conjugated anti-mouse IgG was used as the secondary antibody (Santa Cruz Biotechnologies, USA). Measurements were performed using a CyAn ADP flow cytometer (Beckman & Coulter, USA) and data analysis was performed with Kaluza software version 1.2 (Beckman & Coulter).

Immunofluorescence

BHK cells were infected with NSR-Gn at a multiplicity of infection (MOI) of 0.5 in 24-well plates. Control BHK cells and Rep-Gn cells were seeded as well. After 48 hours of incubation at 37°C and 5% CO_2, cell monolayers were washed with PBS Ca/Mg and fixed with 4% (w/v) paraformaldehyde for 15 min. The cells were washed three times with PBS and permeabilized, when required, with 1% Triton for 5 min. Cells were washed three times with washing buffer (PBS, 0.05% v/v Tween-20) followed by a 30 min incubation in washing buffer containing 5% FCS. Cells were incubated with mAb 4-39-cc, which specifically recognizes Gn [51], for 1 h at 37°C. The cells were subsequently washed three times with washing buffer and incubated with Texas Red-conjugated anti-mouse IgG2b (Beckman & Coulter). Cell nuclei were stained with DAPI staining (Invitrogen), according to the manufacturers' instructions. Images were taken with an AMG EVOSfl fluorescent microscope.

Preparation of the vaccine

To produce replicon particles containing the S genome segment encoding either eGFP (S-eGFP) or Gn (S-Gn), cells of the corresponding replicon cell lines were seeded in T150 cell culture flasks at a density of $7x10^6$ cells/flask in GMEM(10). Medium was exchanged the next day for 18 ml Opti-MEM and the cells were transfected with a mixture of 14 μg of the pCAGGS-M plasmid DNA and 40 μl JetPEI transfection reagent (Polyplus-transfection SA, France) in 2000 μl saline, following the manufacturers' instructions. Culture medium was harvested the day after transfection and cleared from cell debris by centrifugation at 4500 x*g* for 15 min. When required, replicon particles were concentrated by ultra-centrifugation at 64 000 *xg* for 2.5 h and resuspended in GMEM(5). NSR-Gn replicon particles to be used for the vaccination of sheep were diluted in Opti-MEM, yielding a high dose ($10^{6.3}$ $TCID_{50}$/ml), a medium dose ($10^{4.6}$ $TCID_{50}$/ml) and a low dose ($10^{4.0}$ $TCID_{50}$/ml). The indicated NSR-Gn titers were determined after vaccination.

Vaccination of mice

Six week old female BALB/cAnCrl mice (Charles River Laboratories) were housed in two groups of 6 animals and one group of 4 animals and were kept in type III filter top cages under BSL-3 conditions. Mice were allowed to acclimatize for 6 days, after which the groups of 6 animals were vaccinated with a titer of $5x10^5$ $TCID_{50}$ of either the NSR vaccine or the NSR-Gn vaccine. The group of four mice was mock vaccinated with GMEM(5). Vaccines were administered in a volume of 50 μl by injection into the thigh muscle using a 25 gauge, 16 mm needle. The

mice were observed daily and no signs of illness were recorded. Blood samples were obtained on -1, 13, 22 and 29 days post vaccination (DPV). On DPV 29, the mice were euthanized by cervical dislocation and spleens were collected.

Enzyme-linked immunospot (ELISPOT) assay

A mouse interferon (IFN)-γ antibody pair, consisting of a capture and a detection antibody (Becton Dickinson, USA) was used to determine the number of IFN-γ secreting spleen cells. MultiScreen$_{HTS}$ (Millipore, USA) plates were coated overnight with the capture antibody at 4°C. Unbound antibody was removed by washing with PBS. The plates were then blocked with cRPMI (RPMI 1640, Invitrogen, supplemented with 10% FCS, 1% L-glutamine, 0.1% BME and 1% penicillin/streptomycin) for 30 min at room temperature (RT). Spleens were collected from mice immediately after euthanization and spleen cells were isolated as described [52]. Briefly, spleens were gently crushed in 70 μm cell strainer (BD Bioscience) and the cells were collected by washing the strainer with cRPMI. The red blood cells were depleted by incubation of the splenocytes in 5 ml ACK cell lysis buffer (Invitrogen) for 5 minutes. Cells were washed with cRPMI and the cell counts were determined with a Scepter cell counter (Millipore), supplied with 40 μm sensor. The splenocytes were seeded at a density of $5x10^5$ cells/well in cRPMI in triplicate and stimulated overnight at 37°C and 5% CO_2 with a peptide derived from the Gn protein: SYAHHRTLL [53], a peptide derived from GFP: HYLSTQSAL [54] or a peptide derived from the nucleoprotein of influenza virus: TYQRTRALV [55]. Peptides were synthesized by the Genscript Corporation (USA) and used at a concentration of 5 μg/ml. Additionally, the ectodomain of the Gn glycoprotein [56] was used for stimulation at a concentration of 10 μg/ml. Concanavalin A (1 μg/ml) was used as a positive control and cRPMI as a negative control. After 10 h of stimulation, the cells were removed with chilled water and the plates were extensively washed with washing buffer (PBS, 0.05% v/v Tween-20). Detection of spots was performed by incubation of the plates with biotinylated detection antibodies for 2 h at room temperature, followed by 3 washing steps and an incubation with alkaline phosphatase (ALP)-conjugated streptavidin (Mabtech, Sweden) for 1 h at RT. Plates were washed 4 times with washing buffer and 3 times with PBS to remove residual Tween-20. Spots were developed with BCIP/NBT chromagen (Thermo scientific, USA) for 20 min. Plates were dried in a dry incubator at 37°C. Spots were counted with a CTL ImmunoSpot apparatus. The number of specific spots was determined as the average of three repetitions from each sample after the average number of the respective negative controls was subtracted.

Virus neutralization test with mouse sera

Virus neutralization tests (VNT) with mouse sera were performed as previously described [45], with some modifications. Briefly, sera were diluted 1:10 in a 96-well plate and then serially diluted (2-fold) in a volume of 25 µl in GMEM(5). Another 25 µl of GMEM(5), containing ~50 NSR particles was added to each serum dilution and incubated for 1.5 h at 37°C. Next, 30, 000 BHK cells in 50 µl of GMEM(5) were added to each well. After 36-48 hours, the NSR neutralization was determined and titers were calculated using the Spearman-Kärber method [57, 58]. All samples were tested in triplicate.

Vaccination and challenge of lambs

Texel crossbreed lambs were divided into four groups of eight animals. At 9-10 weeks of age, lambs were vaccinated via the intramuscular route (right thigh) with a high-dose (HD, $10^{6.3}$ $TCID_{50}$/ml), medium dose (MD, $10^{4.6}$ $TCID_{50}$/ml) or low dose (LD, $10^{4.0}$ $TCID_{50}$/ml) of the NSR-Gn vaccine in a 1 ml volume or mock-vaccinated with Opti-MEM. Three weeks after vaccination, all lambs were challenged via the intravenous route with 10^5 $TCID_{50}$ of RVFV rec35/74. Animals were sedated before intravenous inoculation (jugular vein) by intramuscular administration of medetomidine (40µg/kg medetomidine hydrochloride, Sedator®, Eurovet, The Netherlands). For vaccination and challenge, a 18 gauge, 25 mm needles were used. Rectal temperatures were determined daily from the day of arrival until the day of euthanasia. Fever was defined as a rectal temperature above 40.5°C. This threshold was determined as the average plus three times the standard deviation of the rectal temperatures of all animals, measured from -7 to -1 DPC. EDTA blood samples were obtained daily during the first week following challenge and subsequently on 9, 11, 14 and 21 days post challenge (DPC). Serum samples were collected on -21, -14, -7, 0, 7, 14 and 21 DPC. The surviving animals were euthanized three weeks after the challenge by exsanguination, after being anesthetized with 50 mg/kg sodium pentobarbital (Euthasol®, ASTfarma BV, The Netherlands), applied IV. Plasma samples, isolated from the EDTA blood, were analyzed with quantitative real-time PCR as described previously [47]. Virus isolation was performed as previously described [47]. Sera were analysed for the presence of anti-N antibodies with a commercial competition ELISA (ID-VET, France) according to the manufacturers' instructions. Virus neutralization titers were determined by a virus neutralization test (VNT) as described [59] .

Statistical analysis.

Data from the ELISPOT assay and the virus neutralization assay of the mouse experiment were analyzed with the Mann-Whitney non-parametric test, using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Statistical differences with *p*-values<0.05 were considered significant.

Results

Creation of a replicon cell line expressing Gn

To produce replicon particles that express the Gn glycoprotein from the S genome segment, the NSs gene was replaced for a codon-optimized Gn gene (Fig. 1A). A cell line that constitutively maintains the resulting S-Gn genome segment together with the L genome segment was created essentially as described previously [45]. Briefly, BHK-GnGc cells were infected with fowlpox virus expressing T7 polymerase and subsequently transfected with the pUC57 plasmids encoding the L and S-Gn genome segments, together with plasmid pCAGGS-M, which encodes the glycoprotein precursor. Cells were repeatedly passaged and transfected after every cell passage with pCAGGS-M, until more than 98% of the cells became positive for N protein expression, as determined by flow cytometry. The resulting cell line was named Rep-Gn.

The expression and localization of the Gn protein in the Rep-Gn cells was analyzed by immunofluorescence. Consistent with previous reports [60, 61], in permeabilized cells, Gn was mainly distributed perinuclearly, corresponding to Golgi localization (Fig. 1B). In cells with intact cell membranes, Gn was detected at the cell surface (Fig. 1E). Transfection of the Rep-Gn cells with the pCAGGS-M glycoprotein expression plasmid resulted in the production of replicon particles that contain the L and S-Gn segment. The yield of the NSR-Gn particles was determined at 10^7 $TCID_{50}$/ml. These particles were used to infect BHK cells and the synthesis of the Gn protein was analyzed by immunofluorescence. The *de novo* synthesized protein showed a similar distribution as observed in the Rep-Gn cell line (Fig. 1C and F).

Immune responses elicited by NSR and NSR-Gn vaccination of mice

Groups of six mice were vaccinated once via the intramuscular route with either NSR or NSR-Gn, and four mice were mock-vaccinated. Sera were collected the day before vaccination and on DPV 13, 22 and 29. Virus neutralization tests (VNT) revealed the presence of neutralizing antibodies in the sera of the NSR-

Gn-vaccinated animals on DPV 13, and the titers increased until DPV 22, after which they remained unchanged until 29 DPV (Fig. 2A). The VNT titers of the NSR vaccinated group were marginal. No titers were detected in the mock vaccinated group (data not shown).

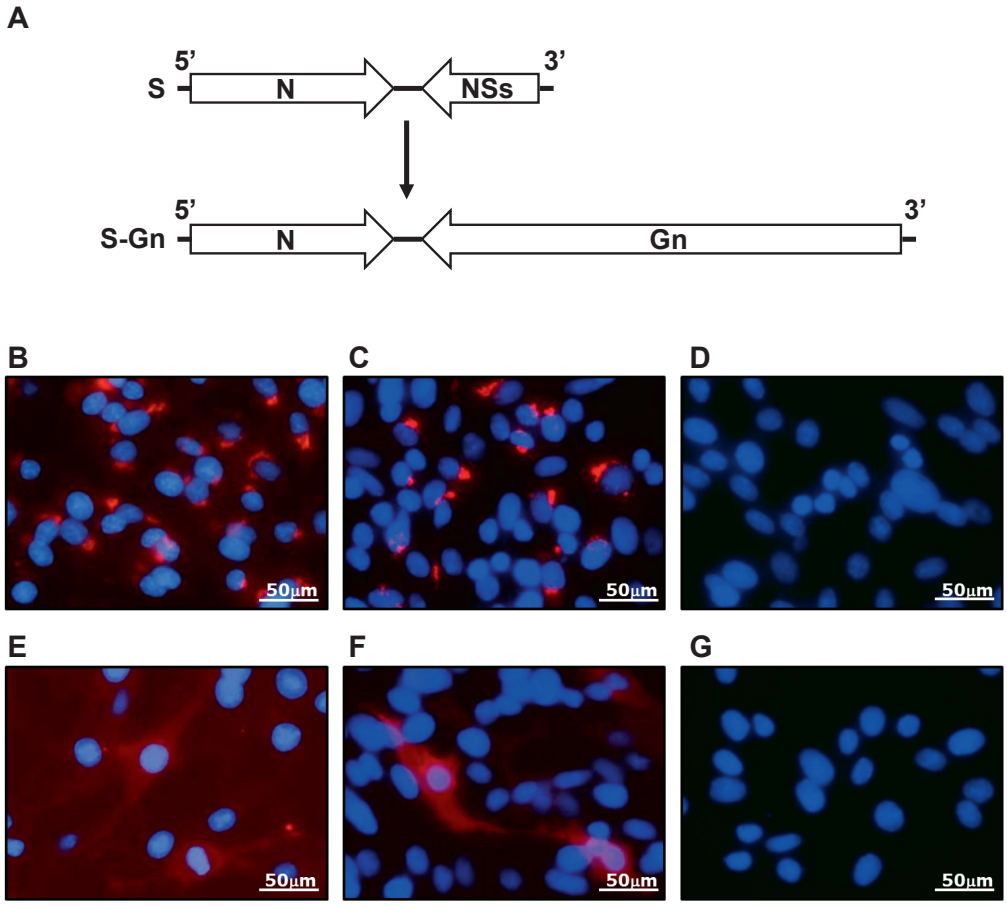

Figure 1. Construction of the S-Gn segment and expression of Gn. (A) Schematic representation of the S segment of RVFV (upper panel) and the S segment in which the NSs gene is replaced for the codon-optimized Gn gene (lower panel). Distribution of Gn in Rep-Gn cells (B and E) and in BHK cells infected with NSR-Gn at an MOI of 0.5 (C and F). Panels D and G represent BHK control cells. Upper panels represent permeabilized cells and lower panels represent nonpermeabilized cells. The cells were stained with an anti-Gn monoclonal antibody and a Texas Red-labeled secondary antibody. Nuclei were visualized by DAPI staining.

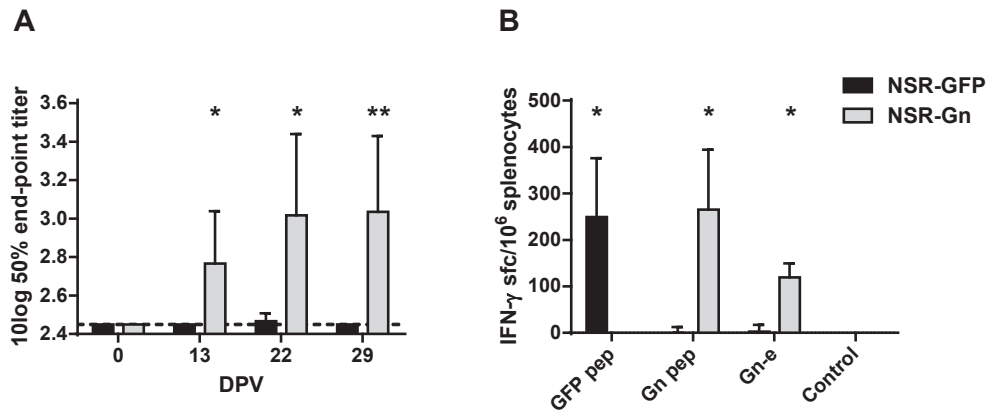

Figure 2. Humoral and cellular immune responses elicited by vaccination of mice with NSR or NSR-Gn. (A) VNT titers in sera collected from mice vaccinated with NSR or NSR-Gn before vaccination (DPV -1) and 13, 22 and 29 DPV. Bars represent average titers (n=6) of each group with standard deviation. The detection limit of the assay is depicted by the interrupted line. (B) Detection of IFN-γ-producing splenocytes isolated from mice vaccinated with NSR or NSR-Gn. Splenocytes were isolated and seeded at a density of $5x10^5$ cells/well in triplicate and stimulated for 12 hours with the indicated peptides or the ectodomain of Gn. Bars represent an average number of IFN-γ producing cells (n=4) per group with standard deviation. The non-parametric Mann-Whitney test was used for statistical analysis and statistical significance between the groups is depicted by asterisks (*p<0.05; **p<0.01).

Spleens were collected on DPV 29 from four mice of each group and from three control mice. Splenocytes were isolated and stimulated with peptides representing previously reported CD8-restricted epitopes of the GFP protein or the RVFV Gn protein. An unrelated peptide derived from the nucleoprotein of influenza virus was used as a negative control. Additionally, the ectodomain of the Gn protein was used for cell stimulation to estimate predominantly the Gn-specific CD4 response. The GFP peptide induced a clear response in splenocytes from all four NSR-vaccinated mice. Analyses with the Gn peptide or the Gn ectodomain revealed significantly higher numbers of IFN-γ producing cells in NSR-Gn-vaccinated mice than in those vaccinated with NSR. The control peptide did not induce a measurable response in splenocytes of any of the mice (Fig. 2B). None of the stimulating agents induced IFN-γ secretion in the spleen cells of the mock vaccinated mice (data not shown).

Sheep dose-titration study

After establishing that expression of Gn from the small genome segment of NSR particles contributes to both cellular and humoral immunity, we proceeded with a sheep study to determine the minimum protective dose. Groups of 8 lambs were vaccinated with either 10^4 $TCID_{50}$/ml (low dose), $10^{4.6}$ $TCID_{50}$/ml (medium dose) or $10^{6.3}$ $TCID_{50}$/ml (high dose) of NSR-Gn. One group of 8 lambs was

included as a challenge control (mock) group. All lambs were challenged at 21 DPV. Consistent with our previous experiments, mock-vaccinated lambs developed fever within 2 days after challenge, which lasted on average for four days. In four lambs of the mock group the fever was multiphasic (Fig. 3). One lamb in this group, C3, died 7 days after challenge. Post mortem examination revealed findings typical for RVF and pneumonia. Two lambs of the low-dose group and two lambs in the medium-dose group developed fever. When fever occurred in vaccinated animals, it was delayed with one day and no secondary peaks were observed. None of the lambs from the high-dose group developed fever. One of the lambs in this group died from esophagus obstruction two days after the vaccination, therefore data from only seven animals are shown.

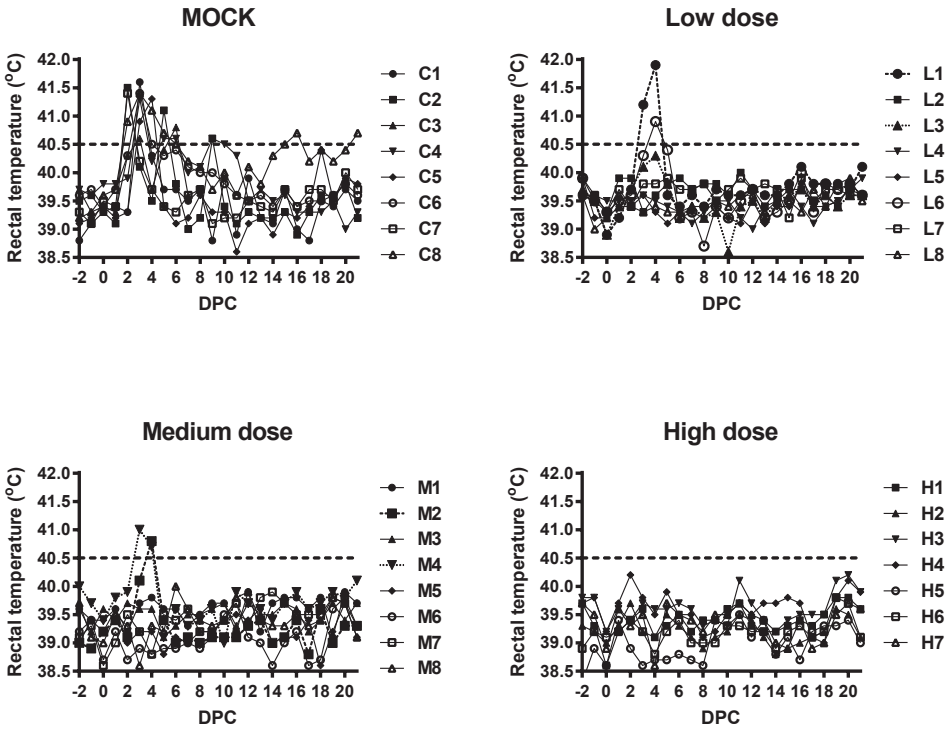

Figure 3. Rectal temperatures of vaccinated and mock-vaccinated lambs before and after challenge with RVFV. Fever was defined as a rectal body temperature above 40.5ºC (interrupted line). Body temperatures of mock-vaccinated lambs (C1-C8) and lambs vaccinated with a low dose (L1-L8), medium dose (M1-M8) or high dose (H1-H7) of NSR-Gn are depicted individually. Lamb C3 died 7 days after challenge, therefore no data is available after day 7.

All mock-vaccinated lambs developed high viremia, reaching maximum values between 10^6 and $10^{9.8}$ RNA copies/ml plasma as detected by PCR. Viremia lasted for 4 to 7 days (Fig. 4). In the low-dose and medium-dose groups, viremia correlated with fever but was of shorter duration compared to the mock group. No viremia was detected in lambs vaccinated with the high dose of NSR-Gn.

The plasma samples with the highest PCR titers from each animal were subjected to virus isolation. Only samples with PCR titers above $10^{5.5}$ RNA copies/ml were used as previous attempts to isolate virus from samples with lower PCR titers were always unsuccessful. Virus was successfully isolated from the plasma of all mock vaccinated animals and from two (L1, L3) and one (M2) plasma samples of the low-dose and medium-dose vaccination groups, respectively (data not shown).

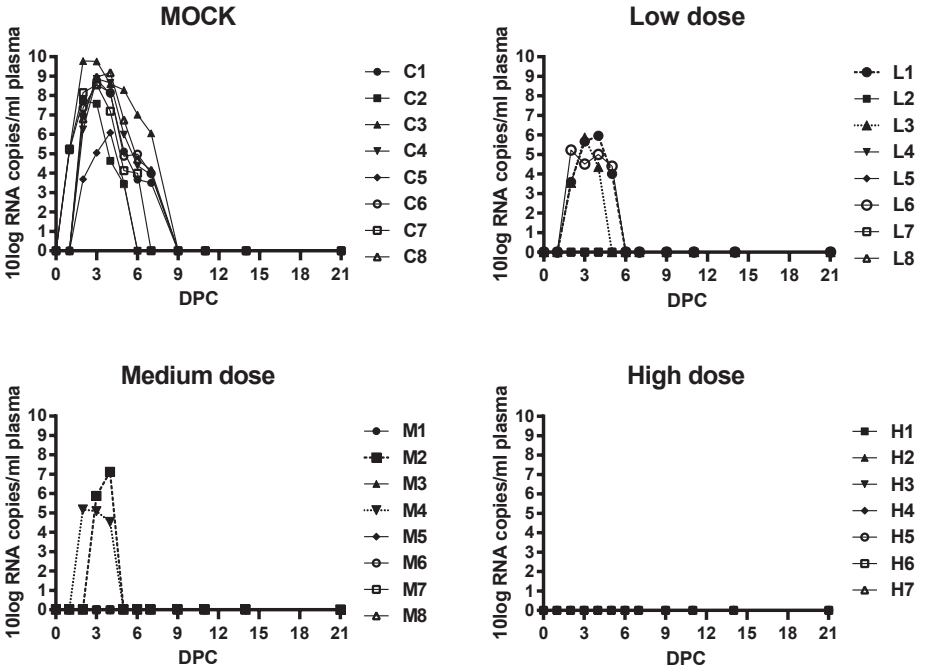

Figure 4. Detection of viral RNA in plasma by qRT-PCR. Plasma samples were collected daily at the first 7 days post challenge (DPC) and subsequently on DPC 9, 11, 14 and 21. Viral RNA copy numbers detected in individual animals of the mock-vaccinated group (C1-C8), low-dose group (L1-L8), medium-dose group (M1-M8) and high-dose group (H1-H7) are depicted. Lamb C3 died 7 days after challenge, therefore no blood samples were available after day 7.

Livers and brains of all animals were tested for the presence of viral RNA with PCR at the end of the experiment. In the mock vaccinated group, viral RNA was found in the livers of three lambs (C3, C4 and C6) and in the brain of one lamb (C8). None of the vaccinated animals was found positive for viral RNA in the organs tested.

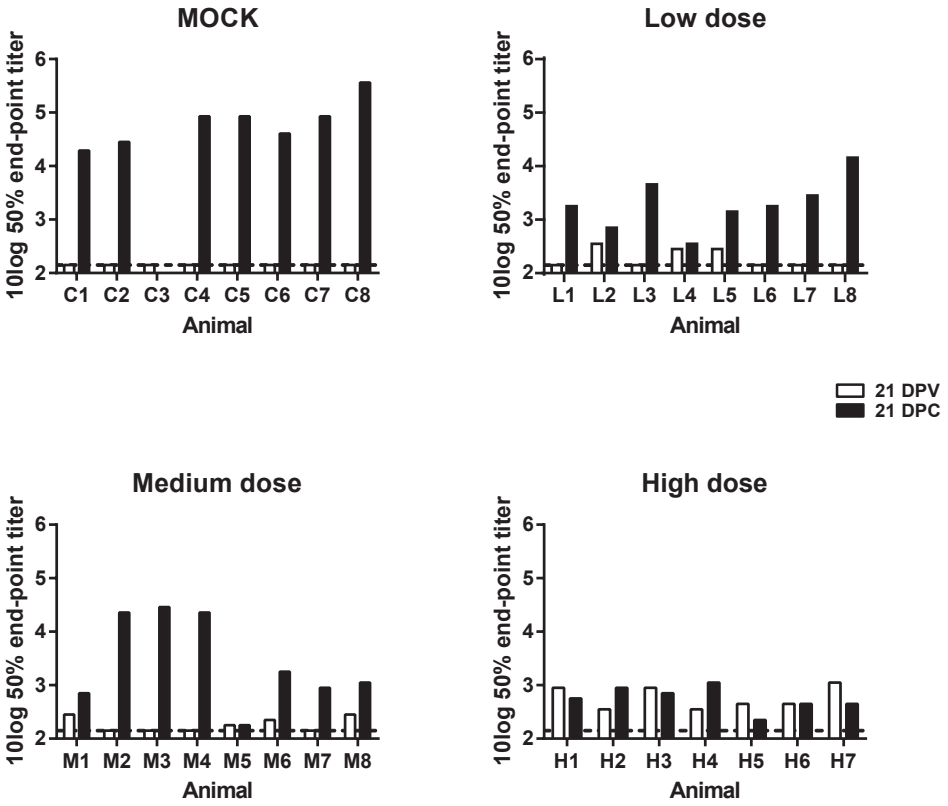

Figure 5. Virus neutralization test (VNT). Sera were obtained from lambs of the mock group, low-dose group, medium-dose group and high-dose group. The white bars represent VNT titers determined 21 days post vaccination (DPV) and the black bars represent the VNT titers determined 21 days post challenge (DPC). Results obtained from analysis of each individual animal from the mock-group (C1-C8), low-dose group (L1-L8), medium-dose group (M1-M8) and high-dose group (H1-H7) are depicted. The detection limit of the assay is represented by an interrupted line. Lamb C3 died 7 days after challenge, therefore no serum samples were collected after day 7.

Antibody responses in sheep

Sera collected from all animals on the day of vaccination, on the day of challenge, and three weeks after challenge were tested for virus neutralizing

activity. Neutralizing antibodies were not detected in any of the sera collected on the day of vaccination (data not shown) and no neutralizing antibodies were detected in the sera of the mock-vaccinated animals collected three weeks after vaccination. Three animals vaccinated with a low dose of NSR-Gn and four animals that received a medium dose developed neutralizing antibodies after vaccination (Fig. 5). Lambs in these groups generally displayed an increase in the neutralizing antibody titers after challenge, being more prominent in animals that did not have neutralizing antibodies before challenge. All animals that received a high dose of NSR-Gn developed neutralizing antibodies and antibody levels were highest in this group before the challenge infection. These neutralizing antibody levels did not increase after challenge. The animals of the mock group developed the highest VNT titers upon challenge.

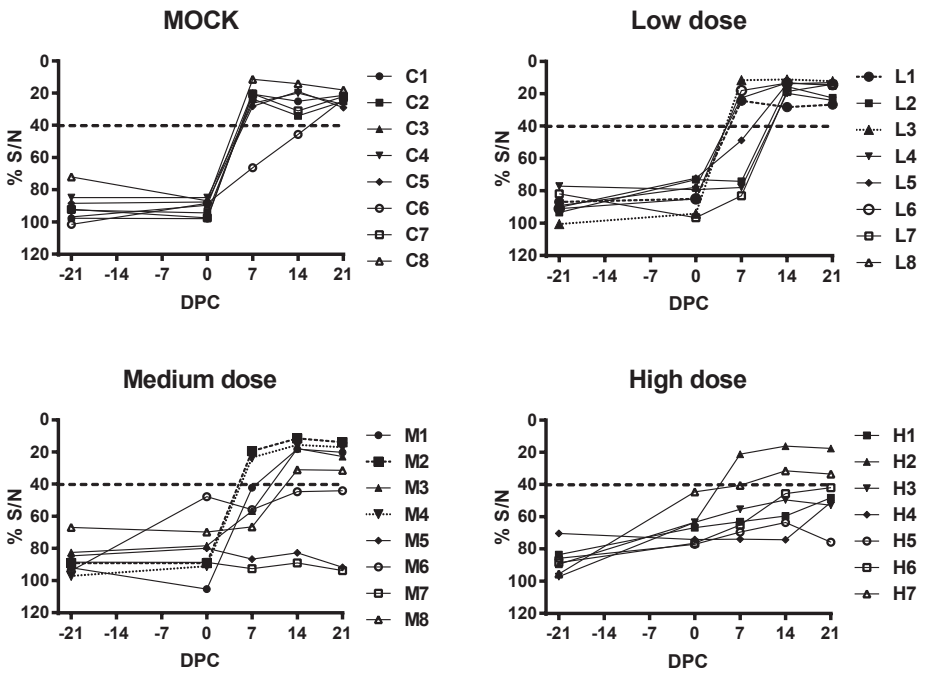

Figure 6. Detection of anti-N antibodies by ELISA. Sera were obtained 7, 14 and 21 days post vaccination (DPV) and at 0, 7, 14 and 21 days post challenge (DPC). Titers are expressed as percentage competition ratio of the optical densities (OD) of the sample and the OD of the negative control (%S/N). All values lower than 40% are considered positive, between 40-50% are considered doubtful and above 50% are considered negative. The 40% and 50% cut-offs are represented by solid and interrupted lines, respectively. Results obtained from analysis of each individual animal from the mock-group (C1-C8), low-dose group (L1-L8), medium-dose group (M1-M8) or high-dose group (H1-H7) are depicted. Lamb C3 died 7 days after challenge, therefore no serum samples were collected after day 7.

In addition to determining the VNT titers, sera were analyzed for the presence of antibodies against the N protein using the commercial ID Screen® Rift Valley fever Competition ELISA kit (ID-Vet, Montpellier, France). Seven of the eight sera from the mock-vaccinated animals were scored positive already on day 7 after challenge and in one animal a delayed increase in the antibody response was observed (Fig. 6). In the low-dose group, four animals developed an anti-N response similar to that observed in challenged mock-vaccinated animals and in four other animals the antibody response was delayed by 7 days (Fig. 6). By DPC 21, all sera were found positive for anti-N antibodies. Five sera of the lambs vaccinated with the medium dose were found positive for anti-N antibodies, one was doubtful and in two animals no anti-N antibodies were detected at 21 DPC. In the high-dose group, two samples were found positive, two were deemed doubtful and three were found negative for the presence of anti-N antibodies at 21 DPC. Although the serum samples from this group were scored negative before the challenge, there was a clear increase in the %S/N values, as compared to the mock group and the low- and medium-dose groups.

Discussion

Vaccines based on replicons can optimally combine the efficacy of live vaccines with the safety of inactivated or subunit vaccines. Vaccination with the recently developed NSR replicon vaccine was shown to reduce challenge virus viremia in lambs to levels undetectable by virus isolation. Low levels of viral RNA were, however, detected in the blood of challenged animals by a highly sensitive qRT-PCR. We recently found that RVFV can be transferred to the fetus in gestating ewes even when viremia is not detectable by qRT-PCR. We therefore aimed to improve our NSR vaccine so that sterile immunity can be achieved. To this end, the Gn glycoprotein, which is the major target for neutralizing antibodies, was introduced in the NSR genome.

Memory T-cell responses were analyzed using spleen cells of mice that were vaccinated with NSR expressing eGFP (NSR) or NSR expressing Gn (NSR-Gn). Stimulation of splenocytes with a GFP-derived peptide resulted in IFN-γ production by spleen cells of the NSR-vaccinated group. As the eGFP protein is not present in the NSR particle, we conclude that the observed response results from eGFP production *in vivo*. Importantly, a Gn-derived peptide stimulated significantly higher numbers of IFN-γ producing splenocytes in cultures obtained from NSR-Gn-vaccinated mice, compared to cultures derived from NSR-

vaccinated mice. Although the Gn protein is incorporated in the NSR particle, expression of the Gn gene from the NSR genome resulted in a superior and sustained memory CD8 response. Soluble ectodomain of Gn (Gne) was also used for splenocyte stimulation. Soluble proteins are internalized by antigen presenting cells by endocytosis and are presented mainly by MHC-II [62, 63]. The observed stimulation by Gne therefore likely represents a $CD4^+$ T-cell response. Once again, splenocytes collected from NSR-Gn-vaccinated animals displayed the strongest response, indicating that the *in vivo* expression of Gn contributes substantially to the immunogenicity of the NSR-Gn vaccine. In addition, the VNT titers of NSR-Gn-vaccinated animals were readily detectable by 13 DPV, while the titers of the NSR group were marginal. This result demonstrates that expression of Gn by NSR-Gn not only promotes priming of IFN-γ secreting T-cells, but also stimulates neutralizing antibody responses. The combined results of this experiment underscore the added value of Gn expression from the NSR genome.

We previously demonstrated that a single vaccination with 10^7 $TCID_{50}$ of NSR protects lambs from viremia and clinical signs, although protection was not sterile [47]. Since we anticipated superior efficacy of the NSR-Gn vaccine, we continued with determining the minimal protective dose of this vaccine in lambs. Vaccination with a low dose ($10^{4.0}$ $TCID_{50}$/ml) prevented detectable viremia in five lambs and strongly reduced viremia in the remaining three animals. No viremia was detected in 6 of 8 lambs that were vaccinated with a medium dose ($10^{4.6}$ $TCID_{50}$/ml), whereas two lambs did develop low-level viremia. It is important to note, however, that neutralizing antibodies were boosted after challenge in these two groups. A sharp increase in the anti-N response was noted in the low-dose group after challenge, similar to that observed in the mock group. The group of lambs vaccinated with a medium dose displayed a more variable pattern, with two animals not developing anti-N antibodies and two others showing only a minor increase in the anti-N responses after challenge. The observed boosts of anti-N antibodies as well as neutralizing antibodies after challenge suggests that virus replication occurred at a low level in the lambs of these two groups. Some lambs of the low-dose and medium-dose groups did not develop detectable levels of neutralizing antibodies after vaccination, but were nevertheless solidly protected from challenge infection. The observed protection in these animals is therefore attributed to T-cell responses. No viral RNA was detected upon challenge in any of the lambs vaccinated with the high dose ($10^{6.3}$ $TCID_{50}$/ml) of NSR-Gn. This result correlates with the absence of an anamnestic antibody response after challenge.

Taken together, these data suggest that challenge virus was not able to replicate in these lambs and that sterile immunity was achieved.

Although a clear dose-response was demonstrated in this study, it is interesting to note that even vaccination with a low dose of NSR-Gn can provide sterile immunity in lambs. After inoculation of NSR-Gn in the muscle, the nonspreading virus can infect not only muscle cells, but also macrophages and dendritic cells. RVFV was recently shown to use dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) as a receptor, which is present on both dendritic cells and macrophages [64]. DCs and macrophages are possibly used by RVFV as "trojan horses" to migrate to secondary sites of replication, such as the lymph nodes. While doing so, the virus must prevent the activation of these immune cells, in which the NSs protein is likely to play a role. Since NSs is lacking from NSR-Gn particles, these particles are probably unable to prevent activation of the macrophages and DCs, thereby facilitating a potent priming of the immune response. We propose that vaccination with a low dose of NSR-Gn can provide sterile immunity if a sufficient number of macrophages and dendritic cells are infected by NSR-Gn. If this hypothesis proves to be correct, it will be interesting to target dermal dendritic cells by intradermal vaccination with the goal to lower the protective dose of the NSR-Gn vaccine.

In conclusion, here we demonstrate that expression of the Gn protein from the NSR genome results in improved cellular and humoral immune responses and that the resulting NSR-Gn vaccine provides sterile immunity in lambs after a single vaccination. Given its high efficacy and safety profile, it would be valuable to evaluate the use of the NSR-Gn vaccine as a human vaccine as well.

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Conflict of interest

J. Kortekaas and R. J. M. Moormann are inventers of WIPO Patent Application WO/2012/039607 "Methods to produce bunyavirus replicon particles".

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4

Comparative efficacy of two next-generation Rift Valley fever vaccines

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Abstract

Rift Valley fever virus (RVFV) is a re-emerging zoonotic bunyavirus of the genus *Phlebovirus*. A natural isolate containing a large attenuating deletion in the small (S) genome segment previously yielded a highly effective vaccine virus, named Clone 13. The deletion in the S segment abrogates expression of the NSs protein, which is the major virulence factor of the virus. To develop a vaccine of even higher safety, a virus named R566 was created by natural laboratory reassortment. The R566 virus combines the S segment of the Clone 13 virus with additional attenuating mutations on the other two genome segments M and L, derived from the previously created MP-12 vaccine virus. To achieve the same objective, a nonspreading RVFV (NSR-Gn) was created by reverse-genetics, which not only lacks the NSs gene but also the complete M genome segment. We have now compared the vaccine efficacies of these two next-generation vaccines and included the Clone 13 vaccine as a control for optimal efficacy. Groups of eight lambs were vaccinated once and challenged three weeks later. All mock-vaccinated lambs developed high fever and viremia and three lambs did not survive the infection. As expected, lambs vaccinated with Clone 13 were protected from viremia and clinical signs. Two lambs vaccinated with R566 developed mild fever after challenge infection, which was associated with low levels of viral RNA in the blood, whereas vaccination with the NSR-Gn vaccine completely prevented viremia and clinical signs.

Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne *Phlebovirus* of the *Bunyaviridae* family that causes recurrent outbreaks among ruminants, particularly sheep. The virus is endemic to the African continent, the Arabian Peninsula and several islands off the eastern coast of southern Africa. Abortion storms and high mortalities among newborn animals are characteristic features of RVFV outbreaks, although losses among adult animals can also be considerable. Transmission of the virus among ruminants occurs via mosquito vectors, whereas humans can be infected via either mosquito bite or via contact with bodily fluids released during the slaughtering of diseased animals. Infections in humans generally manifest as mild, transient disease, but a small percentage of patients develop serious complications, such as ocular impediments, hemorrhagic fever or encephalitis. Considering the zoonotic nature of RVFV, vaccination of livestock will benefit both animal and human health [1].

The first veterinary RVFV vaccine was developed by Smithburn in 1949 [2]. After serial intracerebral passage in mice, a mutant virus was isolated with strongly reduced hepatotropism. Although the resulting Smithburn strain is attenuated, its residual virulence is well recognized [3, 4]. To create a virus with a more attenuated profile, Caplen and co-workers cultivated the virulent RVFV strain ZH548, isolated during the Egyptian outbreak of 1977, in the presence of the mutagen 5-fluorouracil [5]. This procedure yielded the mutagenized MP-12 strain which was derived from the parental virulent strain after 12 successive passages in the presence of the mutagen. Later studies demonstrated that the MP-12 virus contains attenuating mutations in each of the three genome segments [6].

The safety and efficacy of MP-12 were evaluated in several extensive animal trials. These experiments suggested that the vaccine can be safely applied in lambs, calves and ewes after the first trimester of gestation [7-11]. In a recent study, vaccination of ewes during the first trimester of gestation with either MP-12 or a corresponding recombinant virus did not cause untoward effects in the ewes, although one of four ewes of each group was found to carry a dead fetus at the end of the experiment [9]. However, it has remained unclear whether these fetal mortalities were caused by the vaccine viruses. Nevertheless, efforts are continuing to further attenuate the MP-12 virus by reverse-genetics [9, 12].

In 1995, a natural nonpathogenic RVFV isolate was described, named Clone 13, which contains a large (70%) internal deletion in the NSs gene of the small (S) genome segment [13]. Studies on the Clone 13 virus demonstrated that the NSs protein suppresses type-I interferon induction [14, 15], which was later attributed to its interaction with SAP30 of the repressor complex [16]. Subsequent studies demonstrated that the NSs protein is multifunctional by i) acting as a general inhibitor of transcription by sequestering p44 and XPB [17], causing degradation of p62 [18] as well as of the other TFIIH subunits like p52, p34 and cdk7 [17]; ii) promoting the degradation of PKR [19, 20], iii) modifying gene expression to impair the hosts coagulation cascade [21] and iv) interacting with gamma satellite pericentromeric sequences, provoking abnormal nuclei during cell division, a function of NSs possibly responsible for RVFV-mediated teratogenesis [22]. In addition, NSs was shown to be responsible for cell cycle arrest at either G0/G1 or S phase, as well as DNA damage response via the ATM protease [23, 24]. These extensive studies have demonstrated that the NSs protein is the primary virulence factor of the virus and thereby revealed the molecular basis of the avirulent phenotype of Clone 13. The Clone 13 virus was shown to be highly effective and safe in gestating sheep [25] and calves [26]

and was marketed in 2010 by the Onderstepoort Biological Products Company in South Africa.

Despite the high safety profile of the Clone 13 virus, efforts are continuing to create vaccines with an even stronger safety signature. To this end, scientists of Institut Pasteur combined the attenuating mutations on the L and M segments of MP-12 with the S segment of the Clone 13 virus. The resulting reassortant virus, named R566, contains attenuating mutations on each of the three genome segments [27].

In an alternative strategy to develop a vaccine that optimally combines efficacy and safety, a novel vaccine based on a nonspreading RVFV, named NSR-Gn, was recently developed at CVI-WUR [28]. NSR-Gn particles are capable of infecting cells of the vaccinated animal, thereby triggering innate and adaptive immune responsive very similar to those elicited by live-attenuated vaccine viruses. The particles are however optimally safe, since they are incapable of producing progeny virions, due to the absence of the M genome segment. The NSR-Gn vaccine was recently demonstrated to confer sterile immunity in lambs after a single vaccination [28]. In the current study, the vaccine efficacies of R566 and NSR-Gn were compared, using Clone 13 vaccination as a control for optimal efficacy.

Materials and Methods

Ethics statement

All animal experiments were conducted in accordance with the Dutch Law on Animal Experiments (Wod, ID number BWBR0003081) and approved by the Animal Ethics Committee of CVI-Lelystad. To minimize suffering of the animals during our vaccination challenge experiment, lambs were humanely euthanized when they could no longer be stimulated to drink, feed or stand.

Preparation of the vaccines

The Clone 13 [13] and the NSR-Gn [28] vaccines were previously reported in literature. The R566 vaccine virus was created as follows: Vero cells were grown as described [29] and co-infected with MP-12 and Clone 13 at a multiplicity of infection of 1 and 0.1, respectively. Viruses released in the culture medium were collected at 48 h post infection when a complete cytopathic effect was observed as described [30]. The viruses were titrated by plaque assay using the standard method described earlier [31]. Plaques were picked, amplified in Vero cells and

screened for NSs expression by immunostaining with NSs-specific antibodies [17]. The origins of the L, M and S segments were determined by RT-PCR amplification and sequencing as described [30]. Each selected reassortant was sub-cloned three times by plaque assay and the sequences verified at the first and third cycle.

The R566 and Clone 13 vaccine strains were cultured on Vero cells grown in 490 cm^2 roller bottles. The culture medium consisted of a combination of Glasgow's and Eagles modified minimal essential medium (MEM) supplemented with fetal bovine serum and antibiotics. Vaccines were diluted in culture medium to achieve titers appropriate for vaccination.

Preparation of the challenge virus

The molecularly cloned 35/74 (rec35/74) virus was derived from the sequence of strain 35/74 and was titrated on baby hamster kidney (BHK) cells as tissue culture infective dose 50 ($TCID_{50}$) using the Spearman-Kärber algorithm [32, 33]. The virus was handled under biosafety level-3 laboratory conditions in class-III biosafety cabinets.

Vaccination and challenge

Thirty-two lambs of the Romane (INRA 401) breed, 12-14 weeks of age at the moment of vaccination were purchased from Institut National de la Recherche Agronomique (INRA, Nouzilly, France). Lambs were randomized according to gender and age into 4 groups of 8 individuals each and submitted to general health inspection before the start of the study. The Clone 13 vaccine was administered subcutaneously in the right axilla at a dose of 10^5 pfu in a volume of 1 ml. The R566 vaccine was administered subcutaneously in the right axilla with a dose of 10^6 pfu in a volume of 2 ml. The NSR-Gn vaccine was administered via the intramuscular route (neck muscle) at a dose of 10^6 $TCID_{50}$ in a volume of 1 ml. Control lambs were inoculated with 2 ml phosphate buffered saline (PBS, GIBCO™, Carlsbad, CA, USA) subcutaneously in the right axilla and 1 ml of PBS in the neck muscle.

Three weeks after vaccination, all lambs were sedated by intramuscular administration of medetomidine (40 µg/kg medetomidine hydrochloride, Sedator, Eurovet, The Netherlands) and subsequently challenged by administrating 1 ml culture medium containing 10^5 $TCID_{50}$ of molecularly cloned RVFV strain 35/74 in the *vena jugularis*. The rescue of the challenge virus was previously reported [34]. For vaccination and challenge, 18 gauge, 25 mm needles were used. Blood samples for the preparation of sera were collected

starting on day 6 before vaccination (DPV -6), on the day of vaccination (DPV 0) and subsequently every week until the end of the experiment. EDTA blood samples were collected on DPV -6, 0 and subsequently daily from DPV 21 (the day of challenge) to DPV 32, and on DPV 35 and 42. Body weights were determined on DPV -6 and weekly starting on DPV 0. Rectal body temperatures were determined daily. The lambs that survived the challenge were euthanized 3 weeks after challenge (DPV 42) by intravenous administration of 50 mg/kg sodium pentobarbital (Euthasol®, ASTfarma, The Netherlands) and subsequent exsanguination. At necropsy, spleen and liver were collected and stored at -80˚C.

Diagnostic assays

Quantitative real-time PCR (qRT-PCR), virus isolation, virus neutralization tests and ELISAs were performed as described [35]. Briefly, RNA isolated from plasma was used for quantitative Taqman qRT-PCR. RNA was isolated using the QuickGene DNA tissue kit S (DT-S, Fuji Photo Film Europe GmbH, Dusseldorf, Germany) following the manufacturers' instructions with modifications [35]. The LightCycler RNA Amplification Kit HybProbe (Roche, Almere, The Netherlands) was used and primers, probes and cycling conditions were used as described previously [36].

For virus isolation, BHK cells were incubated with plasma samples diluted 1:1 in culture medium with appropriate supplements, including heparin to prevent clotting of the plasma. After 1h incubation, the inocula were replaced with fresh culture medium. After five days, the plates were scored for cytopathic effect.

Antibody responses were analyzed using the commercial ID Screen® Rift Valley Fever Competition ELISA (ID-VET, Montpellier, France), which detects antibodies against the N protein of RVFV.

Virus neutralization tests were performed by incubating ~200 $TCID_{50}$ of challenge virus with two-fold serial dilutions of sera in 96-well plates. After 2.5h incubation at room temperature, 40,000 BHK cells were added per well. After 4 to 5 days incubation at 37°C, the cultures were scored for cytopathic effect. Fifty percent end point titres were calculated using the Spearman–Kärber algorithm.

Immunohistochemistry

Organ samples for histopathology were fixed in 10% phosphate-buffered formalin for a minimum of 48h before routine processing into paraffin. Paraffin blocks were cut into 4 µm sections, collected on aminopropyltriethoxysilane-coated glass slides and dried for at least 48-h in a 37ºC incubator. Sections were

deparaffinized in xylene and rehydrated in graded alcohols while endogenous peroxidase activity was blocked in methanol/H_2O_2. Pretreatment consisted of 15 min autoclaving at 121ºC in citrate buffer (pH 6.0). After cooling down, sections were incubated for 60 minutes with the monoclonal antibody 4-D4, which is directed against the Gn protein of RVFV (kindly provided by Dr. Connie Schmaljohn, USAMRIID) [37]. Mouse Envision horseradish peroxidase (Dakopatts, Denmark) was used as the secondary antibody. Peroxidase activity was detected using a diaminobenzidine substrate.

Results

Creation of R566

Vero cells were co-infected with MP-12 and Clone 13 at a multiplicity of infection of 1 and 0.1, respectively. Viruses released in the culture medium were collected at 48h post infection when a complete cytopathic effect was observed. The viral yield was then plaque purified and a first screening was performed on the basis of the ability or inability to express NSs which was evaluated by immunostaining using NSs-specific antibodies as described [17]. The origins of the L, M and S segments were determined by RT-PCR amplification and sequencing as described [30]. The eight expected genotypes were found, some of them being more frequent than others. Three cycles of plaque purification were performed with reassortants possessing the S segment of Clone 13. Full genome sequencing confirmed that Reassortant 566 (R566) combines the L and M segments of MP-12 with the S segment of Clone 13, and thereby contains attenuating mutations on each genome segment. Virus stocks of R566 were prepared at a low multiplicity of infection 10^{-3} pfu per cell, collected 72 h post infected and stored at -80°C.

Vaccination and challenge

After one week acclimatization, lambs were vaccinated with a dose of 10^6 $TCID_{50}$ of NSR-Gn, 10^6 pfu of R566 or 10^5 pfu of Clone 13. The R566 and Clone 13 vaccines were administered via subcutaneous route, whereas the NSR-Gn vaccine was administered via intramuscular route.

One animal in the control group (animal code: M2) displayed elevated body temperatures from 17 days post vaccination (DPV), and most likely suffered from a pneumonia. None of the other animals displayed elevated body temperatures or clinical signs before challenge infection. Unfortunately, one of the NSR-Gn vaccinated lambs died after administration of anesthetics. Autopsy

of this animal demonstrated pulmonary congestion and edema, suggesting that the animal died of shock.

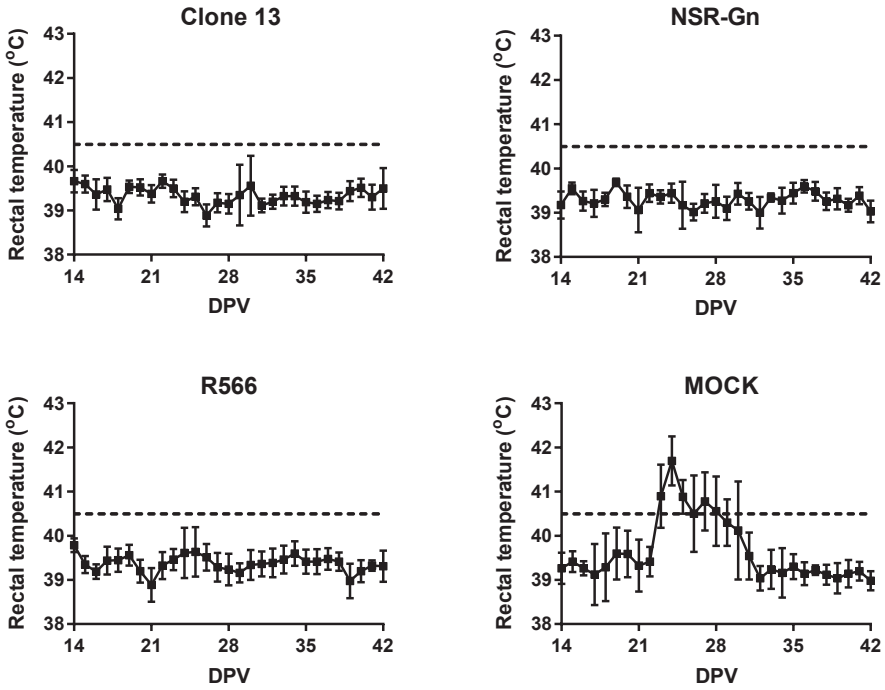

Figure 1. Rectal temperatures of vaccinated and mock-vaccinated (Mock) lambs before and after challenge with RVFV. Rectal body temperatures (ºC) were determined daily. Fever was defined as a body temperature above 40.5ºC (interrupted line). Rectal body temperatures of vaccinated lambs are depicted as averages (*n* = 8) with SD. Rectal body temperatures of NSR-Gn-vaccinated lambs determined after DPV 21 are depicted as averages of 7 determinations. Rectal body temperatures of mock-vaccinated lambs determined after DPV 25 and 27 are depicted as averages of 6 and 5 measurements, respectively, since lambs from this group died on these days.

After challenge infection, all unvaccinated lambs developed high fever, which peaked at two days post challenge (DPC) with an average temperature of almost 42ºC (Fig. 1). Three lambs from this group did not survive the infection. The first lamb to succumb on DPC 3 was lamb M2. This lamb already suffered from an underlying condition, which is believed to have exacerbated the clinical manifestation. Additional fatalities occurred on DPC 4 (M6) and DPC 6 (M7). Post mortem examination of lamb M2 revealed a catarrhal bronchopneumonia involving both apical lobes and part of the middle lobes. The liver was swollen and displayed a patchy pattern of pale and dark areas. Edema and hemorrhages

were observed in the mesenterium at the junction with the intestines. A large volume of hemorrhagic fluid was present in the abdominal cavity. Autopsy of lamb M6 revealed a severely swollen liver with yellowish color and friable consistency. Icterus was also noted. The abdominal cavity contained a large volume of slightly hemorrhagic fluid. Pulmonary edema was also noted. Lamb M7, which died on DPC 6, revealed signs of shock. Pulmonary edema and icterus were noted and the peritoneum, splenic capsule, pleura and heart revealed many petechiae. The liver was severely swollen, yellowish in color and had a friable consistency. Hyperemia and congestion was noted in the small intestines. The large intestines contained hemorrhagic contents.

Vaccinated lambs did not develop fever, with the exception of one lamb that was vaccinated with Clone 13. This lamb (C8) developed fever on DPCs 8 and 9 (data not shown). qRT-PCR analyses did not reveal viral RNA in the plasma of this lamb at these time points, suggesting that fever was not caused by the challenge virus. None of the vaccinated lambs developed any clinical signs during the study period.

To monitor viremia, plasma samples were first analyzed by qRT-PCR and selected plasma samples were used for virus isolation. High levels of viral RNA were detected by qRT-PCR on plasma samples collected from mock-vaccinated animals (Fig. 2). No challenge virus RNA was detected by qRT-PCR in any of the samples collected from lambs vaccinated with either Clone 13 or NSR-Gn. In contrast, low levels of challenge virus RNA were detected in plasma samples from three R566-vaccinated lambs on DPC 1 (R4) and DPC 3 (R1 and R3, Fig. 2) and two of these lambs (R1 and R3) displayed elevated body temperatures (40.4 - 40.5ºC) on DPC 3 and 4 (data not shown).

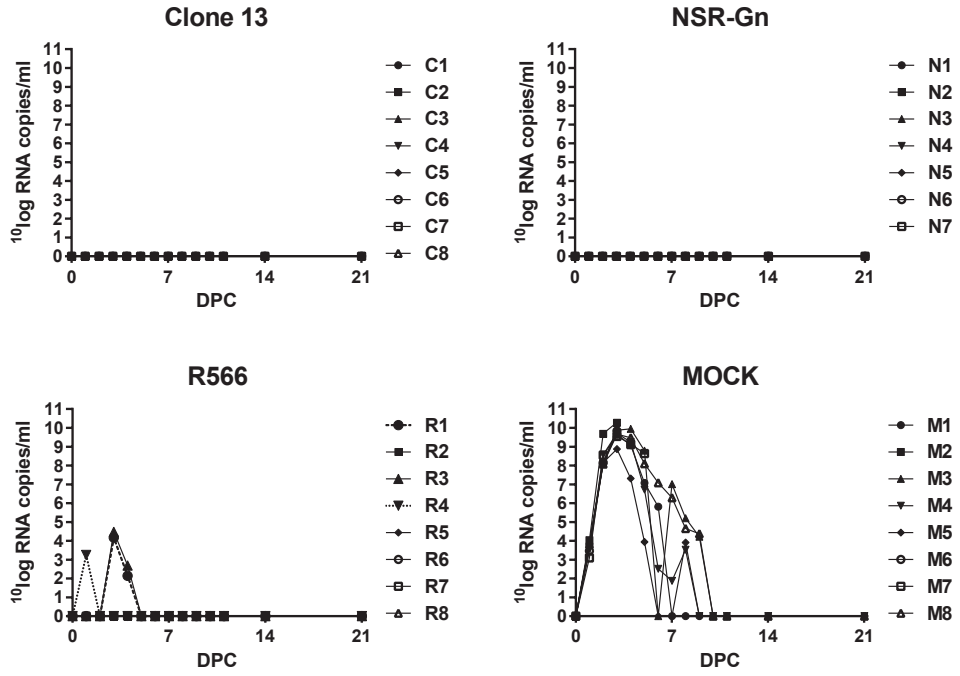

Figure 2. Monitoring of viremia in vaccinated and mock-vaccinated lambs by qRT-PCR. Viral RNA was detected by qRT-PCR in plasma samples obtained at different days post challenge (DPC) with RVFV.

Virus isolations were performed with samples collected at the moments of peak viremia, as determined by qRT-PCR. These analyses demonstrated a 10^3-10^4-fold difference in sensitivity between virus isolation and qRT-PCR (Fig. 3). Virus was readily isolated from plasma samples collected from mock-vaccinated lambs, whereas all plasma samples collected from vaccinated lambs were negative in virus isolation.

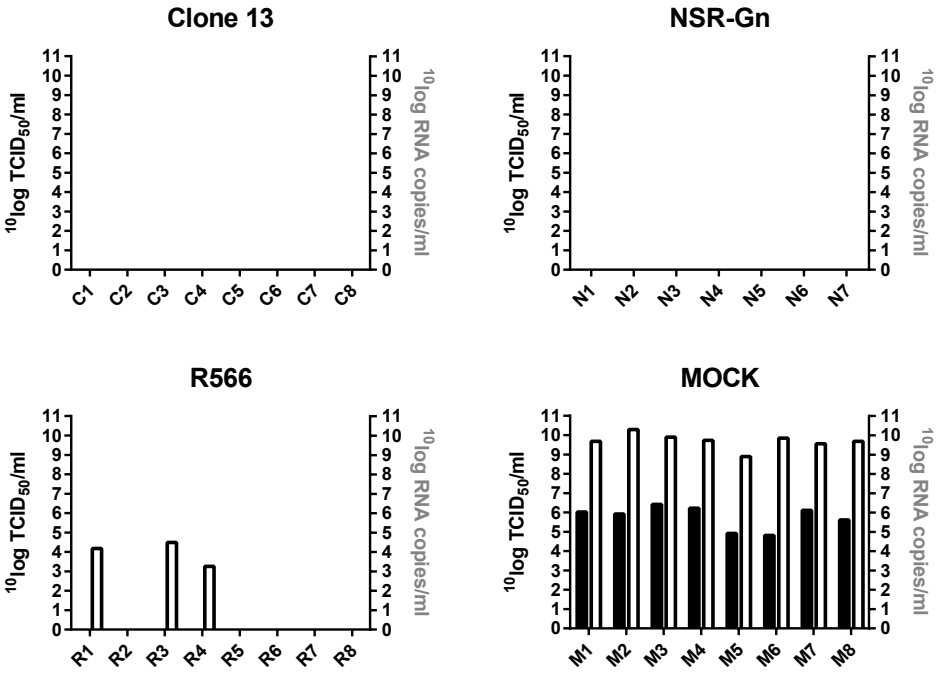

Figure 3. Virus isolations at peak moments of viremia. Virus isolations were performed on plasma samples collected at peak moments of viremia. The same samples were analysed by qRT-PCR. Results from virus isolations and qRT-PCR analysis are depicted in black and grey, respectively.

The body weights of the lambs were determined weekly. Only the animals in the mock-vaccinated group lost weight in the first week after challenge infection. Surviving lambs from this group gradually gained weight until the end of the experiment (Fig. 4).

At necropsy or at the end of the experimental period, samples from livers and spleens were collected to determine the presence of viral RNA. The organs of the three mock-vaccinated lambs that succumbed to the infection revealed the highest RNA levels (M2, M6 and M7, Fig. 5). Mock-vaccinated lambs M3, M4 and M8 were also positive for viral RNA in both livers and spleens, whereas the livers from lambs M1 and M5 did not contain detectable levels of viral RNA at the end of the experimental period (Fig. 5). Interestingly, two of the three R566-vaccinated lambs (R1 and R3) that contained low levels of challenge virus RNA in the blood at DPC 3 were positive for viral RNA in the spleens at the end of the experimental period.

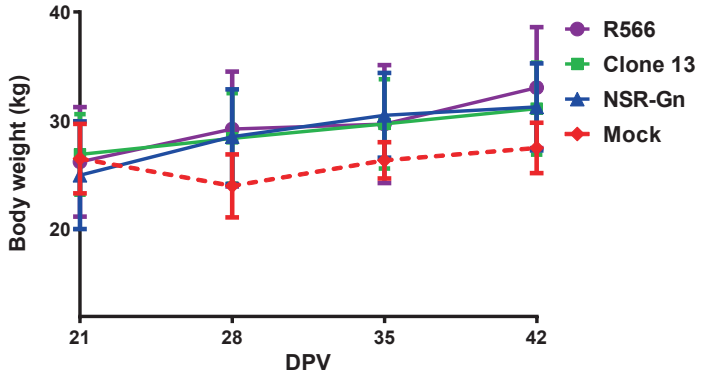

Figure 4. Body weights of mock-vaccinated and vaccinated lambs after challenge infection. The challenge infection was performed at 21 days post vaccination (DPV). The average body weight of mock-vaccinated lambs determined at DPV 28 results from 5 measurements, respectively, since three lambs from this group died before this day. Body weights of NSR-Gn-vaccinated lambs are averages of 7 measurements. Errors bars represent standard deviations.

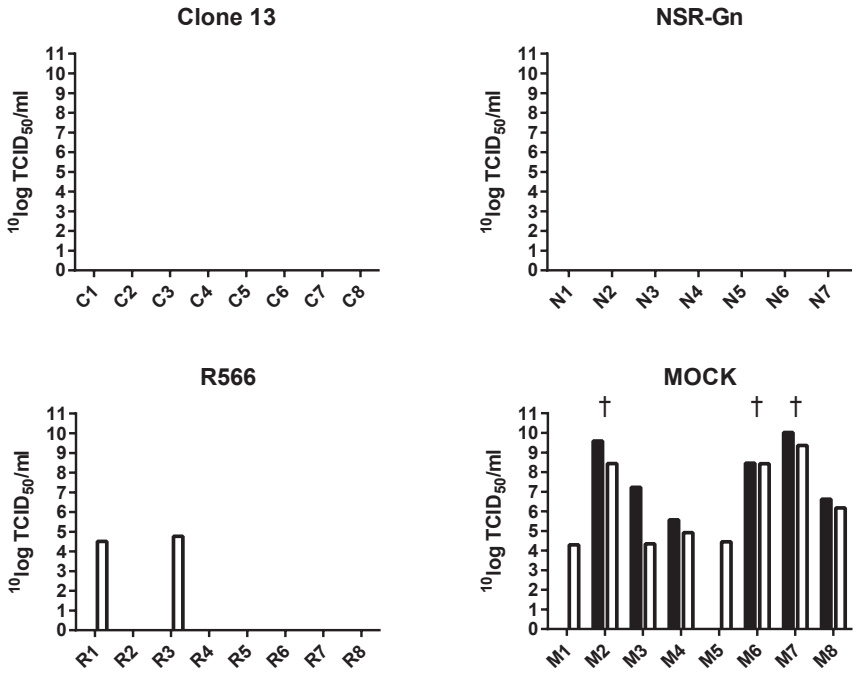

Figure 5. Detection of viral RNA by qRT-PCR in livers (black) and spleens (white). Organ samples were collected at necropsy from lambs that succumbed to the infection (nos. M2, M6 and M7, marked with †) or from surviving lambs at the end of the experimental period.

Attempts to isolate virus from liver samples of surviving lambs collected after euthanasia were unsuccessful, whereas virus was readily isolated from liver samples collected from lambs that succumbed to the infection (data not shown).

Antibody responses

Whereas all lambs showed rises in anti-N antibody levels between DPV 0 and DPV 7 (Fig. 6), neutralizing antibodies appeared in vaccinated animals about two weeks later (Fig. 7). All lambs vaccinated with Clone 13 or NSR-Gn were positive for neutralizing antibodies on the day of challenge (DPV 21), whereas only 4 of the 8 lambs vaccinated with R566 were positive for these antibodies at this time point (Fig. 7). Of note, all three R566-vaccinated lambs that revealed low levels of viral RNA in the blood after challenge infection were negative for neutralizing antibodies on the day of challenge, suggesting that the presence of neutralizing antibodies correlates well with the level of protection.

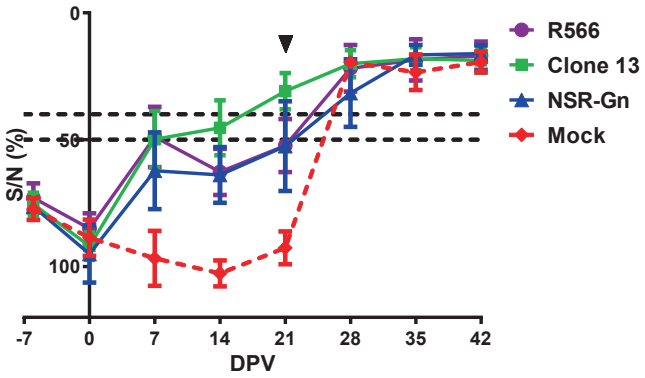

Figure 6. Detection of anti-N antibodies by ELISA. Sera were obtained 7, 14 and 21 days post vaccination (DPV) and at 0, 7, 14 and 21 days post challenge (DPC), corresponding to DPV 21, 28, 35 and 42. Titers are expressed as percentage competition ratio of the optical densities (OD) of the sample and the OD of the negative control (% S/N). All values lower than 40% are considered positive, between 40–50% are considered doubtful and above 50% are considered negative. The 40% and 50% boundaries are represented by interrupted lines. The moment of challenge infection is indicated by an arrowhead.

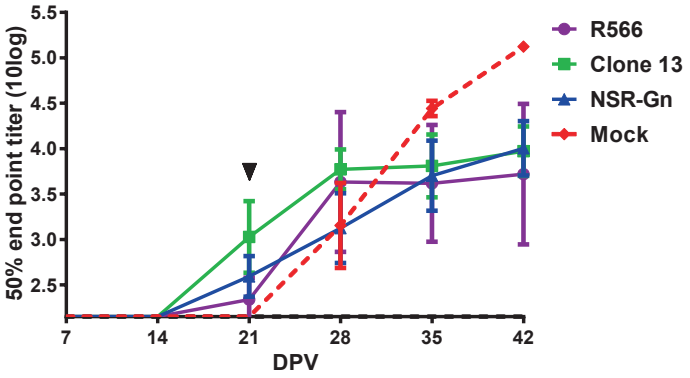

Figure 7. Results from virus neutralization tests performed with sera obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs at different time points after vaccination and challenge infection. Errors bars represent standard deviations. The y-axis crosses at the detection limit of the assay (y=2.15). The moment of challenge infection is indicated with an arrowhead.

Immunohistochemistry

The organs of the two lambs that died on DPCs 3 and 4 revealed typical signs of RVFV infection, whereas the organs of the lamb that died on 6 DPC (M7) showed more dramatic signs of infection. This lamb died unusually late after the challenge infection and immunohistochemistry revealed exceptional amounts of viral antigen in several target organs. The liver displayed a massive necrosis and immunohistochemistry revealed the presence of RVFV antigen throughout the liver parenchyma only sparing a small rim of periportal hepatocytes (Fig. 8). Interestingly, RVF virus also seemed to target the adrenal gland, as revealed by strong staining for RVFV antigen and necrosis of cortical cells. The mononuclear phagocyte system in the liver (Kuppfer cells) in the red pulp of the spleen and in the medullary and cortical sinuses of the portal lymph node stained intensely for RVFV antigen. Antigen was also detected in the intraglomerular mesangial cells in the kidney, in alveolar macrophages in the lung and in macrophages within lymph vessels and blood vessels or capillaries (Fig. 8).

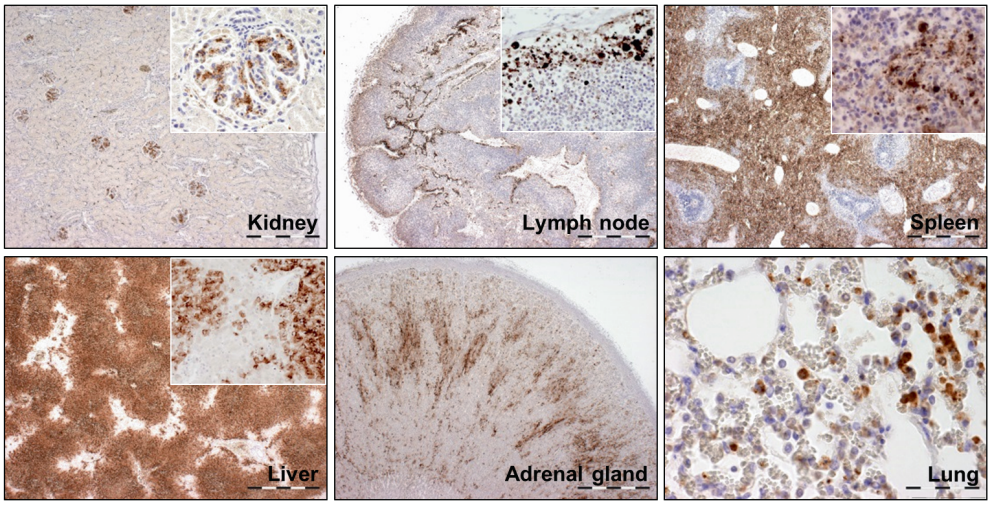

Figure 8. Immunohistochemistry of organ samples collected from a mock-vaccinated lamb that succumbed unusually late after RVFV infection . Organ samples were collected at necropsy from lamb (M7) that succumbed to RVFV infection 6 days after infection. Organ samples were fixed in 10% phosphate-buffered formalin and processing into paraffin. Paraffin blocks were cut into 4 µm sections and prepared for immunohistochemistry with anti-Gn monoclonal antibody 4-D4. Bars represent 1 mm (adrenal gland, liver, spleen, lymph node), 0.5 mm (kidney) or 50 µm (lung). Insets reveal higher magnifications. To demonstrate specificity of the staining, the insets of liver and spleen pictures reveal areas were staining was absent.

Discussion

In the past decade, several experimental RVFV vaccines were developed, of which some were already evaluated in the natural target species. It is difficult to compare the results of these animal studies, since no standardized animal models are available. To properly determine which of these vaccine candidates is most effective and thereby warrants further development for commercialization, the vaccines should be compared side by side in animal trials. We here report the results of a comparative trial in which a next-generation vaccine provided by Institut Pasteur, named R566, was compared with the NSR-Gn vaccine, provided by CVI-Lelystad. The Clone 13 vaccine was included as a control for optimal efficacy.

The results of our study suggest that the Clone 13 and NSR-Gn vaccines are more efficacious than the R566 vaccine. Vaccination with Clone 13 or NSR-Gn completely prevented challenge virus viremia, as determined by both virus isolation and the most sensitive qRT-PCR currently available, whereas low levels of challenge virus RNA were detected in the blood of three R566-vaccinated lambs. It is important to note, however, that no challenge virus was isolated

from any of the blood samples collected from R566-vaccinated lambs. We therefore expect that vaccination with R566 will protect lambs and adult sheep from disease. Nevertheless, it must be considered that a low level of viremia could result in transmission of the virus to the highly susceptible fetus. Given the desire to have one vaccine available to protect ruminants during all physiological stages, including pregnancy, a next-generation vaccine that completely prevents viremia is preferred for registration trials in Europe and other areas that are at risk of future incursions.

We recently reported the results from a dose-titration study with the NSR-Gn vaccine [28]. This previous trial was performed with Texel crossbreed lambs, 12-13 weeks of age at the moment of challenge, whereas in the current study, we made use of Romane lambs (INRA 401 breed), which were 13-15 weeks of age at the moment of challenge. Although these two trials cannot be compared directly, there are some indications that the challenge infections were more severe in the present study. Viremia levels in mock-vaccinated lambs were higher, and three lambs did not survive the infection, whereas one mock-vaccinated lamb died in our previous study [28]. Viral RNA was detected in the livers of three lambs in the previous work, whereas in the present study, almost all organs samples collected from mock-vaccinated lambs were positive for viral RNA. These findings are possibly attributed to a difference in susceptibility of Texel crossbreed and Romane lambs. Despite the more severe manifestation of RVFV infection in the present study, NSR-Gn vaccination was again shown to completely prevent viremia, morbidity and mortality.

Acknowledgements

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Conflict of Interest

J. Kortekaas and R. J. M. Moormann are inventors of WIPO Patent Application WO/2012/039607 "Methods to produce bunyavirus replicon particles". The

present work was aimed to select a lead vaccine candidate for production and marketing by MSD-AH.

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5

Evaluation of nonspreading Rift Valley fever virus as a vaccine vector using influenza virus hemagglutinin as a model antigen

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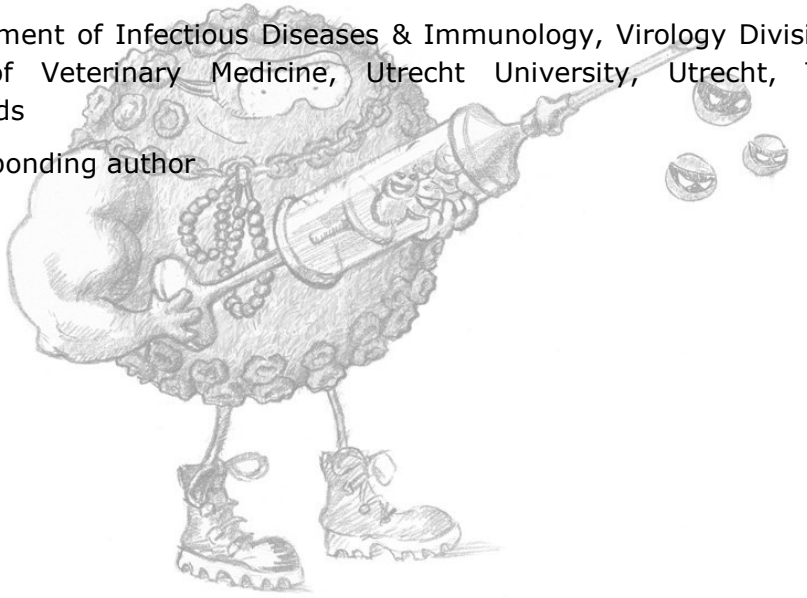

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Abstract

Virus replicon particles are capable of infection, genome replication and gene expression, but are unable to produce progeny virions, rendering their use inherently safe. By virtue of this unique combination of features, replicon particles hold great promise for vaccine applications. We previously developed replicon particles of Rift Valley fever virus (RVFV) and demonstrated their high efficacy as a RVFV vaccine in the natural target species. We have now investigated the feasibility of using this nonspreading RVFV (NSR) as a vaccine vector using influenza virus hemagglutinin as a model antigen. NSR particles were designed to express either the full-length hemagglutinin of influenza A virus H1N1 (NSR-HA) or the respective soluble ectodomain (NSR-sHA). The efficacies of the two NSR vector vaccines, applied via either the intramuscular or the intranasal route, were evaluated. A single vaccination with NSR-HA protected all mice from a lethal challenge dose, while vaccination with NSR-sHA was not protective. Interestingly, whereas intramuscular vaccination elicited superior systemic immune responses, intranasal vaccination provided optimal clinical protection.

Introduction

Vector vaccines based on RNA virus replicons are designed to optimally combine efficacy with safety. These nonspreading viruses lack at least one gene encoding a structural protein, rendering them unable to produce progeny virions and spread beyond the site of initial infection. The viral genome is however able to replicate in the infected cell, thereby providing "danger signals", the recognition of which elicits innate and adaptive immune responses without the need of co-stimulatory adjuvants. Altogether, these properties provide replicon particle-based vaccines a unique status in vaccinology.

The best-studied replicon platforms originate from representatives of the *Togaviridae* and *Flaviviridae* families, which have positive-strand RNA genomes. These replicons have shown promise as vaccines against various pathogens and also as therapeutics against cancer [1-3]. Positive-strand RNA genomes, however, are prone to mutation and recombination events [4-6]. In contrast, genomes of negative-strand RNA viruses are much more stable and recombination events among these viruses are much less common [7, 8]. Replicon particles based on the negative-strand RNA virus vesicular stomatitis virus (VSV) have shown promise in various studies. These replicons, however, seem to perform poorly when applied via the intranasal route [9].

Here, we report the establishment of a new vector platform that is based on previously created Rift Valley fever virus (RVFV) replicon particles [10], here referred to as nonspreading Rift Valley fever virus (NSR). RVFV belongs to the *Bunyaviridae* family and comprises a tri-partite negative-strand RNA genome [11]. The large (L) genome segment encodes the viral polymerase. The medium (M) segment encodes the viral surface glycoproteins Gn and Gc, a 78-kDa protein of unknown function [12, 13] and a 14-kDa protein named NSm, which was reported to have anti-apoptotic properties [14]. The small (S) segment encodes the nucleocapsid (N) protein and a non-structural protein named NSs. The NSs protein functions as an antagonist of host innate immunity and is considered the major virulence factor [15, 16]. NSR particles resemble the authentic virus in structure, but lack the NSs gene and are deprived of the M segment, rendering them avirulent and incapable of producing progeny particles.

We previously created NSR particles that express the enhanced green fluorescent protein (eGFP) gene from the NSs locus to facilitate monitoring of infection (NSR-eGFP) [10]. In the present work, the NSs gene was replaced by genes encoding either the full-length hemagglutinin (HA) protein of influenza A virus A/California/04/2009 (H1N1) or the corresponding soluble HA ectodomain, thereby creating NSR-HA and NSR-sHA, respectively. The HA hemagglutinin was selected as the model antigen because it has already been evaluated in previously developed vector-based influenza vaccines allowing us to gain first insights into the relative efficacy of our system [3, 17, 18].

In the present work, vaccination with NSR-sHA provided no protection when administered via either the intramuscular (IM) or the intranasal (IN) route, whereas a single vaccination with NSR-HA provided solid protection in a mouse model of lethal influenza. Although vaccination with NSR-HA via both routes was highly effective, protection was optimal when the vaccine was delivered via the IN route. Analyses of antibody and T-cell responses demonstrated that the NSR vaccines induce immune responses with polarised Th1 profiles.

Materials and Methods

Challenge virus

Swine-origin influenza virus A/Netherlands/602/09 (H1N1) was used as challenge virus in this study [19]. Mouse adaptation was obtained by three passages in mouse lungs [20].

Preparation of the vaccines

NSR-sHA, NSR-HA and NSR-eGFP vaccine preparations were produced as described previously [21], diluted in phosphate-buffered saline and kept at -80^{o}C until use.

Immunogenicity and vaccine efficacy in mice

Six-week-old female BALB/cAnCrl mice (Charles River Laboratories) were housed in five groups of six mice each (Table 1, experiment 1) or five groups of ten mice and one group of five mice (Table 1, experiment 2). The animals were kept in type-III filter top cages under BSL-3 conditions. After acclimatization for 6 days, two groups of mice from experiment 1 received NSR-HA vaccine via either the IM or the IN route, another two groups received NSR-sHA vaccine via either the IM or the IN route and one group received a control NSR-eGFP vaccine via both routes. All vaccines were administered at a dose of 10^7 $TCID_{50}$/mouse. Mice from experiment 1 were euthanized 28 days after vaccination and spleens were collected for analysis of memory T-cell responses (Fig. 1). Mice from experiment 2 were treated according to the same vaccination protocol as those in experiment 1 but included an additional group of 5 animals, which was mock-vaccinated with PBS via both routes.

Table 1. Vaccine constructs and vaccination routes

	Vaccine[a,b]	Vaccination route[c]	Challenge	Number of mice
Experiment 1	NSR-HA	IM	no	6
	NSR-HA	IN	no	6
	NSR-sHA	IM	no	6
	NSR-sHA	IN	no	6
	NSR-GFP	IM+IN	no	6
Experiment 2	NSR-HA	IM	yes	10
	NSR-HA	IN	yes	10
	NSR-sHA	IM	yes	10
	NSR-sHA	IN	yes	10
	NSR-GFP	IM+IN	yes	10
	Mock (PBS)	IM+IN	no	5

[a] NSR, nonspreading Rift Valley fever virus

[b] A dose of 107 TCID50/mouse was used for all vaccinations

[c] IM, intramuscular; IN, intranasal

The mice from experiment 2 were challenged 3 weeks after vaccination by IN administration of 25x LD50 (equal to 5.6 log10 $TCID_{50}$) of mouse-adapted influenza virus. Challenged mice were monitored twice per day in the first week

and daily during the second week after the challenge infection and survival, body weights and clinical scores were recorded. The following clinical scoring system was used: score 0 = no clinical signs; 1 = rough coat; 2 = rough coat, less reactive, passive during handling; 3 = rough coat, rolled up, laboured breathing, passive during handling; 4 = dead. Mice with clinical score of 3 at two consecutive observations or a body weight loss of more than 20% as compared to the body weight on the day before the challenge were considered to have reached the humane end point and were euthanized by cervical dislocation. Two weeks after challenge, all surviving mice were euthanized. Blood samples were collected on the day before vaccination (day -1) and on the day before the challenge (day 20, Fig. 1).

All vaccines were administered in a volume of 50 µl. IM vaccinations were performed by injection into the thigh muscle using a ∅ 0.30 mm, 12 mm needle. IN vaccinations were performed by equal distribution of the vaccine between both nostrils. All IN vaccinations were preceded by anaesthesia with 7 mg/kg xylazine and 70 mg/kg ketamine, applied intraperitoneally with a 25 gauge, 16 mm needle. Blood samples were collected by puncture of the facial vein.

All animal experiments were conducted in accordance with the Dutch Law on Animal Experiments (Wod, ID number BWBR0003081) and approved by the Animal Ethics Committee of the Central Veterinary Institute of the Wageningen UR (Permit Number: 2013105).

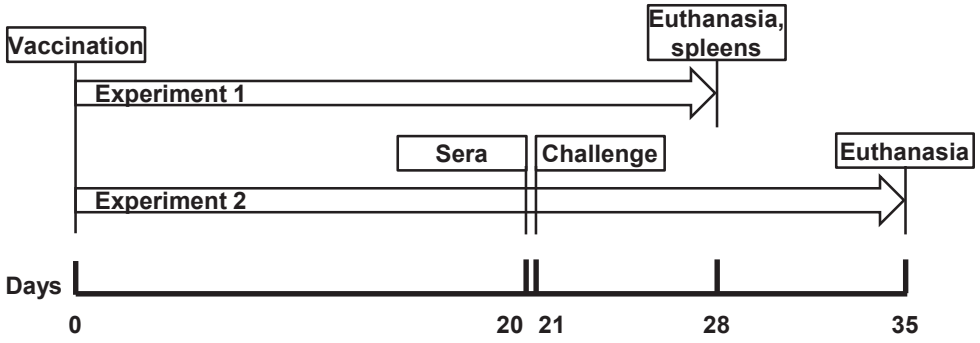

Figure 1. Time lines of vaccination experiments 1 and 2.

ELISA

Sera were tested for the presence of HA-specific IgG and IgA antibodies, as well as for IgG1 and IgG2α isotypes in indirect ELISA. A soluble HA ectodomain derived from influenza virus A/California/04/2009 (H1N1) (residues 17 to 523), fused to a T4 foldon trimerization domain [22] and a 3xFLAG tag was used as antigen. The HA ectodomain was expressed in HEK293T cells [23] and purified from the cell culture supernatant using anti-FLAG M2 magnetic beads according to the instructions of the manufacturer (Sigma).

Starting at dilution 1:40, two-fold serial dilutions were prepared of each mouse serum and optical densities were determined for all dilutions. To determine the end-point titers, cut-offs were calculated according to the method described by Frey *et al.* [24] at a level of confidence of 95%, using negative sera collected from the same mice on the day before the vaccination. Ten negative samples per plate were used for the IgG and IgA tests and four negative samples were used for the IgG1 and IgG2α tests.

Detailed description of the ELISA method is provided in the Supplementary data.

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assays were performed as described previously [18]. Briefly, the serum samples were heat-inactivated at 56^{o}C for 30 min and then treated overnight with Vibrio cholera-derived neuraminidase (Roche) at 37^{o}C. Inactivation of the neuraminidase was achieved by incubation at 56^{o}C for 30 min after which sera were pre-treated with turkey red blood cells. Starting at 1:12.5, serial two-fold dilutions of the sera were prepared and tested with 4 hemagglutination units (HAU) of inactivated H1N1 virus (A/Neth/602/09).

Enzyme-linked immunospot (ELISPOT) assay

Spleen lymphocytes were tested in an ELISPOT assay as previously described [21] and the number of interferon gamma secreting cells was determined after stimulation with: 5 µg/ml of soluble HA ectodomain (described in the ELISA section); 5 µg/ml of a peptide derived from the transmembrane region of the HA protein (IYSTVASSL [25]); or 5 µg/ml of a peptide derived from GFP (HYLSTQSAL [26]). Peptides were synthesized by the Genscript Corporation (Piscataway, NJ, USA). Each sample was tested in triplicate, the average numbers of spots were calculated and normalized with the averages of background signals.

Quantification of cytokines

Spleen cells were cultured in 96 wells plates at a density of 10^6 cells/well in duplicate. Cells were stimulated with soluble HA ectodomains (described in the ELISA section) or cRPMI for 48 h. Cell-free supernatants were harvested, pooled per mouse and investigated for the presence of IFN-γ, IL-2, IL-4, IL-5 and IL-10. The cytokines were detected by the luminex xMAP technology, using a Milliplex® map kit (Millipore) according to the manufacturers' instructions. For the measurements, a Luminex 100 instrument was used.

Statistical analyses

Maximum body weight losses were analysed with two-way analysis of variance (ANOVA) with least significant difference (LSD) adjustment for multiple comparisons to determine the separate effects of the vaccine treatments and administration routes. One-way ANOVA with Dunnett t post-hoc test was used to compare the vaccination groups with either the mock-challenged control group or the NSR-eGFP control group. ELISA titres were log-transformed and analysed with two-way ANOVA with LSD adjustment for multiple comparisons. Log-transformed titres were assumed to be normally distributed. Sera with an ELISA titre <40 were assigned a value of 20 for the analysis. The influence of the vaccine type (NSR-HA or NSR-sHA) and vaccination routes (IM or IN) on IFN-γ production as detected by ELISPOT, were analysed with two-way analysis of variance (ANOVA) with LSD adjustment for multiple comparisons. Differences between the vaccinated and control groups were analysed with a one-way ANOVA with Dunnett t post-hoc test. Extreme outliers were replaced with values that differ by 1 from the next highest or lowest value. Statistical tests were performed, using the SPSS software version 20 (IBM) and differences with *p*-values <0.05 were considered significant.

Results

Characterization of the NSR vaccines

After developing the NSR vaccine constructs, expression of the foreign genes and expected protein localization were confirmed by Western blotting and immunofluorescence, respectively (Supplementary data).

NSR-HA protects mice from a lethal dose of influenza virus H1N1

Vaccine efficacies of the NSR-HA and NSR-sHA were tested in a mouse model of lethal influenza (Fig. 1, Table 1). After challenge infection, the control mice that

were vaccinated with NSR-eGFP developed severe respiratory illness and displayed significant weight loss. Four days post challenge (DPC) all these mice either succumbed to the infection or were euthanized because the humane endpoint was reached (Fig. 2A). The mice vaccinated with NSR-sHA also did not survive the challenge infection. However, death was delayed with one day in half of the IM-vaccinated mice, and with one to three days in all IN-vaccinated mice, compared to the control group (Fig. 2A). In addition, also the development of clinical signs was somewhat delayed in mice vaccinated with NSR-sHA. This effect was more pronounced after IN vaccination (Fig. 2B). In sharp contrast, the mice vaccinated with NSR-HA did not develop any clinical signs (Fig. 2A and B).

Monitoring of maximal body weight loss suggested that the NSR-HA vaccine performed better than the NSR-sHA vaccine and that vaccination via the IN route was more effective than vaccination via the IM route (Fig. 2C). Weight loss in NSR-sHA-vaccinated mice did not significantly differ from the control mice vaccinated with NSR-eGFP. On the other hand, weight loss in mice vaccinated with NSR-HA via the IN route did not significantly differ from unchallenged healthy control mice (Fig. 2C).

Antibody responses

HA-specific IgG antibodies were detected in all vaccinated animals before challenge infection. The average titers of the IM-vaccinated animals were higher than those of the IN-vaccinated animals (Fig. 3A), but differences in antibody titers did not correlate with the expressed antigen (HA or sHA). Investigation of the IgG isotypes revealed that IgG2α antibodies had a pattern very similar to that of the total IgG (Fig. 3D). Vaccines administered via the IM route elicited higher IgG2α responses than those administered via the IN route and the responses were not influenced by the type of HA expressed. On the contrary, IgG1 antibodies were more abundant after NSR-sHA vaccination, while the administration route was of no influence (Fig. 3C). HA-specific serum IgA antibodies were less abundant than IgG antibodies, but the titers correlated with the IgG titers (Fig. 3B). In the sera of mock-vaccinated and NSR-eGFP-vaccinated control mice, no HA-specific IgG or IgA antibodies were detected (data not shown).

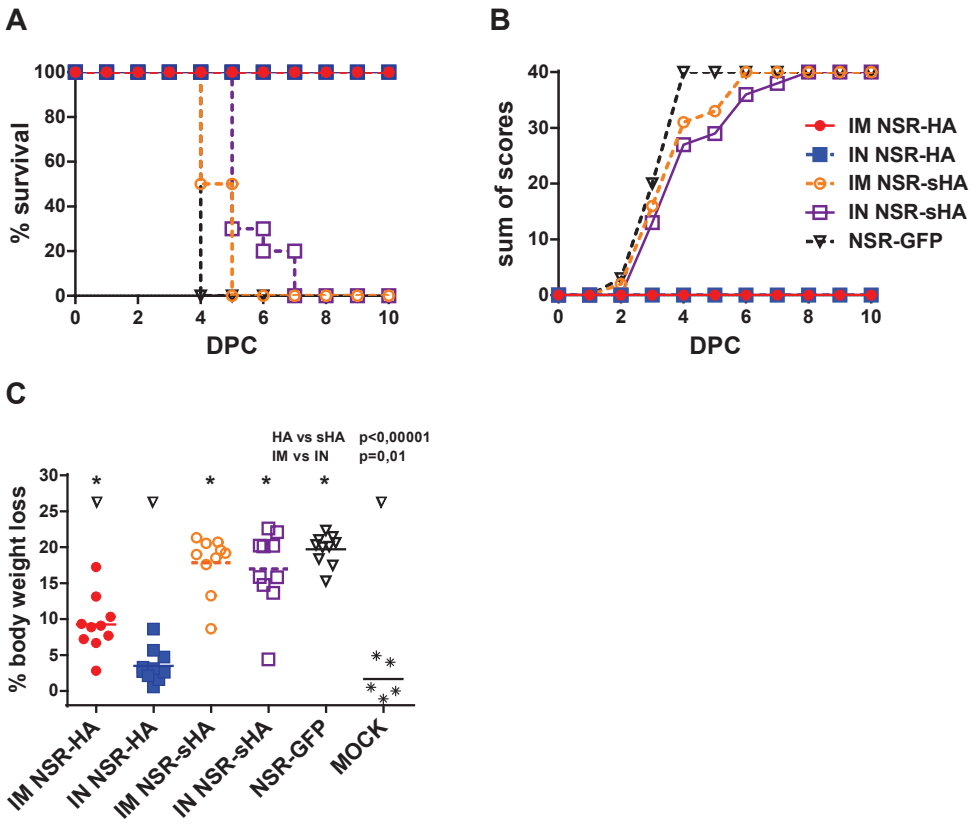

Figure 2. Vaccine efficacy of NSR particles expressing influenza virus HA proteins. (A) Kaplan-Meier survival curve of the percentage of surviving mice. DPC = days post challenge. (B) Total clinical scores, expressed as sums of the scores of all mice per group per day. (C) Maximal body weight loss after challenge infection, expressed as percentage of the weight of each mouse on the day before the challenge. The symbols depict individual measurements and the horizontal lines represent the group means. Statistical significance of the effect of the vaccine type (NSR-HA vs NSR-sHA) or the administration route (IM vs IN) is shown. Differences between vaccinated groups and MOCK group are depicted with an asterisk (*, $p<0.001$) and between vaccinated groups and NSR-GFP group with an inverted triangle (▽, $p<0.001$).

In the groups of mice vaccinated with NSR-HA, all mice vaccinated via the IM route and 7/9 mice vaccinated via IN route developed HI-titres (Fig. 3E). The average titer in the group vaccinated via IM route was slightly higher than that in the group vaccinated via IN route. In the groups vaccinated with NSR-sHA, 2/10 IM-vaccinated mice developed HI titres. These mice displayed the lowest weight loss. Also 2/10 IN-vaccinated mice developed HI titres (Fig. 3E). These mice were among the three animals with the longest survival time (Fig. 2A). No HI-titres were detected in mice from the control groups (Fig. 3E).

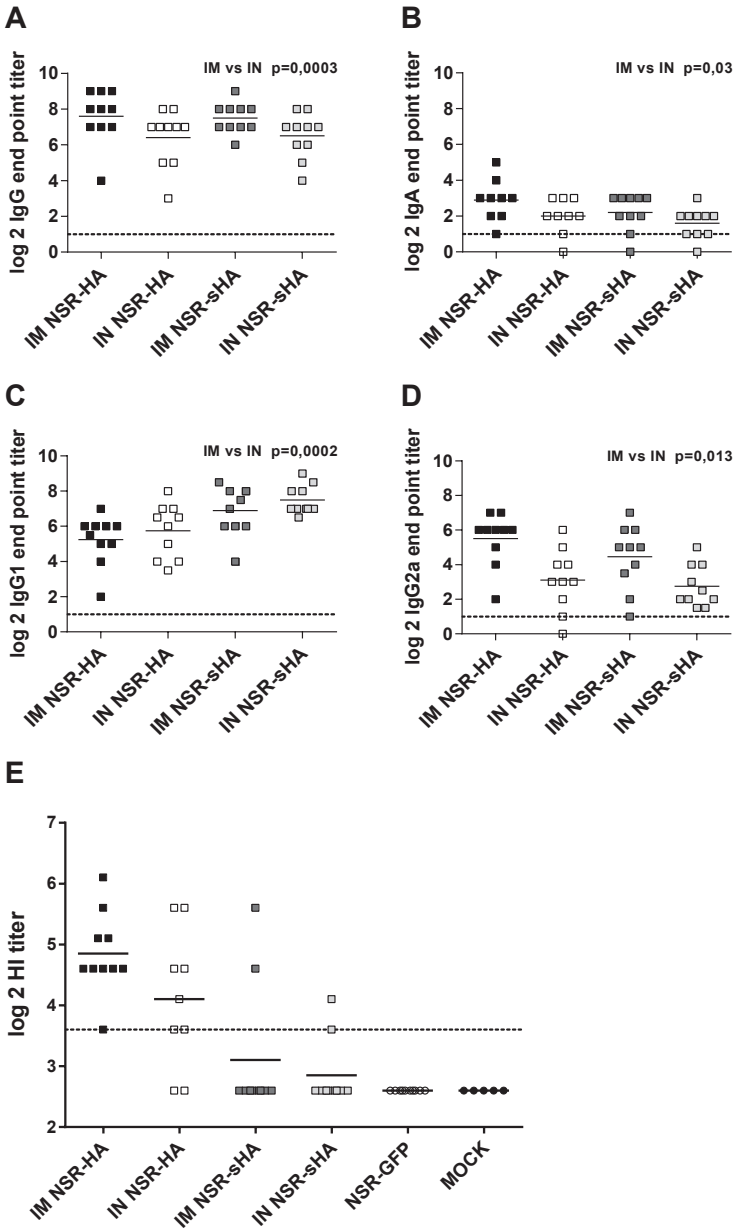

Figure 3. HA-specific antibodies induced by NSR-based influenza vaccines. Sera were tested for the presence of HA-specific IgG (A), IgA (B), IgG1 (C) and IgG2α (D) antibodies by ELISA and for hemagglutination inhibiting (HI) antibodies (E). Groups consisted of 10 animals, with the exception of the following groups in panel B: IM NSR-HA, IN NSR-HA, where n=9 and in panel E: IN NSR-HA, where n=9. Symbols represent individual measurements and the averages are depicted with horizontal lines. The interrupted line depicts the detection limit of the test. (A-D) Titers are expressed as log 2 of the end point titers. Statistical significance of the influence of the vaccine type (NSR-HA vs NSR-sHA) or the administration route (IM vs IN) is shown. (E) HI titers expressed as log 2 of the reciprocal of the highest serum dilution showing HI.

NSR vaccines induce specific IFN-γ responses and predominantly Th1 cytokines

The potencies of the two vaccines to induce memory T-cell immune response were investigated by measuring the specific IFN-γ responses in spleen cells. For stimulation, soluble HA protein or peptides containing CD8-restricted epitopes, derived from the eGFP protein or the transmembrane anchor of the HA protein were used. The HA protein induced IFN-γ response in splenocytes of all mice that had received NSR-HA or NSR-sHA vaccines. These responses were higher in mice vaccinated with NSR-HA than in mice vaccinated with NSR-sHA and higher after IM vaccination as compared to IN vaccination. All groups vaccinated against influenza had significantly higher IFN-γ responses than the control group vaccinated with NSR-eGFP (Fig. 4A). Stimulation with the peptide derived from the transmembrane region of the HA protein induced an IFN-γ response in all mice vaccinated with NSR-HA via the IM route (Fig. 4B) and in only 2/6 mice that were vaccinated via the IN route. As expected, no response was observed in the NSR-sHA-vaccinated mice, as the NSR-sHA vaccine lacks the transmembrane anchor of HA. Stimulation with the eGFP-derived peptide resulted in a marked response in NSR-eGFP vaccinated mice, while in the other four groups no specific responses were detected (Fig. 4C).

To investigate the Th1/Th2 polarization, splenocyte supernatants were subjected to a multiplex cytokine assay with IFN-γ and IL-2 representing the Th1 cytokines and IL-4, IL-5 and IL-10 representing the Th2 cytokines. Both vaccines primed marked Th1 memory responses and weak Th2 responses which, in the case of IL-4 and IL-5, were close to background levels (Fig. 4D). Mice vaccinated with the NSR-HA vaccine displayed the highest IFN-γ and IL-2 responses, both after IM and IN vaccination.

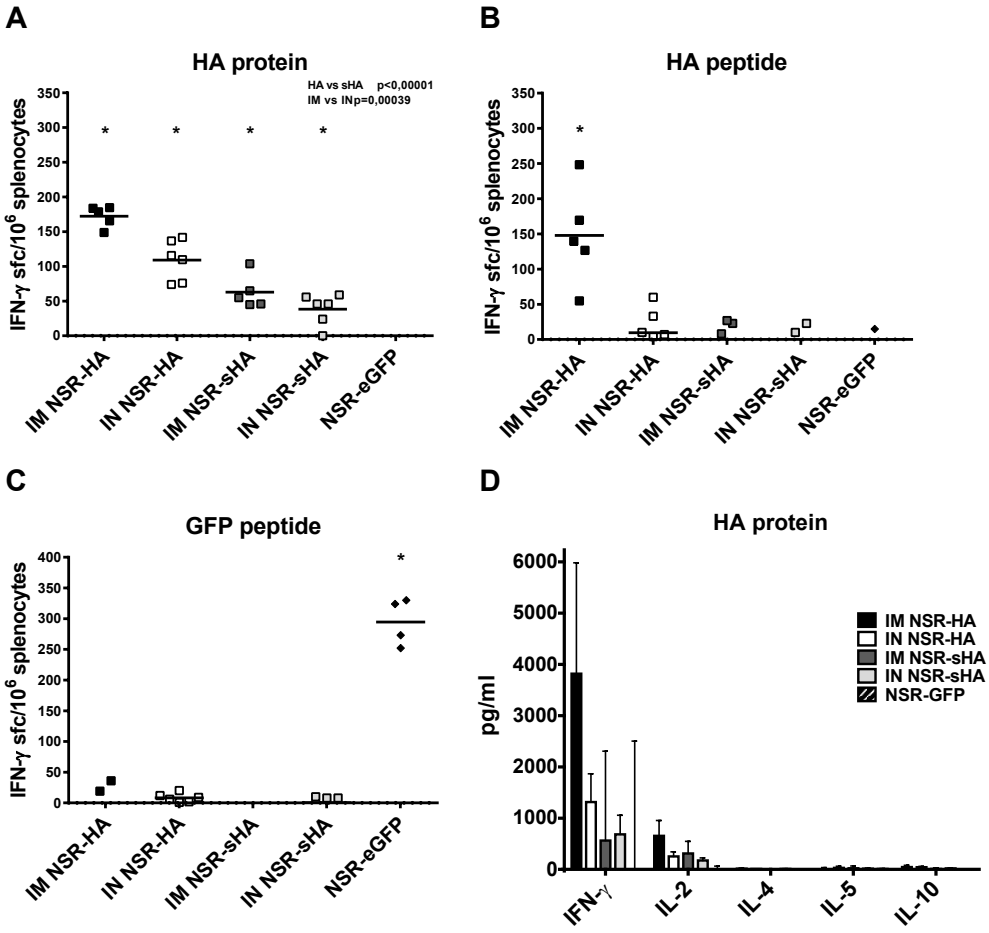

Figure 4. Cytokine responses of splenocytes of vaccinated mice. (A-C) ELISPOT assay for IFN-γ. Symbols represent individual counts of IFN-γ spot forming cells (sfc) and the group averages are depicted with horizontal lines (IM NSR-HA and NSR-sHA, n=5; IN NSR-HA and NSR-sHA, n=6; NSR-eGFP, n=4). Statistical significance of the influence of the vaccine type (NSR-HA vs NSR-sHA) or the administration route (IM vs IN) is shown (panel A). Differences between the test groups and the control group (NSR-eGFP) are denoted with an asterisk (A-C;*, p<0.001). (D) Results obtained from a multiplex cytokine assay for IFN-γ, IL-2, IL-4, IL-5 and IL-10. Bars represent the average cytokine concentrations (pg/ml) per group with a standard deviation.

Discussion

The remarkable efficacy of NSR particles in protecting the natural target species against RVFV warranted the present study on the applicability of the NSR system as a novel vector vaccine platform. The high infectivity of RVFV when delivered via aerosols or intranasal droplets [27] explains our particular interest in the

mucosal application. Therefore we investigated the feasibility of using NSR particles as a vector against a respiratory infection, using a mouse model of lethal influenza. To that end, NSR particles were designed to express the influenza HA protein or its corresponding ectodomain (sHA) and the efficacies of these two vaccines were compared when delivered intranasally or intramuscularly.

It was interesting to find that the type of HA antigen expressed had a strong impact on efficacy. All mice were protected by a single vaccination with the NSR-HA vaccine, whereas the NSR-sHA vaccine did not provide protection. A similar striking difference was observed in the vaccine-induced hemagglutination inhibition (HI) titers, the presence of which is known to correlate with protection. Vaccination with NSR-HA induced HI titers in almost all mice, while only a few mice developed these antibodies after vaccination with NSR-sHA.

Our finding that full-length HA is a more effective antigen than sHA when expressed from a viral vaccine vector is in agreement with a previous study by Cornelissen *et al.* [18]. In that study, vaccination with a Newcastle disease virus (NDV) expressing full-length HA partially protected mice from a lethal challenge with influenza virus H5N1, whereas vaccination with an NDV expressing the corresponding HA ectodomain was not protective. In contrast with our results, the NDV-based vector vaccine failed to induce detectable levels of HA-specific HI antibodies in mice.

To gain insight into the type of immune response elicited by NSR-HA or NSR-sHA vaccination, the isotypes of antibodies were determined. These analyses demonstrated that IgG1 antibodies were more abundant than IgG2α subtypes. This effect was more pronounced in the NSR-sHA vaccinated animals which developed higher IgG1 titers than the NSR-HA vaccinated animals. . However, it is important to note that the sensitivities of the antibody conjugates used to detect the respective IgG1 or IgG2α antibodies were not identical. Detection of IgG1 antibodies was about eight times more sensitive than the detection of IgG2α antibodies (data not shown). Taking this into account, together with the observation that the IgG2α titer distribution across the groups resembled that of total IgG, suggest that IgG2α was the predominant antibody isotype. Since IgG1 antibodies are associated with Th2 type immune responses, while IgG2α antibodies are associated with Th1 type responses [28], we conclude that vaccination with NSR elicits a predominant Th1 immune response. Further confirmation of this notion was provided by the markedly higher amounts of the Th1 cytokines IFN-γ and IL-2, as compared to the Th2 cytokines IL-4, IL-5 and IL-10, released by splenocytes after stimulation with specific antigens.

The efficacies of NSR-HA and NSR-sHA vaccines were compared to determine differences in immunogenicity of the antigens when expressed from the NSR genome. Soluble trimeric forms of HA were reported to be immunogenic in mice [29], chicken [30] and pigs [31] and to elicit a balanced Th1/Th2 response in mice [29], while cell-associated proteins are expected to elicit a predominant Th1 immune response. It was therefore surprising to find that regardless of the higher IgG1 antibody titers in the NSR-sHA vaccinated animals as compared to the NSR-HA vaccinated animals, the cytokine responses were strongly dominated by Th1 cytokines in all vaccination groups. Moreover, NSR-HA was superior in inducing both HI antibodies and cellular IFN-γ responses which correlated with protection. From these data we conclude that full-length, membrane- anchored HA is more potent in eliciting protective immunity than soluble trimeric HA when expressed by the NSR vector.

Comparison of the vaccination routes revealed that IM vaccination induced higher systemic humoral and cellular immune responses. However, clinical scores and body weights data collectively demonstrate superior performance of the IN vaccination route. This seeming discrepancy between immune response and protection may be attributed to the induction of a solid local immune response at the mucosal surface of the respiratory tract of the vaccinated mice. Indeed, RVFV has been shown to be highly infectious and immunogenic when administered through the respiratory tract via aerosols or intranasal droplets [27, 32-35]. Further investigation of local humoral and cellular responses and their duration is needed to elucidate the correlates of protection elicited by NSR vaccination. Nevertheless, the results presented here suggest that NSR can be used as a powerful platform for mucosal vaccination against respiratory diseases.

Acknowledgements

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Conflict of Interest

J. Kortekaas and R. J. M. Moormann are inventers of WIPO Patent Application WO/2012/039607 “Methods to produce bunyavirus replicon particles”.

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Supplementary data

Materials and methods

Plasmids

The plasmid encoding the complete RVFV L genome segment and the plasmid encoding the S genome segment of which the NSs gene is replaced by the eGFP gene (pUC57-L and pUC57-S-eGFP, respectively) were described previously [1]. In the current work, the eGFP gene of the S segment was replaced by either the gene encoding the full-length hemagglutinin (HA) protein or its ectodomain (aa 17-523), thereby creating S-HA and S-sHA respectively. Sequences were derived from influenza virus A/California/04/2009 (H1N1) (GenBank™ accession no. FJ966082) and were codon-optimized for optimal expression in mammalian cells. The sHA ectodomain sequence is preceded by a CD5 signal sequence and followed by a GCN4 leucine zipper motif and a single Strep-Tag (Fig. S1) [2]. The presence of a GCN4 leucine zipper results in trimerization of the sHA protein [3]. The plasmid pCAGGS-M encodes the complete glycoprotein precursor of RVFV under CMV immediate enhancer/β-actin (CAG) promoter control [4].

Cells and growth conditions

BHK cells were grown in Glasgow minimal essential medium (GMEM; Invitrogen), supplemented with 4% tryptose phosphate broth (Invitrogen), 1% minimum essential medium nonessential amino acids (MEM NEAA, Invitrogen), 1% Penicillin-Streptomycin (Invitrogen) and 5% fetal bovine serum (FBS; Bodinco). BHK-GnGc cells [1] and derivatives thereof were grown in the above described medium supplemented with 10% FBS and 1 mg/ml Geneticin (G-418; Promega).

Creation of replicon cell lines expressing HA or sHA

Replicon cell lines were created essentially as described previously [1]. Briefly, BHK-GnGc cells were infected with fowlpox virus expressing T7 polymerase [5] and subsequently transfected with plasmids encoding the L and S-HA or S-sHA genome segments, as well as the glycoprotein expression plasmid pCAGGS-M. Cells were repeatedly passaged and transfected with pCAGGS-M upon every passage, until virtually all the cells became positive for the N protein, as verified by flow cytometry. The resulting cell lines are referred to as Rep-HA and Rep-sHA, respectively.

Production of replicon particles

To produce NSR particles expressing either the sHA or the HA protein (NSR-sHA or NSR-HA respectively), $7x10^6$ of Rep-sHA or Rep-HA cells were seeded in 150 cm^2 tissue culture flasks and transfected the following day with 14 µg of pCAGGS-M, using 40 µl of JetPEI transfection reagent (Polyplus) in 20 ml Opti-MEM® medium (Invitrogen). Supernatants were harvested after 24 h and cleared from cell debris by centrifugation at 4500 x*g* for 15 min and stored at 4°C. The titers were determined on BHK cells as described [1].

Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Rep-HA, Rep-sHA or Rep-eGFP cells were seeded in 6-well plates in Optimem, 10^6 cells/well, and incubated for 24 h at 37°C and 5% CO_2. BHK cells were seeded at the same density and were infected 2 h later with NSR-GFP, NSR-HA or NSR-sHA at a multiplicity of infection (m.o.i) of 3 in Optimem. After 24 h, supernatants were harvested and cells were collected in standard Laemmli sample buffer. Proteins present in the cell lysates and the supernatants were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 4-12% Bis-Tris NuPAGE gels (Invitrogen) and transferred to Whatman® Protran® nitrocellulose membranes (Sigma-Aldrich). After blocking, the membranes were incubated with rabbit polyclonal antibody specific for the HA protein of influenza A virus H1N1 (A/California/04/2009) (Sino Biological Inc., Beijing, China). Subsequently, membranes were washed and incubated for 1 h with anti-rabbit IgG conjugated to HRP (Dako). After another washing step, proteins were detected with Amersham™ ECL™ Prime Western blotting detection reagents (GE Healthcare). All incubation and washing steps were performed in incubation buffer (1% skim milk and 0.05% Tween 20 in Tris-buffered saline (TBS)).

Immunofluorescence

Rep-HA, Rep-sHA and BHK cells infected with NSR-eGFP, NSR-HA or NSR-sHA with an m.o.i. of 0.5 were incubated for 24 h at 37°C and 5% CO_2 and subjected to immunofluorescent staining described previously [6]. A primary mouse monoclonal antibody (mAb) specific for the HA protein of influenza virus A/California/04/2009 (H1N1) (Sino Biological Inc.) and a secondary Texas-red-conjugated anti-mouse antibody (Beckman & Coulter) were used to visualize the HA protein in the cells. Cell nuclei were stained with DAPI staining (Invitrogen), according manufacturers' instructions. Images were taken with an AMG EVOSfl fluorescent microscope.

ELISA

ELISA plates (Greiner Bio-One) were coated overnight at 4°C with 50 µl of the soluble HA protein diluted in coating buffer (0,05M $NaHCO_3$ and 0,05M Na_2CO_3, pH 9.6), at a concentration of 0.1 µg/ml for detection of IgG, IgG1 and IgG2α antibodies or 0.4 µg/ml for detection of IgA antibodies. Plates were washed and incubated with 100 µl blocking buffer (PBS, supplemented with 2% bovine serum albumin (BSA) and 0.05% Tween-20) for 1 h. Mouse sera, serially diluted in PBS supplemented with 0.2% BSA and 0.05% Tween-20, were added to the plates in 50 µl volume/well and incubated for 1 h. Plates were washed and subsequently incubated with anti-mouse IgG, IgG1, IgG2α or IgA antibodies conjugated to HRP (DAKO) for 1 h. After another wash, antibody binding was detected by adding 50 µl of substrate buffer (0,1M sodium acetate and 2,1% citric acid, pH 6.0), supplemented with 1% of TMB substrate (stock solution 10 mg/ml TMB in DMSO) and 0.005% H_2O_2. Substrate conversion was terminated by adding 25 µl of 0,5 M H_2SO_4 and the extinction was measured at 450 nm using a Multiscan EX ELISA reader (Thermo Scientific). All incubations were performed at room temperature. Between the incubation steps, plates were washed three times with water supplemented with 0.01% Tween-80.

Results

Creation of replicon cell lines constitutively expressing influenza virus HA genes

We and others [1, 7] have previously shown that the NSs gene of the S segment of RVFV can be substituted by a foreign gene without hindering viral genome replication. Here, in the place of the NSs gene, we introduced genes encoding either full-length influenza virus HA or its corresponding soluble ectodomain (sHA). The full-length HA protein contains a transmembrane region that anchors the protein into the cell membrane. The corresponding ectodomain (sHA) lacks a transmembrane region and was therefore expected to be secreted from the cell. The S segments containing the HA genes were named S-HA and S-sHA, respectively (Fig. S1).

Previously, replicon cell lines were described that constitutively maintain the RVFV S and L genome segments [1]. Using the same strategy, replicon cell lines constitutively maintaining L and S-sHA or S-HA genome segments were created. To monitor expression of the recombinant HA proteins, cells and supernatants from the replicon cell lines were analysed by SDS-PAGE and Western blotting. A

replicon cell line expressing eGFP (Rep-eGFP) served as a negative control. The HA protein was detected in the cell fraction, consistent with its expected association with the cellular membrane. As expected, the sHA protein was predominantly found in the supernatant (Fig. S2A, left panel). We noticed that the sHA protein, when isolated from the cell fraction, migrated faster in the gel as compared to the secreted sHA protein, most probably as a result of differences in the maturation of N-linked glycans. It is also worthwhile to note that some HA protein was detected in the supernatant of Rep-HA cells, suggesting the formation and secretion of virus-like particles comprised of HA.

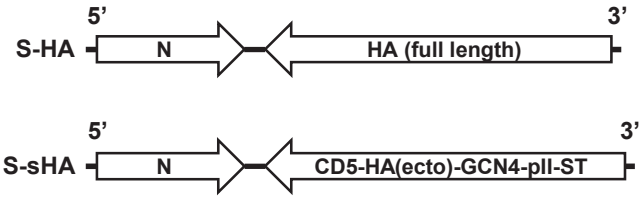


Figure S1. Schematic representation of the RVFV S-HA and S-sHA segments in antigenomic-sense orientation. The sHA ectodomain is fused to a CD5 signal sequence on its N-terminus and a trimerization domain (GCN4-pII) and a Strep-Tag (ST) on its C-terminus.

The replicon cell lines were used to produce NSR-HA and NSR-sHA particles as previously described [1, 6]. Of note, whereas transfection of the Rep-eGFP cell line generally yields titers of 10^8 $TCID_{50}$/ml, transfection of the Rep-HA and Rep-sHA cell lines resulted in lower yields, generally around 10^7 $TCID_{50}$/ml.

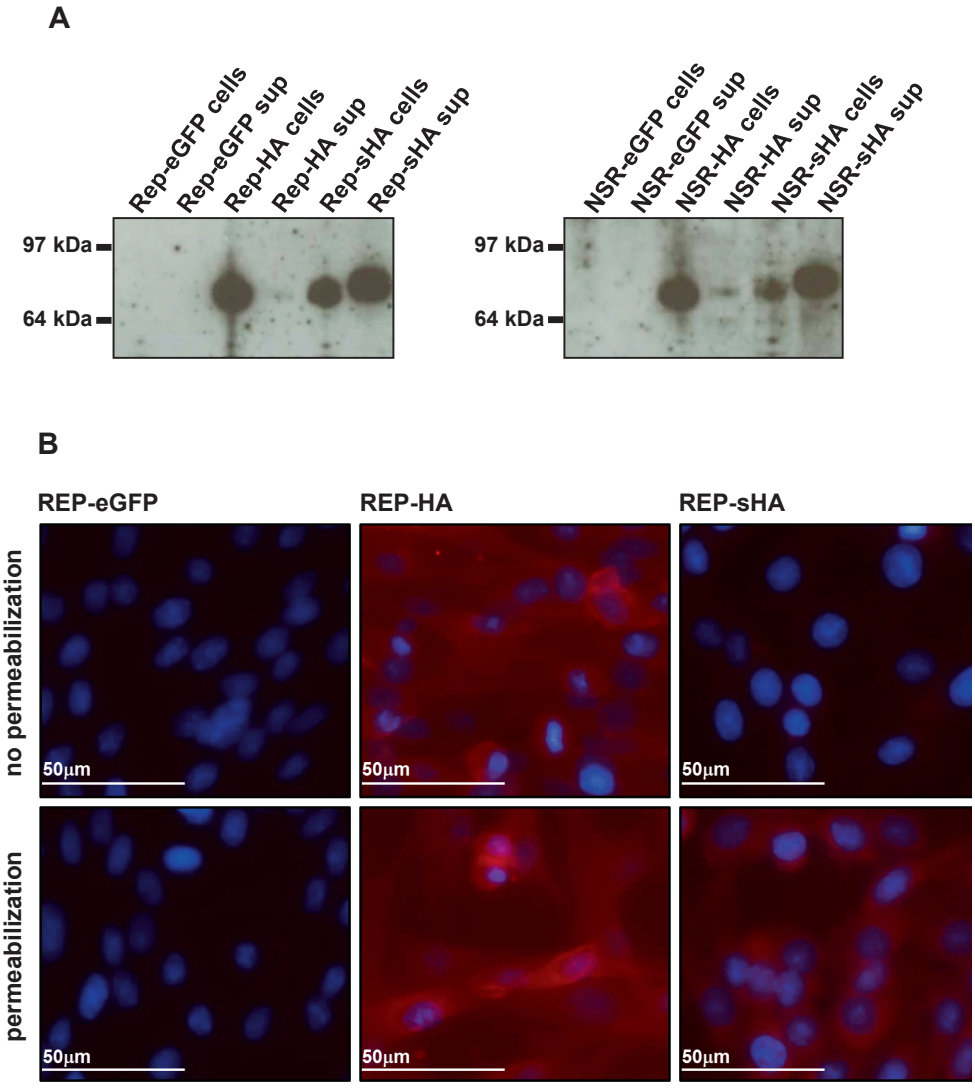
Oligomerization of the HA protein

To determine the oligomeric structure of the HA protein, we used a water-soluble cross-linker (BS3) that reacts with primary amines, thereby covalently linking proteins together. Exposure of HA oligomers to BS3 results in fixation of the oligomers so that they can be analysed on denaturating SDS-PAGE, followed by a Western blot. HA protein that was cross-linked with BS3 appeared as a single band around 200 kDa, while the native protein was denaturated and was detected at around 65 kDa.

Expression of HA genes after infection by NSR-HA and NSR-sHA

Infection of BHK cells with NSR-HA and NSR-sHA particles resulted in *de novo* expression of the HA proteins, as revealed by Western blotting analysis. The full-length HA protein was predominantly detected in the cell fraction and the sHA

protein in both cell and supernatant fractions (Fig. S2A, right panel). These results resemble those from analysis of the corresponding replicon cell lines.



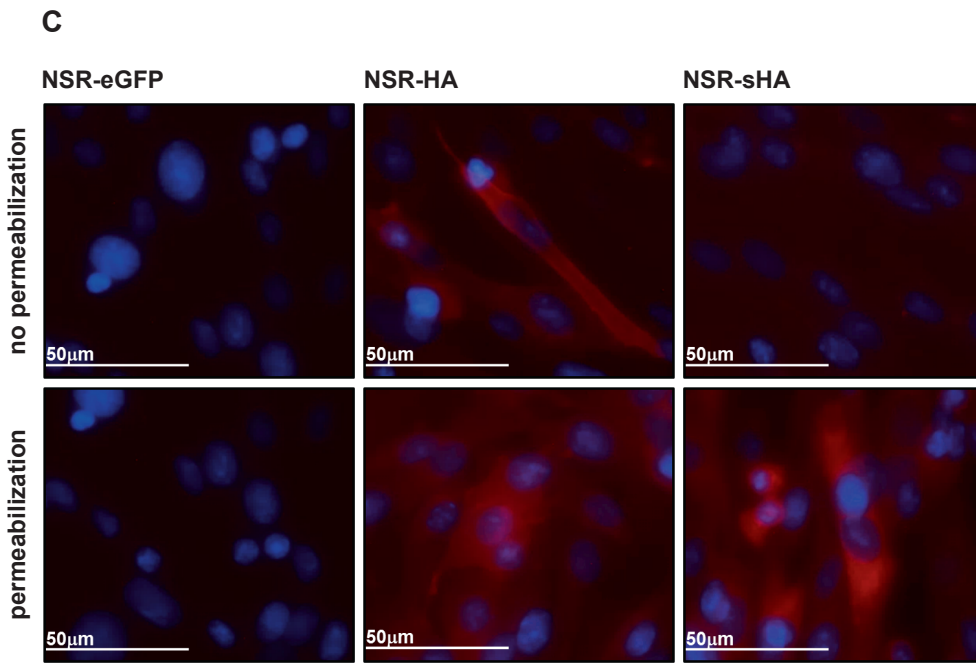


Figure S2. Expression of influenza virus HA proteins from the NSR genome. (A) Western blot analysis of lysates from replicon cell lines expressing eGFP, HA or sHA (left panel) and of BHK cells infected with NSR-eGFP, NSR-HA or NSR-sHA. HA proteins were visualized by a primary HA-specific polyclonal antibody and a secondary HRP-conjugated antibody. (B) Immunofluorescence of replicon cells expressing eGFP, HA or sHA and (C) BHK cells infected with NSR-eGFP, NSR-HA or NSR-sHA. Upper panels depict non-permeabilized cells and lower panels depict permeabilized cells. Cell nuclei were visualized with DAPI.

Cellular localization of HA proteins

The expression and cellular localization of HA and sHA proteins were further analysed by immunofluorescence. HA-specific staining of both permeabilized and non-permeabilized cells demonstrated that the full-length protein localizes to the cell surface (Fig. S2B and C, middle panels). As expected, the sHA protein was detected exclusively after permeabilization of the cells (Fig. S2B and C, right panels).

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6

Nonspreading Rift Valley fever virus infection of human dendritic cells results in downregulation of CD83 and full maturation of bystander cells

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Abstract

Vaccines based on nonspreading Rift Valley fever virus (NSR) induce strong humoral and robust cellular immune responses with pronounced Th1 polarization. The present work was aimed to gain insight into the molecular mechanisms of NSR-mediated immunity. Recent studies have demonstrated that wild-type Rift Valley fever virus efficiently targets and replicates in dendritic cells (DCs). We found that NSR infection of cultured human DCs results in maturation of DCs, characterized by surface upregulation of CD40, CD80, CD86, MHC-I and MHC-II and secretion of the proinflamatory cytokines IFN-β, IL-6 and TNF. Interestingly, expression of the most prominent marker of DC maturation, CD83, was consistently downregulated at 24 hours post infection. Remarkably, NSR infection also completely abrogated CD83 upregulation by LPS. Downregulation of CD83 was not associated with reduced mRNA levels or impaired CD83 mRNA transport from the nucleus and could not be prevented by inhibition of the proteasome or endocytic degradation pathways, suggesting that suppression occurs at the translational level. In contrast to infected cells, bystander DCs displayed full maturation as evidenced by upregulation of CD83. Our results indicate that bystander DCs play an important role in NSR-mediated immunity.

Introduction

Rift Valley fever virus (RVFV) replicon particles, also known as nonspreading RVFV (NSR), resemble authentic RVFV by structure and infectivity [1]. They retain the genes encoding proteins necessary for viral RNA amplification, but are deprived of the gene encoding the structural glycoproteins, required for the generation of progeny virions. In addition, NSR particles lack the gene encoding the nonstructural NSs protein, which counteracts innate immune responses [2-5]. The absence of the NSs gene adds to the safety profile of NSR and provides an expression slot for a protein of interest. These combined features render NSR an intrinsically safe and powerful platform for the development of vaccines.

NSR proved to be highly efficacious when used as a RVF vaccine both in mice and in sheep, the latter being the main natural target species of the virus [1, 6]. A single vaccination with similar replicon particles, developed by Dodd and co-workers, resulted in systemic induction of interferon-stimulated genes as early as 12 h post vaccination and initiation of an antiviral state that protected mice from lethal RVFV challenge already 24 hours post vaccination [7]. The efficacy of the NSR vaccine was further improved by introducing in the NSR genome the gene encoding the glycoprotein Gn, which is the dominant target of neutralizing

antibodies. A single vaccination with the resulting NSR-Gn vaccine provided sterile protection against RVFV challenge in lambs [8, 9]. More recently, we developed NSR particles encoding the hemagglutinin (HA) of the influenza virus. These particles protected mice from a lethal dose of influenza virus after a single intranasal or intramuscular administration [10]. Vaccination with NSR was consistently associated with neutralizing antibody responses and robust T-cell responses with strong Th1 polarization [1, 6, 8-10]. The ability of NSR to induce strong cellular immune responses was recently confirmed by controlling outgrowth of tumor cells in mice by vaccination with NSR particles that expressed a single tumor-associated CD8-restricted epitope [11]. The remarkable efficacy of the NSR vaccine prompted further studies on the molecular mechanisms of NSR-mediated immunity.

Recent findings by Lozach *et al.* demonstrated that wild-type RVFV can efficiently infect human DCs, using dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) as a receptor [12]. Infection of DCs resulted in generation of high titers of progeny virions. In another study, RVFV was shown to specifically target cells of the monocyte/macrophage/dendritic cell lineages in mice [13]. These data suggest that the interaction of RVFV with DCs plays an important role in the pathogenesis of RVF. Innate immune responses resulting from RVFV infection of bone marrow-derived macrophages are efficiently counteracted by the NSs protein [14], and it is plausible that NSs has a similar function in DCs. However, infection of DCs with NSR particles lacking NSs should result in full-blown antiviral responses, which likely contribute to vaccine efficacy.

DCs are key players in the initiation and regulation of immune responses. Immature DCs are equipped with a broad range of pattern recognition receptors and are very effective in recognizing various pathogen-associated molecular patterns (PAMPs). When contact with a PAMP occurs, DCs start to mature. During this process, the cells undergo changes in their morphology, migratory capability, expression of surface molecules and function [15]. The cells migrate from areas of antigen uptake to T-cell areas of secondary lymphoid organs, where they present antigen-derived peptides and instruct epitope-specific naïve T-cells to develop their effector function [16]. The maturation of DCs is associated with increased expression of surface molecules, such as MHC-I and MHC-II, which are involved in antigen presentation, as well as CD86, CD80, CD40 and CD54, which act as co-stimulators in T-cell activation [17, 18]. The most characteristic marker of fully matured human DCs is CD83 [19, 20]. Although the exact mechanism of action and the specific ligand of CD83 remain

to be elucidated, surface expression of this molecule on DCs is critical for priming naïve T cells [21, 22].

In the present study, we investigated the interaction between NSR and human DCs. We found that DCs are efficiently infected and tolerate viral genome replication and protein expression. The cells exhibited evidence of maturation, manifested by morphological changes, secretion of the proinflamatory cytokines IFN-β, IL-6 and TNF and upregulation of the surface molecules CD40, CD80, CD86, MHC-I and MHC-II. Surprisingly, while bystander DCs displayed upregulation of CD83, suggestive of full maturation, infected DCs exhibited a gradual downregulation of CD83. This effect was not associated with corresponding downregulation of CD83 mRNA or defects in mRNA transport from the nucleus. Neither the proteasomal nor the endocytic degradation pathways seemed to be involved in the decrease in CD83 levels, suggesting that NSR-mediated downregulation of CD83 expression occurs at the translational level. The incomplete maturation of NSR-infected DCs and full maturation of bystander DCs suggest that only the latter play an active role in NSR-mediated immunity.

Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and frozen until use. PBMCs were used as a source of monocyte-derived dendritic cells (MoDCs). Immature DCs were cultured as previously described [23]. Briefly, PBMCs were seeded in standard culture 48 or 96-wells plates in X-VIVO-15 medium with gentamycin (Lonza), supplemented with 2% heat-inactivated and 0.2 μm-filtered FCS (Bodinco). Cells were allowed to adhere for 1 h. Non-adherent cells were subsequently removed by washing with PBS and adherent cells were cultured in serum-free X-VIVO-15, supplemented with 450 U/mL GM-CSF and 300 U/mL IL-4 (Milteny). Medium was refreshed on the second day of incubation. On day 5, cells were stimulated with LPS, NSR or NSRmock diluted in RPMI 1640 with HEPES and glutamine (Gibco), supplemented with 10% FCS. As a negative stimulation, medium was used. The stimulation conditions were selected for optimal infectivity of NSR. DCs were harvested at different time points after stimulation as indicated in the results section, by replacing the growth medium with cold PBS, followed by shaking (450 rpm) of the culture plates for 1 h at 4ºC to detach cells.

Generation of nonspreading RVFV (NSR) and control inoculum (NSRmock)

NSR particles, were generated as previously described [8]. Briefly, NSR replicon cell lines were transfected with a plasmid encoding the RVFV surface glycoproteins Gn and Gc. NSR containing supernatants were harvested the next day and cleared from cell debris by centrifugation at 4500 x g for 15 min. Subsequently NSR particles were purified and concentrated by ultracentrifugation at 64,000 x g for 2.5 h on a 2 ml 25% sucrose cushion, followed by resuspension in Opti-MEM (Invitrogen), supplemented with 0.2% heat-inactivated FCS. Particles were stored at -80ºC until use. For generation of NSRmock, a similar procedure was used, but the replicon cells were transfected with a plasmid that encodes only the Gc protein. Supernatants, harvested after this transfection contain the same media, transfection reagents and cellular metabolism products, but lack infectious NSR particles. The absence of infectious particles in NSRmock control preparation was confirmed by titration on BHK21 cells.

Flow cytometry

The cell-surface phenotypes of unstimulated and stimulated DCs were analysed by flow cytometry using human-specific mAbs: anti-CD40 (clone 5C3) and anti-CD83 (clone HB15e) (eBioscience); anti-CD80 (clone L307.4), anti-CD86 (clone IT2.2) and anti-CD11c (clone B-ly6) (BD); anti-HLA-A,B,C (clone W6/32) and anti-HLA-DR (clone L243) (BioLegend), together with the respective isotype controls. Optimal concentrations of the antibodies were determined prior to flow cytometry assays. The DC population was selected by gating on cells that were double positive for CD11c and MHC-II. Median fluorescence intensity (MFI) was used as measure for expression of the analysed molecules. Data was acquired using Canto II flow cytometer (BD) and analyzed using FlowJo software.

Cytokine assay

PBMCs were seeded in 48-well culture plates and after an initial adherence step for 1 h as described above, were washed vigorously with PBS so that only adhering cells were retained. After 5 days of differentiation, DCs were stimulated with LPS, NSR, NSRmock or medium and after 24h supernatants were harvested, pre-cleared at 6,000 rpm for 10 min and stored at -80ºC until use. Cytokine concentrations were determined using a multiplex assay (eBioscience), according to the manufacturers' instructions using the Luminex 200 system.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting

DCs were harvested 24 h post stimulation, counted and brought to equal concentrations in Pierce IP lysis buffer (Thermo Scientific), supplemented with protease inhibitors (Roche). Samples containing 50,000 cells were either directly denatured in standard Laemmli sample buffer or first pre-treated with peptide-N-Glycosidase F (PNGase F, BioLabs® Inc.) according to the manufacturers' instructions. Proteins present in cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting as previously described [10]. Rat monoclonal anti-human CD83 antibody (clone 1G11, Enzo Life Sciences) and corresponding secondary horseradish peroxidase (HRP)-conjugated antibody were used to detect CD83. The blot with samples without PNGase F treatment was subsequently stripped and re-stained with mouse monoclonal anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 0411) and anti-GFP (clone B-2) antibodies (Santa Cruz) and corresponding secondary HRP-conjugated antibodies.

ELISA to detect soluble CD83

Supernatants from DCs cultures, stimulated with LPS or NSR were harvested and centrifuged at 3,000 x g for 10 min to remove cell debris. Concentrations of soluble CD83 were determined with a commercial ELISA kit (Sino Biological Inc.) according to the manufacturers' instructions. Each sample was tested at two different concentrations in triplicate. A standard curve was generated using serial dilutions of recombinant CD83, provided with the kit.

Proteasome inhibition assay

DCs were stimulated with different stimulae for 8 h after which clasto Lactacystin β-lactone (CLBL) was added at a final concentration of 5 or 10 μM. The solvent of CLBL, dimethylsulfoxide (DMSO), was used as a control. Sixteen h after these treatments, cells were harvested and analyzed by flow cytometry.

Endocytosis inhibition assay

DCs were stimulated with LPS+NSRmock, LPS+NSR or medium, for 6 h or 12 h. Cytochalasin D (Sigma) was subsequently added to a final concentration of 10 μg/ml. The solvent of Cytochalasin D, dimethylsulfoxide (DMSO), was used as a control. Cells were harvested 6, 12 or 18 h after Cytochalasin D/DMSO treatment and analyzed by flow cytometry.

RNA isolation and real-time PCR

After stimulation of DCs for 24 h, culture medium was discarded and cells were immediately lysed with Trizol. Total RNA was isolated with the Direct-zol™ RNA MiniPrep kit (Zymo research) according to the instructions of the manufacturer. 100 ng RNA of each sample was subsequently reverse-transcribed using random primers and Superscript III reverse transcriptase (Promega). Quantitative real-time PCR was performed as previously described [24]. The primer sequences are enlisted in Supplementary Table 1.

Single-molecule RNA fluorescence in situ hybridization (FISH)

DCs were cultured on a CultureWell™ 16 Chambered Coverglass (C-37000, Grace Bio-labs) and stimulated as indicated. 24 h post stimulation cells were fixed with 4% paraformaldehyde (10 min) and permeabilized with 70% ethanol (>1 h at 4°C). Individual Intracellular GAPDH, CD80 and CD83 mRNAs were subsequently visualized using human specific CD80, CD83 and GAPDH cDNA probes labelled with quasar 570 (Supplementary Table 2) according to the Stellaris® FISH method (protocol for adherent cells, Biosearch Technologies) [25]. The oligonucleotides for GAPDH were predesigned by Biosearch Technologies, whereas the oligonucleotides for CD80 and CD83 were designed using the online Stellaris probe design software. Images were generated using an Axioskop 40 (Zeiss) fluorescent microscope with a 1.28 NA 100× oil objective and an AxioCam MRm camera. Raw images were deconvolved and analyzed using Huygens software (Scientific Volume Imaging, Hilversum, The Netherlands).

Statistical analysis

Differences in CD83 surface expression levels were analysed with a Student's T test for paired samples. Differences in mRNA levels of CD80, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and peptidylprolyl isomerase A (PPIA) and differences in CD83 expression in the endocytosis inhibition assay were analyzed using an unpaired Student's T test. Values of $p<0.05$ were considered significant. Analyses were performed with GraphPad Prism® 5.04 software.

Results

NSR infection of human DCs triggers phenotypic maturation

Immature DCs were generated as described in materials and methods and were infected with NSR particles expressing GFP (Fig. 1A). Initial experiments

revealed that GFP expression in DC cultures was detectable already at 6 h post infection (hpi, data not shown). Infection efficiencies varied between different experiments and donors, but exceeded 90% under optimal conditions (data not shown).

Monitoring of NSR infection in time revealed that the number of GFP-positive (GFP+) DCs increased gradually until 24 hpi (Fig. 1B). After that time point, a rapid decrease was noticed and at 48 hpi a 5-fold reduction was observed, compared to GFP+ cell counts at 24 hpi. The decrease in GFP+ cells coincided with a decrease in the total numbers of viable cells.

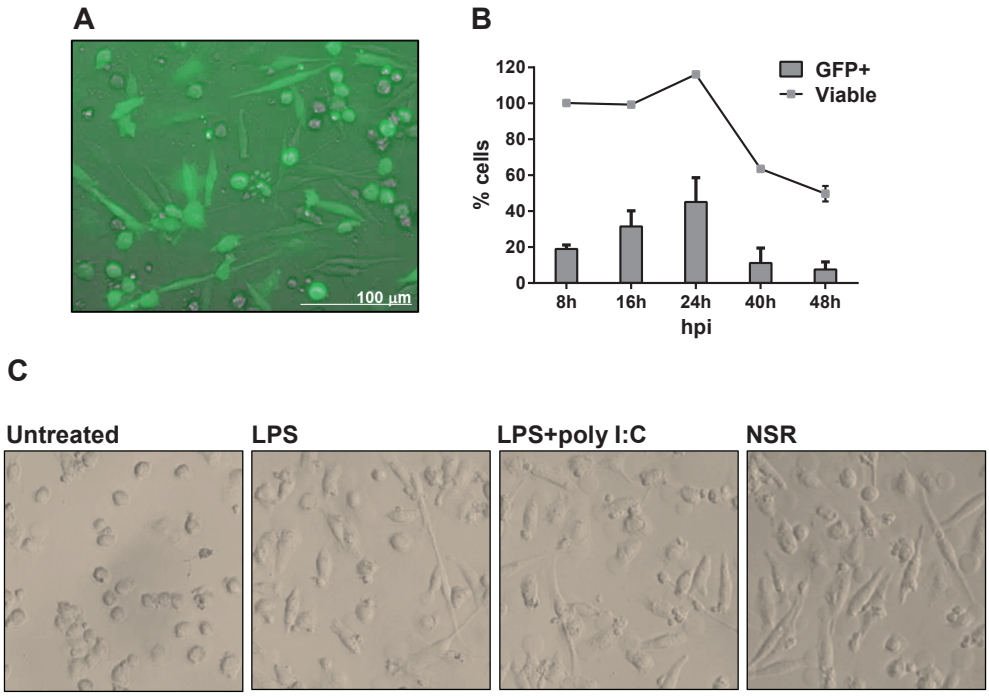

Figure 1. Infection of DCs by NSR. (A) DCs were infected with NSR (left panel) or mock infected (right panel) for 24 h and evaluated for expression of GFP, using EVOS fluorescent microscope. (B) Viability and percentage of infected cells at different time points after infection. Cells were infected with NSR, harvested at the indicated time points and analyzed by flow cytometry for GFP expression (bars) and viability (line) after staining with 7AAD. Viability of the cells was calculated relative to the viability at 8 hpi, which was set at 100%. The data depicts average values from two experiments with cells from two different donors ±SD. (C) Morphology of DCs stimulated with the indicated stimuli at 24 h post treatment.

Infection of DCs with NSR resulted in distinct morphological changes in the cells. Infected DCs acquired flat and stretched shapes, discriminating them from

untreated cells, which remained predominantly round-shaped (Fig. 1C). The phenotype of the infected cells resembled closely that of cells treated with LPS or a combination of LPS and poly(I:C), which are known to trigger DCs maturation [26]. This finding suggests that NSR infection of DCs results in maturation.

Infected DCs secrete proinflammatory cytokines

To evaluate whether NSR infection results in the induction of a proinflamatory response, supernatants of infected DCs were analyzed for the presence of IFN-β, TNF, IL-6 and IL-10. Infection was performed such that more than 90% of the cells were positive for GFP. As expected, control LPS stimulation resulted in the induction of IFN-β, TNF and IL-6, while NSRmock-infected cells did not show any induction of cytokines (Fig. 2). NSR-infected DCs showed relatively strong induction of IFN-β, TNF and IL-6. In contrast, concentrations of IL-10, which is known for its immunosuppressive functions [27, 28] remained low. These results show that NSR is capable of inducing proinflamatory cytokine response in DCs.

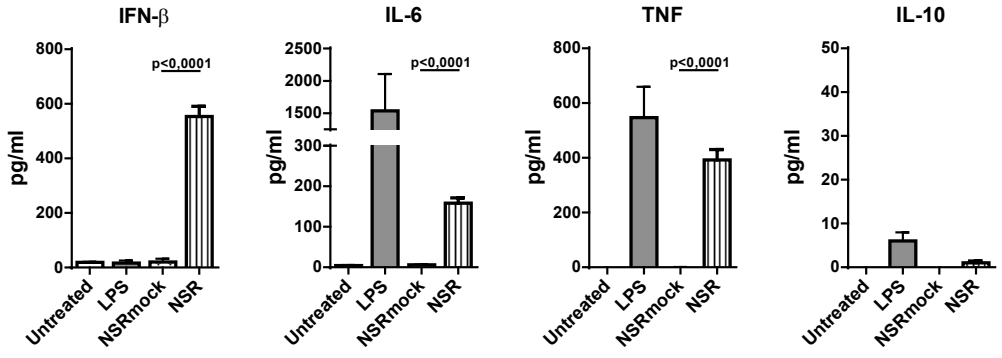

Figure 2. Cytokine secretion by NSR-infected DCs. Supernatants of infected or control-treated DCs were harvested at 24 hpi and analyzed with a luminex-based assay. Bars represent the mean cytokine concentrations ± SD of triplicates with cells from one donor. Statistical significance between infected (NSR) and mock-infected (NSRmock) conditions is indicated (Student's *t* test)

NSR-infected DCs upregulate maturation markers, but only bystander DCs upregulate CD83

Maturation of DCs is associated with upregulation of the surface expression of MHC class I and II molecules and co-stimulatory molecules such as CD80, CD86 and CD40 [17, 18]. The hallmark of mature human DCs is the de novo presentation of CD83 at the cell surface. To test the maturation status of

infected DCs, we analyzed surface expression of MHC-I, MHC-II, CD40, CD80, CD83 and CD86 molecules upon infection.

Flow cytometry analysis of infected, GFP+ DCs at 24 hpi revealed a strong induction of CD80 surface expression when compared to untreated cells or cells incubated with NSRmock (Fig. 3, left and middle panels). A less prominent but still distinct increase was observed in CD40 and MHC-II surface expression, while upregulation of CD86 and MHC-I was minor. Expression levels of all markers in NSR-infected cells resembled (CD80, CD86, MHC-I) or slightly exceeded (CD40, MHC-II) those observed in LPS-stimulated cells. Strikingly, although CD83 expression was strongly upregulated 24 h after LPS stimulation, no upregulation of CD83 was observed in GFP+ cells at this time point.

To evaluate whether the maturation triggered by LPS can counteract the downregulation of CD83 in NSR-infected cells, LPS stimulation was performed simultaneously with NSR infection. Combination of LPS with NSRmock resulted in upregulation of CD83 at the cell surface comparable to that observed after treatment of DCs with LPS only (Fig. 3, right panels). However, incubation of DCs with both LPS and NSR did not result in upregulation of CD83. In contrast, co-inoculation with LPS and NSR resulted in additional upregulation of CD40, CD80, MHC-I and MHC-II surface expression.

Analysis of DCs that were GFP-negative (GFP-) at 24 hpi, revealed that the levels of all surface molecules were elevated when compared with the negative controls, presumably representing a bystander effect resulting from cytokines released by GFP+ cells (Fig. 3). Addition of LPS resulted in further upregulation of all maturation markers.

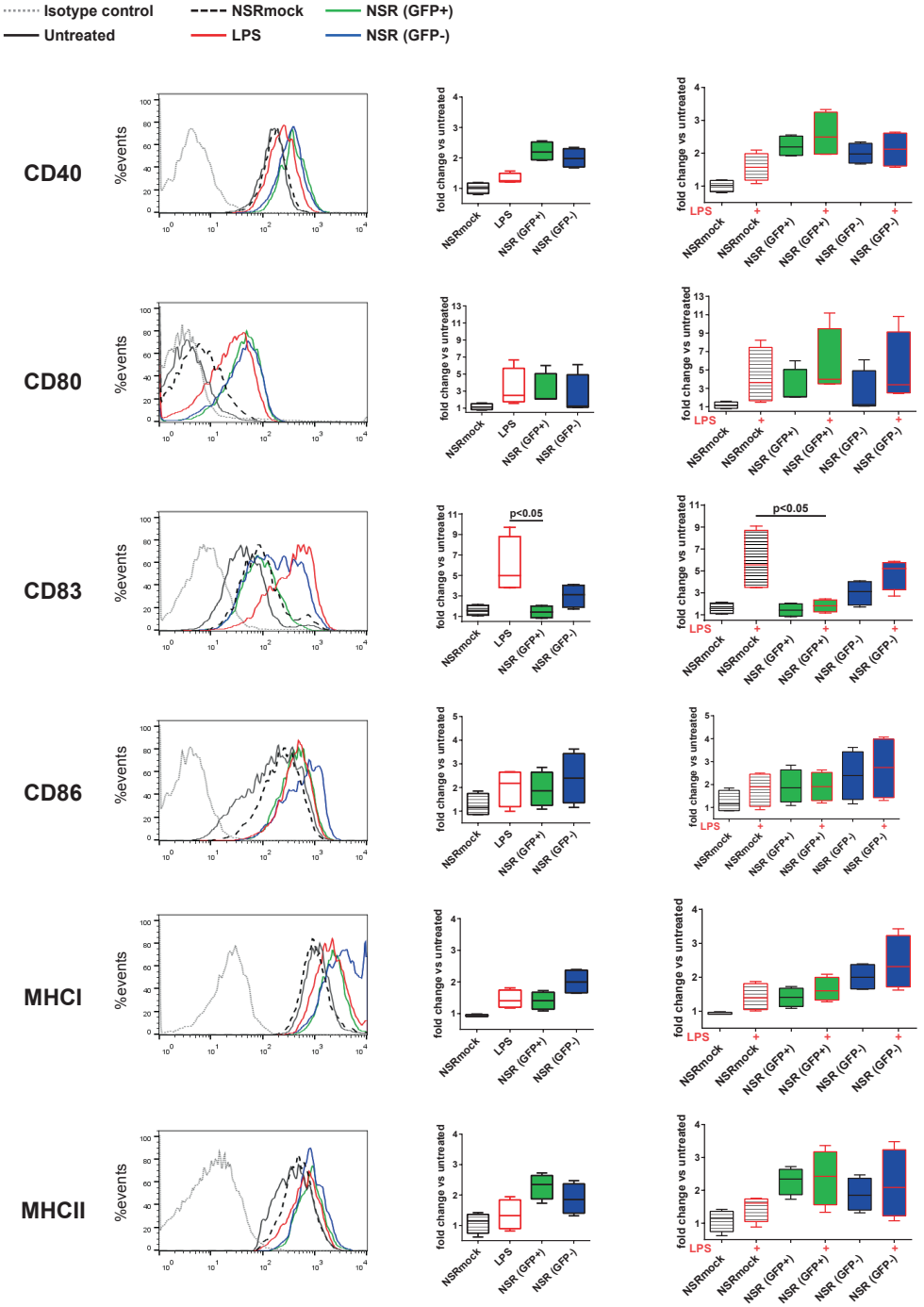

Figure 3. Surface expression of CD40, CD80, CD83, CD86, MHC-I and MHC-II on DCs at 24 h after NSR infection as measured by flow cytometry. Immature DCs were infected with NSR, mock-infected with NSRmock, or stimulated with LPS (left and middle panels). All surface markers in NSR- infected cells are upregulated except CD83. Incubation of DCs with NSR+LPS resulted in an additional increase

in expression of the surface markers (right panels). However, LPS is not able to rescue expression of CD83 in NSR-infected cells. The left panel shows representative histograms of surface markers on cells stimulated with LPS, mock infected cells (NSRmock), cells infected with NSR (GFP+) and uninfected bystander DCs (GFP-). Expression of markers in untreated cells and an irrelevant isotype control are depicted. The middle panels depict summarized data from 4 independent experiments performed with cells from 3 donors. Data in the right panels further elaborates on the data in the middle panel, comparing single NSR treatments with combined LPS and NSR treatments. The box plots depict MFI of the different markers relative to untreated cells. Statistically significant differences of CD83 expression levels are indicated (paired T test).

Kinetics of CD83 expression in NSR-infected DCs

To investigate the dynamics of CD83 surface expression in more detail, DCs were harvested after 4, 8, 12, 16, 24 and 48 hpi and analyzed with flow cytometry. As a reference marker for upregulation, CD80 was used. NSRmock treated cells did not display changes in CD83 surface expression during the whole observation period, while in LPS treated cells upregulation of CD83 was observed already after 4 h and expression levels remained high until the end of the observation period (Fig. 4, upper panel). Similar expression levels were observed after combined LPS+NSRmock treatment. In NSR infected cells, the GFP signal was detectable at 8 hpi. In GFP+ cells, an initial upregulation of CD83 was observed which peaked at 12 hpi. After that time point, CD83 levels decreased gradually and at 24 hpi reached the levels of the NSRmock control. Contrastingly, in GFP- cells, CD83 expression levels were initially comparable to those of control cells and at 12 hpi levels increased and remained high, revealing that CD83 upregulation in bystander DCs depended on the presence of GFP+ cells. When a combination of LPS and NSR was used to stimulate DCs, maximal upregulation of CD83 in GFP+ cells was reached already at 8 hpi, consistent with the presence of LPS. However, as time progressed, CD83 expression decreased, reaching the lowest levels at 24 hpi. In GFP- cells, CD83 levels resembled those in cells treated with LPS and LPS combined with NSRmock. These data indicate that infection with NSR results in initial upregulation of CD83 that is accelerated by the presence of LPS. Later, as viral genome replication advances, CD83 is gradually depleted from the cell surface, counteracting the effect of LPS. Notably, CD83 was dramatically upregulated at 48 hpi in all stimulated cells, including those positive for GFP.

In contrast to CD83, CD80 displayed a gradual upregulation in cells infected with NSR and in cells treated with a combination of LPS and NSR, regardless of the expression of GFP. The dynamics of CD80 expression in GFP+ cells resembled closely that in cells treated with LPS or LPS+NSRmock (Fig. 4, lower panel). A strong increase in the surface expression of CD80 was observed at 48 hpi similar to CD83.

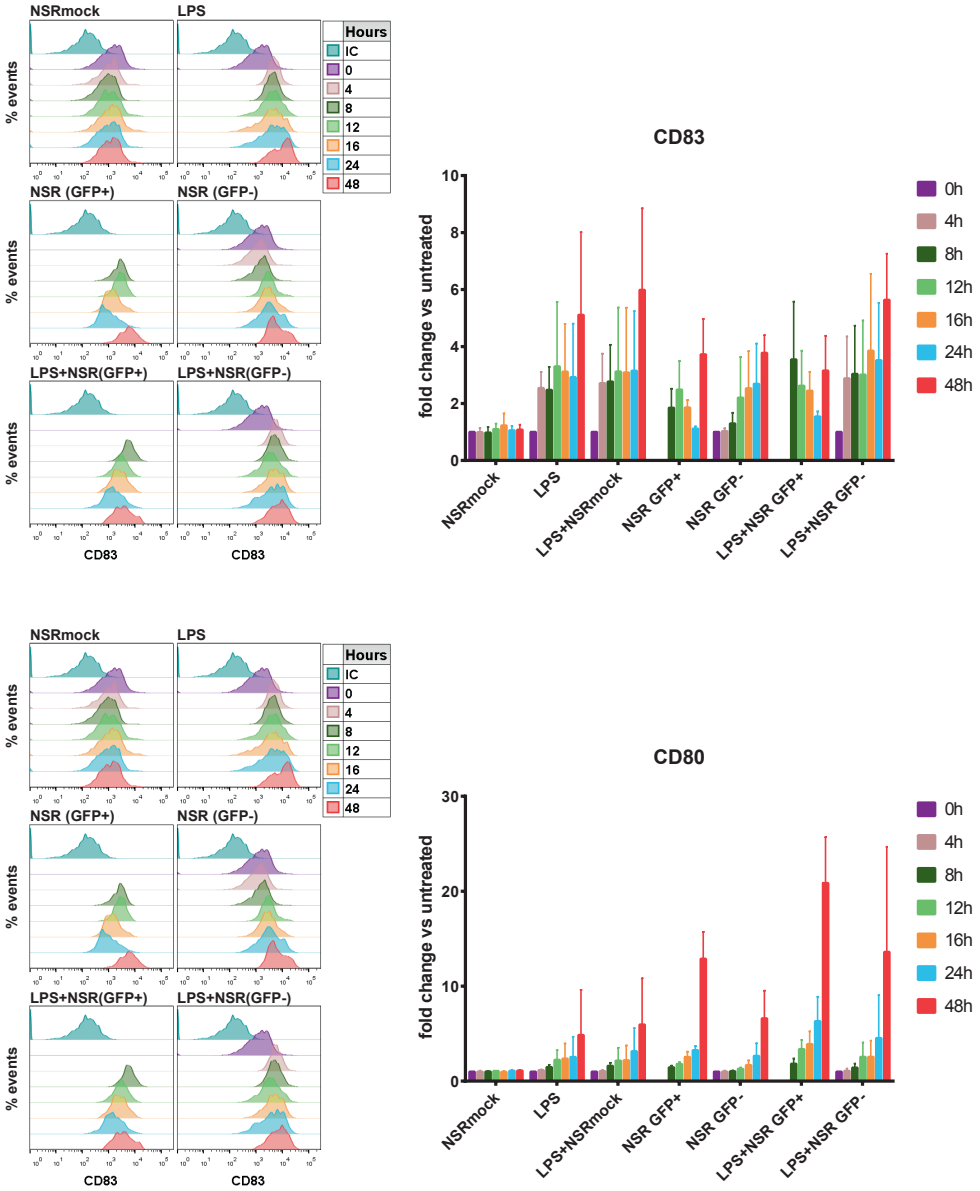

Figure 4. Analysis of CD83 and CD80 surface expression in time. DCs were treated with NSRmock, NSR, LPS, LPS+NSRmock or LPS+NSR and were harvested at 0, 4, 8,12, 16, 24 and 48 h post treatment. Surface expression of CD83 (upper panels) and CD80 (lower panels) were measured by flow cytometry. Left panels show histograms from one representative experiment. Time points are depicted with different colors and the color code is shown at the right. IC – isotype control. Right panels illustrate summarized data from three independent experiments with cells from three different donors. Bars represent means ±SD of the fold change of MFI relative to untreated cells.

CD83 mRNA levels and subcellular distribution are unaffected by NSR-infection

To evaluate whether the reduced CD83 surface expression observed after NSR infection correlated with reduced mRNA levels, we analyzed with qRT-PCR the quantities of CD83 mRNA in DC lysates prepared 24 h post NSR or LPS+NSR treatments, resulting in more than 90% GFP-positive cells. Levels of two house-keeping gene mRNAs, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and peptidylprolyl isomerase A (PPIA), and the level of CD80 mRNA were determined as well and served as controls. The results show that neither NSR infection, nor a combination of LPS stimulation and NSR infection significantly affected the total levels of CD83 mRNA, as compared to control treatments (Fig. 5). In contrast, levels of CD80 mRNA were upregulated by both NSR and LPS+NSR treatments, as well as by LPS treatment alone, respective to the relevant controls. Strikingly, incubation of the DCs with NSR or LPS+NSR resulted in significant decrease in mRNA levels of GAPDH and PPIA.

To be able to discriminate between GFP+ and GFP- in the DC population and to exclude possible arrest of host mRNA nuclear transport, which is a common mechanism used by viruses to counteract cellular antiviral mechanisms [29], we also evaluated subcellular location of GAPDH, CD80 and CD83 mRNAs using a fluorescence in situ hybridization (FISH) technique. With this technique, individual mRNA molecules are visualized, revealing their total amount and cellular location. The overall FISH results corresponded very well with the qRT-PCR data (Fig. 6). The significant decrease in the quantity of GAPDH mRNA, detected with qRT-PCR in NSR infected cells, correlated with strongly reduced number of spots, detected with FISH in GFP+ cells. Interestingly, no reduction in GAPDH mRNA was observed in GFP- cells. CD80 mRNAs levels were induced by LPS stimulation, as well as by NSR infection, both in GFP+ and GFP- cells. Analysis of CD83 mRNA revealed similar total number of spots in all treatment conditions and no differences in mRNA distribution. Collectively, the FISH and qRT-PCR data reveal that the downregulation of the CD83 protein observed after NSR infection was not associated with downregulation of CD83 mRNA levels or arrest of CD83 mRNA transport from the nucleus.

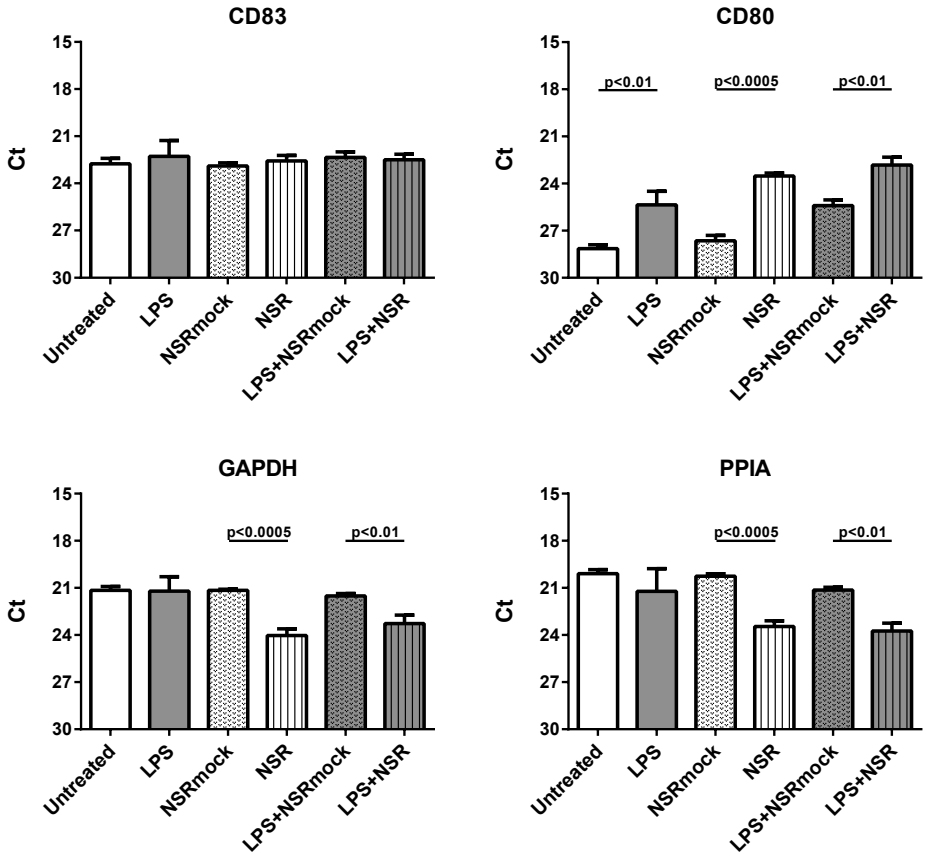

Figure 5. Analysis of mRNAs quantities of CD83, CD80, GAPDH and PPIA. Cell lysates were prepared 24 h after different treatments as indicated and analyzed with qRT-PCR. Bars represent average Ct values from triplicates ±SD with cells from one donor. The experiment is representative for 3 independent experiments with cells from 3 different donors. Relevant statistical significance is indicated (Student's t test).

Surface downregulation of CD83 does not result from release into the growth medium

Sénéchal and co-workers reported downregulation of CD83 in monocyte-derived mature DCs infected with human cytomegalovirus [30]. The downregulation was attributed to release of the protein into the culture medium. This finding was confirmed by Kummer *et al.* [31]. Regarding those reports, we investigated whether the decrease of CD83 downregulation in NSR infected cells resulted from shedding of the protein from the cell surface. To this end, supernatants of

NSR-infected cells and of cells treated with LPS were harvested at 24 hpi and analyzed for the presence of soluble CD83 by ELISA. Quantities of CD83 in the supernatant of LPS stimulated cells were comparable to those previously reported (Fig. 7A) [31]. No difference in soluble CD83 was found between LPS-treated cells and NSR-infected cells, demonstrating that shedding of CD83 into the growth medium does not explain the observed surface downregulation in NSR-infected DCs.

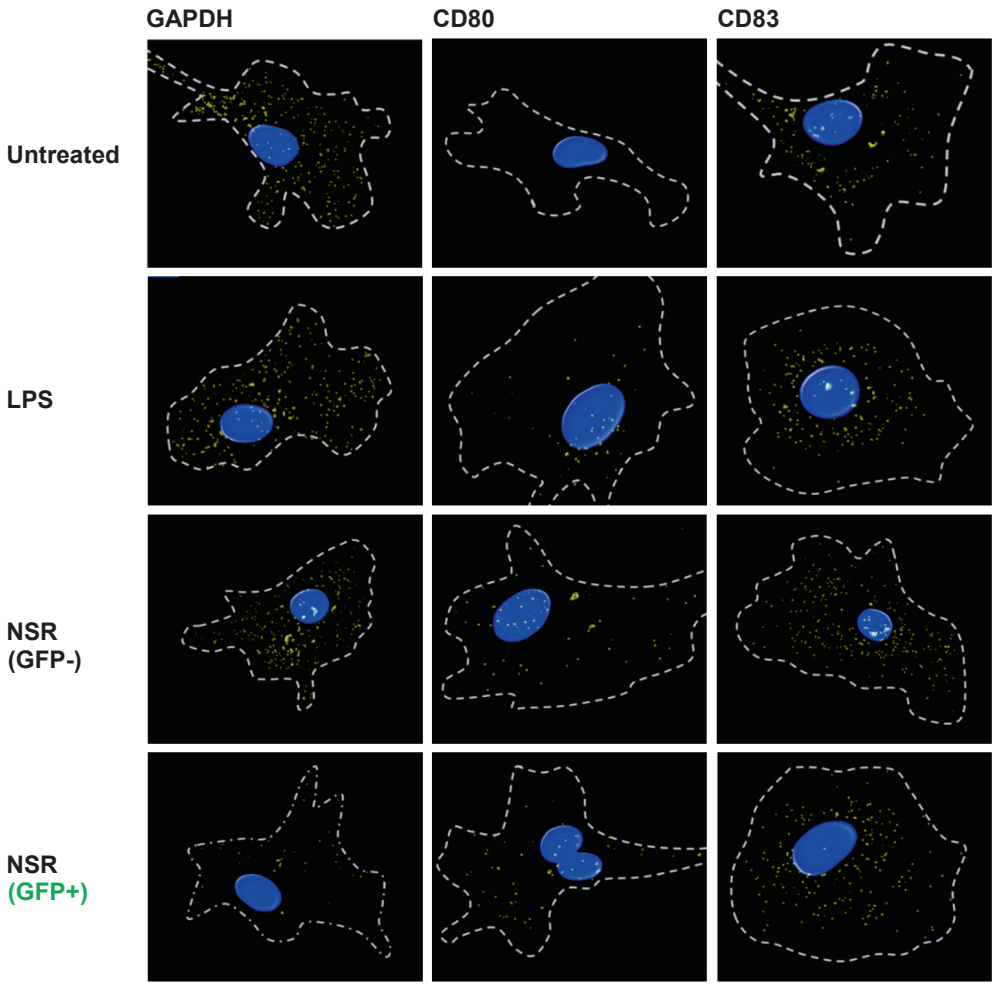

Figure 6. Visualization of GAPDH, CD80 and CD83 mRNAs with fluorescence *in situ* hybridization (FISH). DCs were stimulated for 24 h with LPS, infected with NSR or left untreated and then fixed and subjected to FISH. Shown are representative cells of each treatment condition from two independent experiments with cells from two different donors.

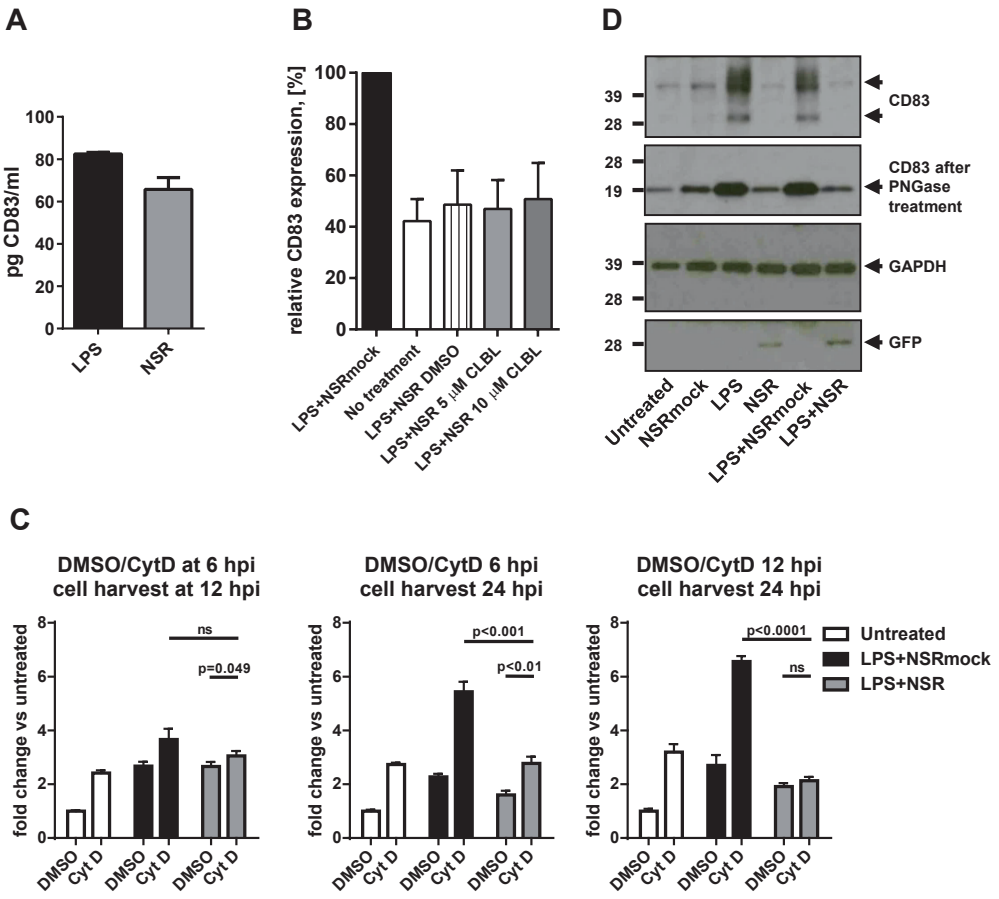

Figure 7. Effect of NSR infection on intracellular and extracellular CD83 levels. (A) Levels of CD83 were determined by ELISA of supernatants from cells harvested 24 h after stimulation with LPS or infection with NSR. Shown is one of two independent experiments performed with cells from two different donors, with similar results. (B) Flow cytometry analysis of CD83 surface expression after inhibition of the proteasome. DCs were stimulated with LPS+NSRmock or LPS+NSR for 8 h and then clasto Lactacystin β-lactone (CLBL) was added at two final concentrations. Control cells were left untreated or treated with DMSO. Cells were analysed at 24 hpi for CD83 expression. Bars represent percentage of MFI in LPS+NSR infected cells, relative to LPS+NSRmock. Averaged values ±SD of two experiments with cells from one donor are shown. (C) Inhibition of endocytosis. DCs were stimulated with LPS+NSRmock, LPS+NSR or left unstimulated. Cytochalasin D (Cyt D) or the solvent DMSO were subsequently added at different time points. The moments of adding Cyt D/DMSO and harvesting of cells are indicated above each graph. Bars represent average fold change of the MFI relative to unstimulated cells treated with DMSO ±SD. Averaged values of three experiments with cells from one donor are depicted. Relevant statistical significances are shown (Student's *t* test) (D) Detection of CD83 in cell lysates by Western blot at 24 hpi. The different treatments are shown above the top panel and the probed proteins are depicted at the right. Molecular weight protein standard is shown at the left. The top blot was stripped and re-probed with antibodies against GAPDH and GFP, which served as loading control and control to confirm NSR infection, respectively. Shown is one out of two independent experiments with cells from two donors.

Inhibition of the proteasomal or endocytic degradation pathways does not prevent NSR-mediated downregulation of CD83

Mature human DCs, infected with herpes simplex virus type 1 (HSV-1), were reported to downregulate CD83 by proteasomal degradation [31]. This process is mediated by the immediate-early protein ICP0 of HSV-1 and is prevented by inhibition of the cellular proteasome machinery. To investigate whether proteasomal degradation of CD83 explains the decreased surface exposure of this molecule after NSR infection, we used the inhibitor clasto Lactacystin β-lactone (CLBL) to supress cellular proteasomal activity. This drug acts selectively and irreversibly on the 20S and 26S subunits of the proteasome without affecting serine and cysteine proteases. We preferred CLBL because earlier experiments demonstrated that this drug did not exert negative effect on RVFV replication, as opposed to another often used alternative drug, MG-132 [2]. As we already showed that CD83 levels increase in the first 8-12h after infection and then gradually decrease, we first incubated DCs with LPS+NSR or LPS+NSRmock for 8 h and then added CLBL in two different concentrations. DMSO, the solvent of CLBL, was used as a control. Analyses were performed at 24 hpi. Addition of CLBL or DMSO to LPS+NSR stimulated cells did not have an effect on CD83 surface expression as compared to LPS+NSRmock stimulated cells (Fig. 7B).

Stable levels of CD83 at the DC surface are maintained by continuous production and recycling of the exposed protein in both immature DCs and mature DCs [32]. Inhibition of endocytosis with cytochalasin D (Cyt D) was shown to promote surface exposure of CD83 in both DCs types. CytD is a drug that depolymerizes F-actin filaments and thereby blocks endocytosis, without affecting the exocytotic pathway [33-35]. Cyt D treatment of infected cells was therefore employed to investigate possible involvement of endocytic degradation pathways in NSR-mediated downregulation of CD83. LPS+NSR co-stimulated cells, control LPS+NSRmock stimulated cells and untreated cells were incubated with Cyt D. Treatment with Cyt D for 6 h, added 6 h after stimulation resulted in an increase in surface-exposed CD83 in all stimulation conditions. However, in infected cells, this increase was the lowest (Fig. 7C, left panel). Longer incubation with Cyt D (18 h) resulted in a further increase of the CD83 quantities, but CD83 levels in cells co-stimulated with LPS+NSR were significantly lower than those in LPS+NSRmock treated cells (Fig. 7C, middle panel). Importantly, when Cyt D was added at 12 h post treatment, it had no effect on CD83 expression levels in infected cells, while in control cells an increase in expression was observed (Fig. 7C, right panel). The combined results

suggest that CD83 downregulation in NSR-infected cells does not involve increased degradation via the proteasomal or endocytic degradation pathways.

CD83 protein levels are strongly reduced in cell lysates of NSR-infected DCs

Finally we evaluated levels of total CD83 protein in cell lysates. As CD83 expression was first induced and then downregulated in NSR infected cells, we hypothesized that these alterations may result from trapping of the molecule inside the cells. Alternatively, translational suppression can lead to the observed downregulation. To test these hypotheses, we analyzed with SDS-PAGE and Western blotting the quantities of CD83 in cell lysates of NSR-infected cells and LPS+NSR co-stimulated cells and compared these with CD83 quantities in untreated cells, cells stimulated with NSRmock, LPS or LPS+NSRmock at 24 hpi. More than 90% of the infected DCs were positive for GFP. In untreated and NSRmock treated cells, a single band of around 42 kDa was visible, corresponding to the known molecular weight of CD83 when present in intracellular protein pools [32] (Fig. 7D). Upon induction with either LPS or LPS+NSRmock, CD83 expression was upregulated as evidenced by the appearance of a lower molecular weight band that corresponds to de novo synthesized CD83, as well as higher molecular weight bands that correspond to high-glycosylated, surface-exposed protein [32]. In both NSR and LPS+NSR treated cells, the detected CD83 protein levels closely resembled those in the untreated and NSRmock treated cells and only the band that corresponds to the preformed protein was visible, while the bands corresponding to the de novo form and the high-glycosylated form were not detected. Digestion of cell lysates with peptide-N-glycosidase F to remove the N-linked carbohydrates revealed a discrete band of deglycosylated protein. Amounts were comparable in untreated, NSRmock, NSR and LPS+NSR treated cells and much higher in cells treated with LPS or LPS+NSRmock. The total CD83 protein quantities in cell lysates correlated well with those measured by flow cytometry. This finding suggests that the observed downregulation of CD83 from the cell surface of NSR infected cells at 24 hpi does not result from trapping of the protein inside the cells, but rather from suppression of protein translation by an as yet unknown mechanism.

Discussion

DCs express various pathogen recognition receptors that are able to sense viral RNA, including Toll-like receptors 3, 7 and 8, and cytoplasmic helicases RIG-I, MDA5 and LGP2 [36]. Of these receptors, RIG-I was shown to play a primary role in cytoplasmic detection of RVFV [37]. Upon recognition of its ligand, RIG-I triggers a signaling cascade resulting in activation of transcription factors NF-κB, IRF3 and 7 [38, 39], which induce cell maturation [40] and production of type-I interferons [41]. This whole cascade of events is purposed to control virus replication in the infected DC and to prepare the cell for efficient antigen presentation. A well-established mechanism to control virus dissemination from infected cells is apoptosis, which can be initiated by RIG-I activation [42]. Based on the aforementioned, we propose that apoptosis of NSR-infected DCs at 48 hpi results from RIG-I activation.

Induction of apoptosis was previously reported to occur upon infection of DCs with replicon particles of the alphavirus Venezuelan equine encephalitis virus [43, 44], which have proven to be highly immunogenic [45]. Moreover, apoptosis of DCs was found to be essential for optimal efficacy of alphavirus replicon-based DNA vaccines in a tumor challenge mouse model [46]. DCs that go into apoptosis as a result of infection with replicons can serve as a source of antigen for cross-presentation, a mechanism that plays an important role in efficient priming of specific CD8+ responses [46, 47].

We observed that NSR-infected DCs did not fully mature, while bystander DCs did, as evidenced by CD83 upregulation. Maturation of bystander DCs is triggered by the presence of apoptotic infected DCs and cytokines and chemokines released as a result of the infection [48-50]. In supernatants of infected cells we detected IFN-β, TNF and IL-6, which are important for activation of antigen-presenting cells [51, 52] and are likely involved in the observed maturation of the bystander cells. Additionally, type-I interferons were shown to augment the efficiency of cross-presentation [53]. Full maturation of bystander cells therefore suggests that these cells play a critical role in the priming of T-cell responses via cross-presentation of antigens, acquired from apoptotic infected cells. In accordance to this assumption, we hypothesize that the GFP+ cells with restored levels of CD83 at 48 hpi represent mature bystander DCs that acquired GFP by phagocytosis of apoptotic NSR-infected DCs.

A surprising finding was the gradual downregulation of CD83 in NSR-infected DCs. Interestingly, NSR infection also prevented CD83 upregulation by LPS.

Downregulation of CD83 is, however, not an unusual consequence of virus infection of DCs. In HIV-1 infected DCs, a reduced surface exposure of CD80, CD86 and CD83 was observed, which was shown to result from downregulation of the respective mRNAs, mediated by the Vpr protein [54]. Human cytomegalovirus infection of mature DCs caused a decreased surface expression of CD83 which coincided with detection of a soluble form of this molecule in the cell culture medium [30]. Herpes simplex virus 1 infection of mature DCs resulted in rapid proteasomal degradation of CD83, mediated by the viral immediate-early protein ICP0 [31, 55]. In all cases, CD83 downregulation correlated with a reduced capacity to stimulate naïve T cells, revealing that targeting of CD83 is employed by these viruses as a specific means of immune evasion.

In NSR-infected cells, CD83 downregulation was not correlated with decreases in mRNA levels. The similar amounts of CD83 mRNA in DCs regardless of the maturation state are consistent with the mRNA pools previously described by Kruse *et al.* [56]. Notably, the mRNA levels of two house-keeping genes were significantly downregulated in infected DCs, suggestive of a general suppression of cellular mRNAs, which can be attributed to early apoptotic events associated with degradation of cellular mRNA [57]. Additionally, viral cap-snatching could be involved, resulting in destabilization of cellular mRNA and accelerated degradation. The unaffected CD83 mRNA levels and the induced levels of CD80 mRNA in infected DCs could be explained by increased stability of mRNAs that encode proteins dedicated to DC maturation and antigen presentation.

The lack of correlation between the stable mRNA levels and the reduction of CD83 in infected cells led us investigate whether CD83 downregulation occurs at the protein level. NSR infection did not result in increased amounts of CD83 in the cell culture medium. Analysis of cell lysates revealed that CD83 levels in NSR-infected cells were low and comparable to the levels in unstimulated cells. This finding suggested that in infected cells the protein was either not produced or very efficiently degraded. Involvement of enhanced degradation was investigated by using inhibitors of the major cellular protein degradation routes. Inhibition of the proteasomal protein degradation route did not restore levels of CD83 in NSR-infected DCs. Inhibition of endocytosis at early time points after infection (6 hpi) only partially restored CD83 levels, while inhibition at later time points (12 hpi) did not result in restoration of CD83 levels. These data demonstrate that downregulation of CD83 does not result from proteasomal degradation or lysosomal degradation following endocytosis. Considering all our findings, we propose that CD83 in NSR-infected cells is downregulated at the

translational level. This notion is further supported by the kinetics of CD83 downregulation. CD83 downregulation occurs relatively slowly, after an initial upregulation that peaks between 8 and 12 hpi. In contrast, HSV-1-mediated downregulation that was shown to depend on proteasomal degradation was already significant within 10 hpi. The initial upregulation after NSR infection can be explained by the presence of protein and mRNA pools of CD83 in immature DCs that are ready to be mobilized upon stimulation [32, 56, 58]. Thus, we propose a model where infection with NSR is sensed by DCs, resulting in surface exposure of CD83 originating from intracellular pools. As viral replication and protein synthesis progresses, an unknown viral and/or cellular factor inhibits translation of CD83. CD83 originating from the protein pools is recycled normally but not replenished, resulting in relatively slow downregulation.

In summary, we demonstrate that NSR infection of monocyte-derived immature DCs results in incomplete maturation, associated with gradual downregulation of CD83. The observed downregulation is attributed to inhibition at the translational level. Infected cells were able to control viral replication by apoptosis, while bystander cells reached a fully matured phenotype. The combined results point to a central role for bystander DCs in NSR-mediated immunity, which will be addressed in future studies.

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Supplementary data

Table S1. Sequences of the primers used for quantification of CD83, CD80, GAPDH and PPIA mRNAs by real-time PCR

mRNA	Forward primer	Reverse primer
CD83	ATGGAGACACCCCAGGAAGAC	TCAGGGAATAGGGCCTTTCA
CD80	CCTCAATTTCTTTCAGCTCTTGGT	GGACAGCGTTGCCACTTCTT
PPIA	CATACGGGTCCTGGCATCTT	TGCCATCCAACCACTCAGTCT
GAPDH	CCATCTTCCAGGAGCGAGATC	GCCTTCTCCATGGTGGTGAA

Table S2. Sequences of the probes used for fluorescence in situ hybridization (FISH)

mRNA	Probe
GAPDH	Human GAPDH (Stellaris FISH probes, SMF-2026-1)
CD83	ctcaggagcagaagctggag caggagaccgtgtagggaac accaccctccaataacttga tttgccccttctgatgatag attgggggcgtcgaaagaac ctgcagagtgcacctgtatg aagtctcttctttacgctgt acaatctccgctctgtattt ctgggaagatactctgtagc tccatgccagctttagaaaa cactagccctaaatgcttat ataccagttctgtcttgtga agaacctgcagaaatcctgc cagtgtaacagacaggcaca ttcagcgtaggctcattctt tgaaggacttcacaggatgc cttccagatgttttcagtga ccacagaaaatggggtggga atgtggtcatgtgatggttt agctgcatacatcgctgaaa catctcaccatagcttttat atatttcccaagacccttt gatgcagaagaggacagctg

	aaacatctgggctggtacag
	cttgatctgtcaatttctcc
	tccttagtaatagcaggacc
	ggggaatagttcatcgactt
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Preliminary evaluation of a bunyavirus vector for cancer immunotherapy

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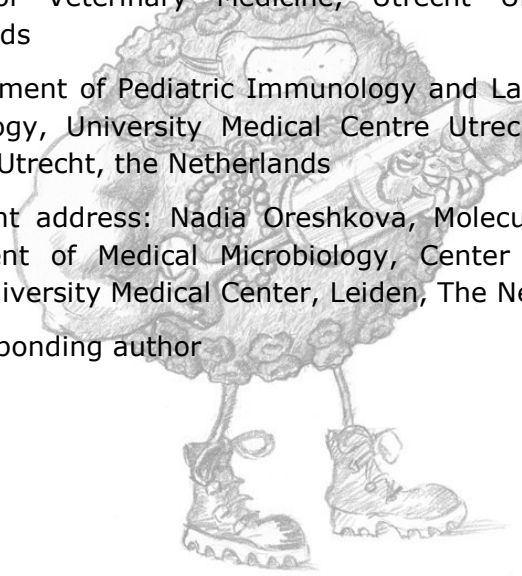

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Abstract

Replicon particles of Rift Valley fever virus, referred to as nonspreading RVFV (NSR), are intrinsically safe and highly immunogenic. Here, we demonstrate that NSR-infected human dendritic cells can activate CD8+ T-cells *in vitro*, and that prophylactic and therapeutic vaccination of mice with NSR encoding a tumor associated CD8 peptide can control outgrowth of lymphoma cells *in vivo*. These results suggest that the NSR system holds promise for cancer immunotherapy.

Dendritic cells (DCs) are the most potent antigen-presenting cells of the body and are instrumental in directing adaptive immune responses against pathogens and tumors. DCs are naturally targeted by many arboviruses. Infection induces DC maturation and presentation of virus-associated antigens, explaining the interest in using these viruses as therapeutic vectors of tumor-associated antigens (TAA). Members of the positive-strand RNA virus families *Togaviridae* and *Flaviviridae* have been extensively evaluated for cancer immunotherapy [1-4], some of which have already entered clinical trials [2]. Remarkably, arboviruses with segmented negative-strand RNA genomes have remained largely unexplored in this field. The genome segments of these viruses form dsRNA panhandles which, in some viruses, contain a 5' triphosphate. This structure is an optimal ligand for the cytoplasmic pattern recognition receptor RIG-I, and thereby an excellent inducer of adjuvanting innate immune responses [5]. We therefore propose that arboviruses with segmented negative-strand RNA genomes that activate RIG-I hold promise for cancer immunotherapy.

Rift Valley fever virus (RVFV), a member of the *Bunyaviridae* family, was recently shown to productively infect immature DCs with high efficiency [6]. Whereas wild type RVFV counteracts innate immune responses via the NSs protein [7], infection of DCs with RVFV variants lacking the NSs protein was shown to result in strong interferon responses triggered by RIG-I signaling [8]. We recently developed RVFV particles that not only lack the NSs gene, but also the glycoprotein-encoding genome segment [9]. The resulting nonspreading RVFV (NSR) particles are unable to produce progeny virions, ensuring their safe application [9]. Moreover, the absence of the NSs gene provides an expression slot for a gene of interest. Vaccination with NSR particles was shown to be highly effective in protecting livestock from RVFV [10-12] and NSR particles expressing the influenza hemagglutinin gene protected mice from lethal influenza [13]. The remarkable efficacy of NSR-based vaccines in both inbred and outbred animals,

and particularly its association with Th1-type immune responses [13], prompted the present study on the use of NSR for cancer immunotherapy.

Initially, we investigated whether NSR-infected human monocyte-derived DCs (MoDCs) can activate CD8+ T-cells *in vitro*. MoDCs were derived from peripheral blood mononuclear cells of healthy donors by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and cultured as previously described [14]. Infection of DCs was remarkably efficient, resulting in over 90% GFP-positive cells under optimal conditions (data not shown). To study the T-cell activation capacity of infected DCs, an NSR variant was constructed that encodes the immunodominant NLVPMVATV epitope of the human cytomegalovirus (HCMV) pp65 (pp65495-503), fused to the C-terminus of GFP (Fig. 1A). NSR particles encoding this fusion protein (NSR-NLV) and particles encoding GFP only (NSR-GFP) were used to infect DCs obtained from a HLA-A2 positive donor. As a positive control, DCs were incubated with synthetic NLVPMVATV peptide (1 μM) and culture medium was used for negative control. After an overnight incubation, HLA-A*0201-restricted, NLVPMVATV-specific CD8+ T-cells [14] were added to the DCs and co-cultured for 4.5 h. T-cells were harvested, stained for surface markers CD3, CD8 and CD107a (LAMP-1) and subsequently intracellularly stained for IFN-γ and TNF. The results of this experiment demonstrate that NSR can be successfully used as a vector to deliver specific immunogenic epitopes to human DCs, which trigger an effector function in corresponding CD8+ T cells (Fig. 1B).

To investigate the feasibility of using NSR for cancer immunotherapy, we made use of C57BL/6 Kh (B6, H-2b) mice and E.G7-OVA cells, a chicken ovalbumin (OVA) gene-transfected clone of mouse lymphoma EL4 cells, obtained from the American Type Culture Collection (Manassas, VA, USA). An NSR variant was constructed that encodes the CD8-restricted SIINFEKL epitope of OVA (OVA257-264) fused to GFP (NSR-OVA) (Fig. 2A). To determine whether NSR-OVA vaccination elicits a cellular immune response specific for this epitope, mice were vaccinated twice via intramuscular route (thigh muscle), with a week interval, with 10^8 $TCID_{50}$ (50% tissue culture infective dose) of NSR-OVA (Fig. 2B). Control mice were vaccinated with NSR-GFP. One week after the second vaccination, spleen cells were collected and evaluated for their ability to produce interferon-γ after stimulation with a synthetic SIINFEKL peptide (10 μg/ml, Invitrogen) in an ELISpot assay [10]. This experiment demonstrated that NSR-OVA vaccination elicits a SIINFEKL-specific cellular immune response (Fig. 2C).

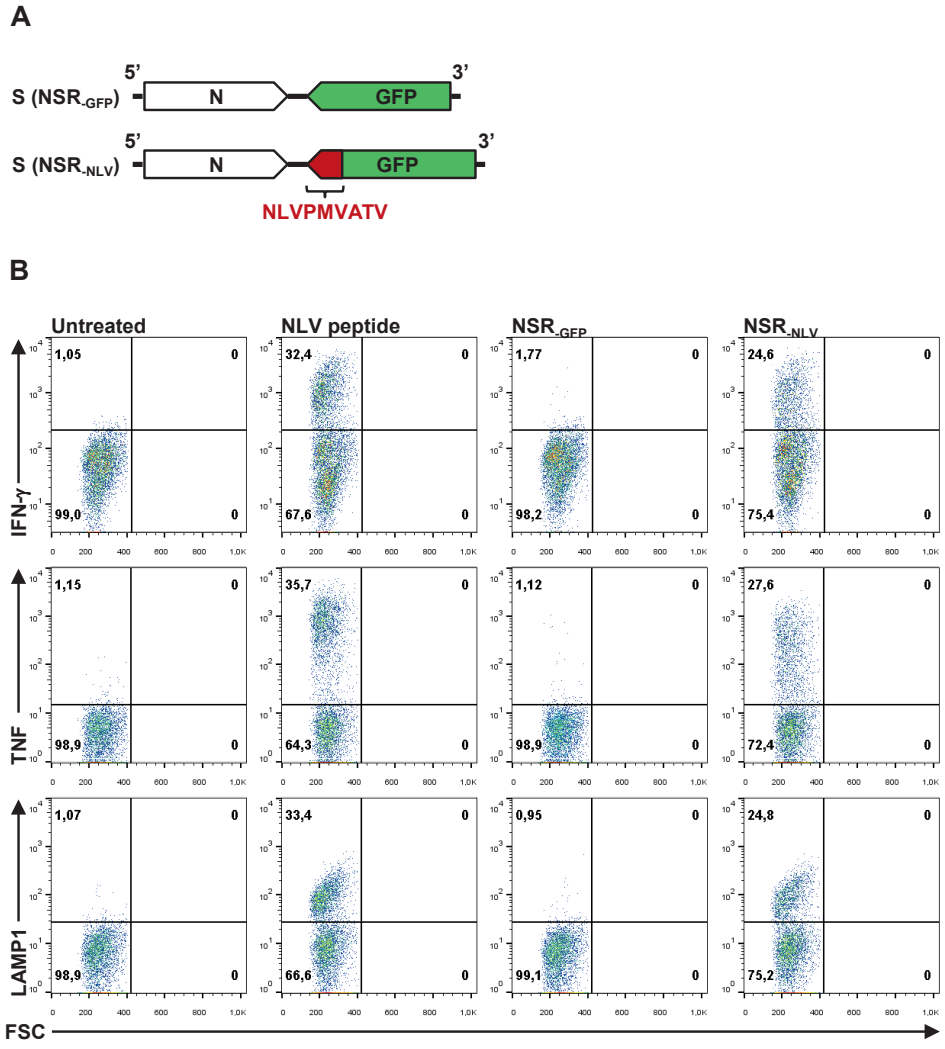

Figure 1. (A) Schematic representation of the NSR S segment encoding GFP (top) or C-terminally fused $pp65_{495-503}$ epitope (bottom) in anti-genomic orientation. (B) NSR-infected human DCs activate antigen-specific $CD8^+$ T-cells. HLA A2+ DCs were infected with $NSR\text{-}_{GFP}$ or $NSR\text{-}_{NLV}$ and were cultured O/N. As negative control, DCs were left untreated; as positive control, DCs were loaded with synthetic peptide $pp65_{495-503}$. A2-restricted $CD8^+$ T cell clone, specific for the $pp65_{495-503}$ epitope, was added for 4.5h in the presence of Golgi stop (Becton & Dickinson), and analyzed for activation. The expression of IFN-γ, TNF and LAMP-1 of CD8/CD3 positive cell population is depicted. All three parameters are induced specifically in $NSR\text{-}_{NLV}$ infected cells and not in $NSR\text{-}_{GFP}$ infected cells. FSC, forward scatter.

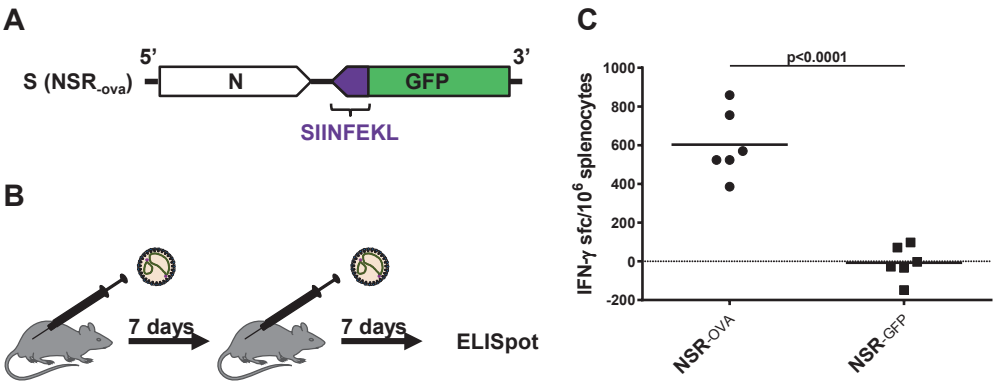

Figure 2. (A) Schematic representation of the NSR S segment encoding the $OVA_{257-264}$ peptide fused to GFP in anti-genomic orientation. (B) Representation of the vaccination regimens with NSR_{-OVA} or control NSR_{-GFP} vaccine. (C) ELISpot assay showing IFN-γ responses of splenocytes collected from mice that were vaccinated with either NSR_{-GFP} or NSR_{-OVA} (n=6). Symbols represent individual counts of IFN-γ spot forming cells. Statistical significance is indicated (Student's t-test).

We finally asked whether prophylactic or therapeutic vaccination with NSR-OVA can reduce outgrowth of E.G7-OVA tumor cells (Fig. 3A). Mice were subcutaneously inoculated with 106 E.G7-OVA cells and euthanized by cervical dislocation when tumor size reached 5,000 mm3. Both prophylactic and therapeutic vaccination via intramuscular route with NSR-OVA resulted in increased survival times, as compared to control mice that had received NSR-GFP (Fig. 3B). Therapeutic vaccination resulted in complete tumor clearance in 2/10 mice, whereas prophylactic vaccination resulted in clearance of tumors in 6/10 mice. As expected, re-cultured tumor cells collected from NSR-GFP-vaccinated mice upon necropsy were found to express OVA, as determined by a commercial OVA ELISA (Agro-Bio). Surprisingly, tumor cells collected from mice vaccinated with NSR-OVA did not express detectable levels of OVA (Fig. 3C). This finding suggests that small numbers of the inoculated cells did not express OVA or lost OVA expression in time, and that tumor cells expressing OVA were efficiently cleared by either prophylactic or therapeutic vaccination.

The present work demonstrates that NSR particles can successfully deliver an immunogenic peptide to human DCs and that these cells are capable of activating CD8+ T cells. In addition, vaccination of mice with NSR expressing a single CD8-restricted epitope resulted in the complete clearance of lymphoma cells expressing the targeted antigen. Future experiments will address the efficacy of NSR vaccination to control outgrowth of tumors expressing self-antigens. Considering that self-antigens are much less immunogenic, NSR

particles will be designed to express not only CD8+ peptides, but also CD4+ and B-cell epitopes.

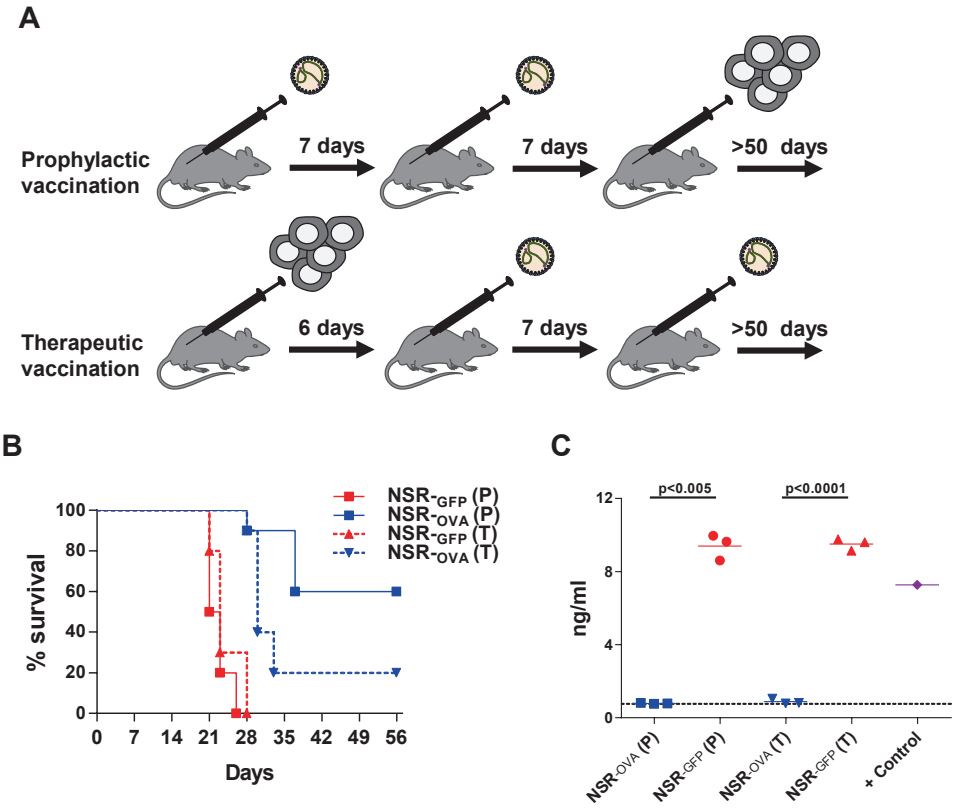

Figure 3. (A) Prophylactic and therapeutic regimens used for vaccination with NSR_{-OVA} or control NSR_{-GFP} vaccine. The primary therapeutic vaccination was applied when more than 50% of the E.G7-OVA-inoculated mice had palpable tumors. (B) Survival curves of mice prophylactically (P, solid lines) or therapeutically (T, interrupted lines) vaccinated with NSR_{-GFP} or NSR_{-OVA}. Statistical analyses were performed with GraphPad Prism. Log-rank (Mantel-Cox) tests revealed statistically significant differences ($P<0.0001$) between NSR_{-GFP} and NSR_{-OVA} vaccinations with either a prophylactic or a therapeutic regimen. (C) Detection of OVA in supernatants of cultured E.G7-OVA cells by ELISA. Cells were recultured for 7 days after being collected from mice (n=3/group) that had reached the humane endpoint. Statistical significance (Student's t test with Welch's correction for unequal variances) is shown. E.G7-OVA cells that were used for the inoculation of mice were used to obtain a positive-control (+ Control) sample.

Acknowledgements

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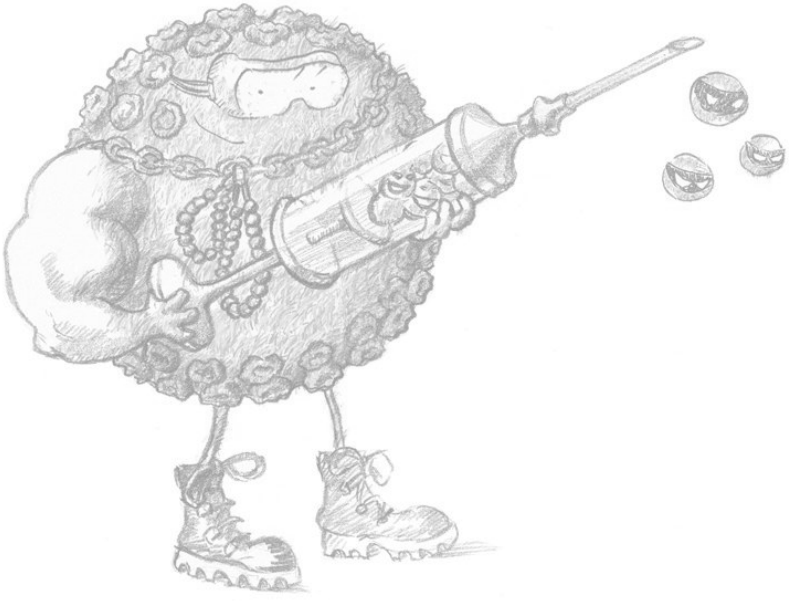
8

General and summarizing discussion



RVF is a disease of serious socio-economic impact. The virus affects ruminants, causing abortions among gestating ewes, goats and cows and mortality among newborns. This results in heavy financial losses for the farming industry and related sectors in affected countries. RVFV infects humans as well. The disease in humans is generally benign, but in some cases complications develop. The most common complication is retinal damage, which may result in temporary or permanent blindness. A small percentage (1-2%) of patients may develop severe complications including hepatic necrosis, hemorrhagic fever or encephalitis. Patients that develop these severe complications may succumb to the infection.

RVFV is confined to the African continent, the Arabian Peninsula and several islands off the cost of Southern Africa, but its potential spread to new territories is of global concern. The existing commercialized vaccines for veterinary use in Africa are not optimal regarding efficacy (inactivated vaccine) or safety (Smithburn vaccine), or still lack well-controlled field study data to confirm their safety (Clone 13). Outside Africa there are no registered vaccines to combat a potential outbreak among ruminants. A vaccine for humans is also not available.

In this thesis, the development of a novel vaccine based on RVFV replicon particles is described, here referred to as nonspreading RVFV (NSR). The aim was to create a vaccine that optimally combines the efficacy of live vaccines with the safety of inactivated vaccines. In this chapter, the development of NSR will be summarized and the advantages for its use as a vaccine to control RVFV in ruminants will be discussed. Our interest in using NSR as a vector vaccine against other diseases will also be explained. Then the molecular mechanisms of NSR-elicited immunity will be discussed and speculations will be presented on possible relevance of our findings for the pathogenesis of the wild-type virus. Finally, the potential use of NSR as a vector for anti-cancer immunotherapy will be discussed.

Development of a system for the production of RVFV replicon particles

The use of reverse-genetics technology led to the development of minigenome systems and systems that can be used to rescue several bunyaviruses (summarized in Chapter 1). By utilizing this technology, our group was the first to develop bunyavirus-based replicon particles, described in Chapter 2 of this thesis and later designated as NSR. Before our studies, Habjan et al showed that a minigenome of RVFV can be packaged into particles [1]. These particles could

infect recipient cells and the encoded reporter gene was successfully expressed when the RVFV L and N proteins were provided by co-transfected plasmids. The advantage of our system is that the NSR particles contain a genome capable of autonomous replication. For the production of NSR particles, we were able to establish a cell line that constitutively maintains replicating L and S segments. The latter was modified to encode the reporter GFP instead of NSs. The cell line expressed GFP for over 100 passages, demonstrating its remarkable stability. Using this replicon cell line, particles can be generated by transfection with just one expression plasmid, encoding the RVFV glycoproteins (Fig. 1). Optimization of the transfection procedure resulted in reproducible yields of 10^8 $TCID_{50}$/ml per transfection.

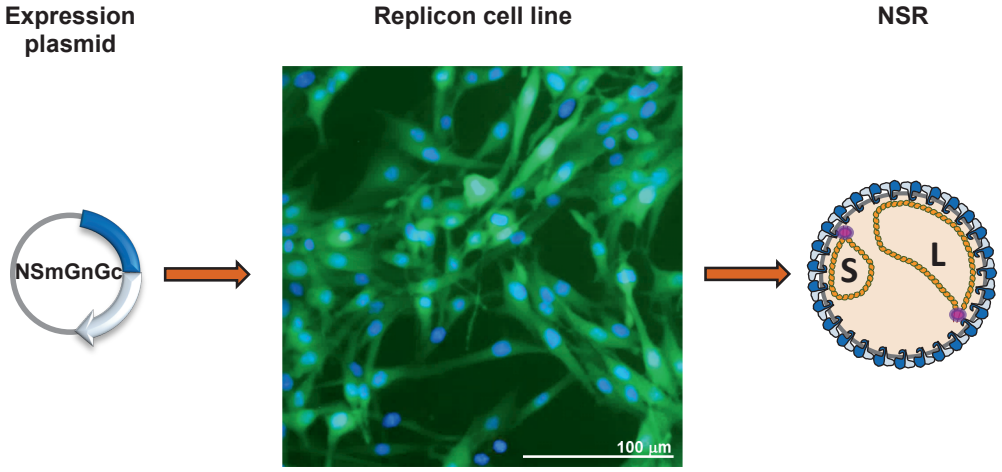


Figure 1. Schematic representation of the production of NSR. The replicon cell line maintains replicating L and S segments and the expression of a reporter (GFP) gene. Transfection with an expression plasmid that encodes the complete open reading frame of the RVFV M segment comprising NSmGnGc results in generation of NSR. The NSR particles can be harvested the day after transfection. An image of the replicon cells under a fluorescent microscope (EVOS) is shown. The nuclei are visualized by DAPI staining.

Shortly after publication of our work, Dodd et al reported the generation of RVFV replicon particles, referred to as VRPRVF [2]. Similar to NSR, VRPRVF comprises the L and the S segments, the latter also modified to encode the reporter GFP instead of NSs. However, production of these particles requires a combination of 3 plasmids, encoding the RVFV L genome segment, the modified S genome segment, and the open reading frame of the M segment with deletion of the

NSm coding region. These plasmids were used to transfect BSR-T7/5 cells and average yields of 10^7 $TCID_{50}$/ml were obtained.

With our system we were able to establish replicon cell lines that express different genes from the NSs locus of the S segment, including Gn of RVFV (Chapter 3) and the hemagglutinin of influenza A virus (Chapter 5). Also the neuraminidase protein of influenza was successfully expressed (unpublished results). Particles from all these cell lines could be produced by a single transfection with an expression plasmid encoding NSmGnGc of RVFV. These data reveal that NSR is a flexible system and that NSR particles can be generated for various vector applications. We noticed, however, that an increase in size of the foreign gene insert reduces the yield of the recombinant NSR particles. This feature should be taken into account when designing genes to be expressed from the NSR genome.

NSR as a vaccine for the prevention of RVF

Vaccines based on live viruses are highly immunogenic and effective after a single vaccination. However, their safety may be of concern because of residual virulence and/or potential to spread to the environment. Inactivated and subunit vaccines are intrinsically safe, but are laborious to produce and often require multiple administrations to induce strong and durable immune responses. Replicon-based vaccines are designed to optimally combine the efficacy of live vaccines with the safety of inactivated vaccines.

The NSR particles developed in our laboratory are deprived of the genes encoding the proteins that constitute the viral envelope. Thereby, these particles are unable to generate progeny virions and cannot spread from the initial site of infection. Additionally, NSR does not encode the major virulence factor, NSs, which counteracts innate immune responses. These characteristics of NSR represent its intrinsically safe nature. On the other hand, NSR is capable of genome replication and viral protein expression in infected cells, triggering innate immune responses followed by a broad and efficient adaptive immune response.

The efficacy of NSR was evaluated in several vaccination-challenge studies. In mice, a single vaccination completely protected against a lethal dose of RVFV (Chapter 2). In lambs, a single vaccination protected against fever and significantly reduced challenge-induced viremia, but low levels of challenge virus RNA were still detected [3]. Although these results suggested that sheep and

even young lambs are protected from clinical disease after a single vaccination with NSR, we anticipated that the highly susceptible ovine fetuses may not be protected after a single vaccination of gestating ewes. The high sensitivity of fetuses to intrauterine infections was demonstrated in a study where vertical transmission was observed, even in the absence of detectable viremia in the ewes [4]. Considering this finding, we assume that sterile immunity is needed to prevent vertical transmission of the virus.

To improve the efficacy of NSR, the Gn gene was introduced into the NSR genome, thereby creating NSR-Gn (Chapter 3). Gn is the major target for neutralizing antibodies and was shown to contain immunodominant T-cell epitopes, at least in mice [5]. Gn encoded by NSR is expressed in infected cells and localizes to the cell surface (Chapter 3). A protein exposed at the cell surface can be recognized by B-cells, resulting in the development of Gn-specific antibodies. Cell-associated protein also serves as an excellent source of antigen for presentation to T-cells. Indeed, NSR-Gn elicited neutralizing antibodies in both lambs and mice and memory T-cell responses in the latter (Chapter 3). A single vaccination with NSR-Gn provided sterile protection in lambs with no detectable viraemia after challenge. NSR-Gn was equally effective as the Clone 13 vaccine in this study and both were more efficacious than R566, another next-generation vaccine based on live-attenuated virus (Chapter 4). The demonstrated efficacy of the NSR-Gn vaccine (Chapters 3 & 4), combined with its intrinsic safety, renders it a particularly promising vaccine for the control of RVF in ruminants.

NSR-Gn can be produced to high titers, but for commercialization, a scaling up of the production is required. A system to produce RVFV-derived VLPs was described by Mandell *et al* [6]. This system, based on suspension-grown HEK-293 cells, was optimized for transfection with an expression plasmid encoding the RVFV glycoproteins and was found suitable for scaling up. Such a system could be employed for production of NSR, using a two-step protocol – first, infection of the HEK-293 cells with NSR-Gn and subsequently transfection with the glycoprotein expression plasmid to generate the NSR-Gn particles. The two-step protocol was successfully used under laboratory conditions (unpublished data). Thus, this system seems a feasible option for industrial-scale production of NSR-Gn. Alternatively, a trans-complementing cell line that stably expresses the glycoproteins of RVFV could be used. Such a system was recently developed by Murakami et al and virus replicon particles were successfully produced [7]. However, the relatively low yields indicate that the system needs further optimization.

NSR as a vector vaccine

Viral vectors are widely explored both for prophylactic vaccination against various infectious diseases and for immunotherapy against cancer. Different vector platforms have been established and evaluated, including, but not limited to those based on poxviruses, adenoviruses, alphaviruses, vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), measles virus and poliovirus [8-10]. These viruses have shown potential as vector platforms and some of them are already being evaluated in human clinical trials [10]. However, the need for safe and effective vector vaccines for mucosal application remains largely unmet. Mucosal vaccines are highly desirable as many pathogens invade the body via mucosal surfaces of the respiratory tract [11, 12].

In this thesis we evaluated the potential of NSR as a new vector platform with a particular focus on mucosal vaccination. NSR contains a negative-strand RNA genome, which has some well-established advantages over other genome types - modular genome organization, stability of the ribonucleoprotein (RNP) complex, no danger of incorporating genes into host genomic DNA and high expression of viral proteins [13]. In addition, as RVFV is largely confined to the African Continent, pre-existing immunity in humans is only found in endemic areas. Most importantly, although RVFV is an arbovirus, infection via the respiratory tract is considered a major infection route in humans [14-16]. Recently, efficient infection via this route was demonstrated in animal trials with rats and nonhuman primates [17-21]. Moreover, infection via aerosols was shown to result in more severe disease [18]. The specified features of RVFV and its capability to infect via the respiratory tract enabled us to use NSR as a vector for administration via the respiratory route, as demonstrated in chapter 5 of this thesis.

We evaluated the vaccine efficacy of NSR expressing the hemagglutinin (HA) of influenza virus (NSR-HA) in a mouse model. Influenza was chosen as a model infection of a well-studied respiratory disease, for which many tools are available to facilitate work with the virus. Moreover, the high immunogenicity of the HA protein renders it very suitable for expression by a vector. Finally, we could make use of a lethal mouse model for influenza infection, developed at CVI.

In an experiment comparing the protective efficacy of the NSR vector when delivered via the intramuscular (IM) or via the intranasal (IN) route, we observed that mice were completely protected from a lethal dose of influenza virus via either route by a single vaccination. Although the IM vaccinated mice

developed higher systemic immune responses in terms of both antibody titers and cellular immunity, these mice experienced a mild weight loss after challenge, while IN vaccinated mice were protected from body weight loss. Our results reveal that the NSR vector was more efficient in preventing influenza after IN administration and suggest that the observed protection resulted mainly from vector-mediated local immunity.

Other negative-strand RNA viruses have been evaluated for IN administration as well. Comparison of the efficacy of oculonasal (ON) versus IM vaccination with Newcastle disease virus (NDV) was reported by Cornelissen *et al* [22]. Similar to NSR, the NDV vector rendered superior protection after a single oculonasal (ON) vaccination, as compared to a single intramuscular vaccination. However, protection after ON vaccination was partial and the surviving mice displayed mild clinical symptoms and a limited body weight loss. Additionally, hemagglutination inhibition (HI) antibodies were not detected in any of the vaccinated mice. In contrast, NSR-HA vaccination elicited antibodies detectable with HI assay in 7 out of 9 mice. Collectively these results suggest that NSR may be a superior vector for respiratory application, as compared to the NDV-based vector.

Another well-studied vector based on a negative-strand RNA virus is the vesicular stomatitis virus (VSV). This vector was shown to be a promising platform in different infection models and was already used in phase I clinical trials [23]. The use of VSV-based vectors against respiratory infections such as influenza and respiratory syncytial virus (RSV) via the intranasal vaccination route was shown to be efficacious in mouse models [23-27]. However, vectors based on live attenuated VSV have been shown to retain a degree of neurovirulence in mice after intranasal administration [24, 28-31] and in non-human primates after intrathalamic administration [32]. Additionally, the IM route was suggested to be superior to IN route for both non-propagating and propagation-competent VSV-based vectors [27, 33]. Accordingly, a single administration of VSV-based vectors via the IN route is not routinely used in vaccination experiments. These data suggest that NSR may be advantageous vector for mucosal vaccination, as compared to VSV, both because of the superior clinical efficacy of IN vaccination as compared to IM vaccination and because of its intrinsic safety.

A paramyxovirus that shows promise as a vector for respiratory application is Sendai virus. This vector is well tolerated in adults and advances towards clinical trials focused on RSV in infants [34]. A disadvantage of the use of this vector is pre-existing immunity in humans as it is antigenically related to human parainfluenza virus type-I, a virus that causes common upper respiratory

infections in adults and children [35]. Pre-existing immunity in the form of maternal antibodies in infants necessitates repeated vaccinations in this age group. In older children or adults, pre-existing immunity may preclude efficacy of the vector. In this regard, NSR is advantageous as the human population is generally free from antibodies against RVFV.

For vaccines purposed to be applied via the respiratory tract, penetration depth is of critical importance for vaccine efficacy. Various studies have suggested that targeting lung tissue can contribute to mucosal immunity against respiratory infections, resulting in the induction of local IgA antibodies and cellular responses in addition to systemic immunity (summarized in [36]). Of note, in preliminary experiments not described in this thesis, the use of NSR expressing a soluble form of the HA protein (sHA) revealed a major importance of the inoculum volume for intranasal vaccination of mice. We observed that a prime-boost vaccination regimen with NSR-sHA applied in a volume of 50 µl protected all vaccinated mice from death and prevented weight loss in 8 out of 10 mice. A volume of 20 µl protected only 4 out of 9 vaccinated mice from death and the surviving mice displayed mild to severe weight loss (Fig. 2). The importance of the vaccine volume in mice has been thoroughly studied by Southam *et al* [37]. The authors of this study showed that larger inocula volumes reach the lung tissue, while smaller volumes remain in the nasal cavity and subsequently can be traced to the digestive tract. These results can explain the dramatic difference observed in our experiments when using different vaccine volumes. Extrapolated to humans, the described findings emphasize the significance of penetration depth for an NSR-based vaccine and indicate that formulations are required that can target the vaccine to the lower respiratory tract mucosa for optimal efficacy.

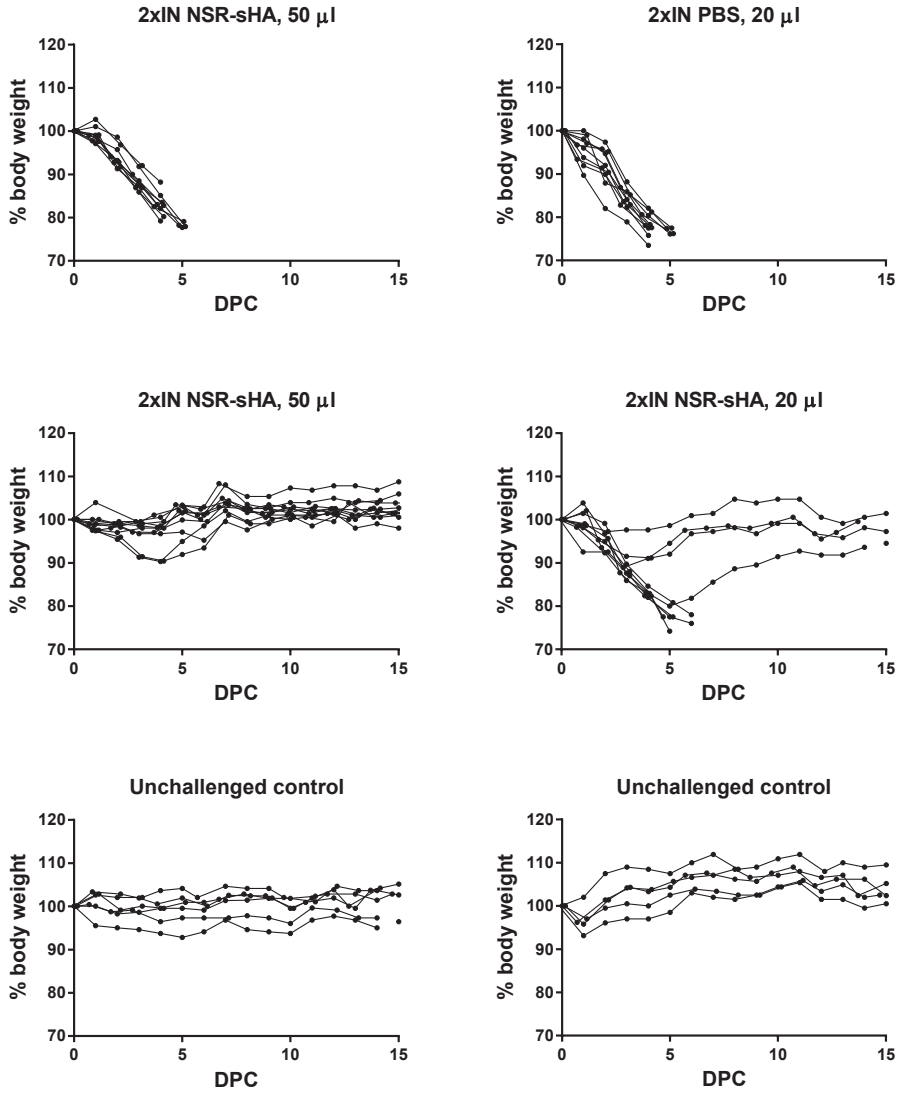


Figure 2. Influence of the volume of intranasally administered NSR. Mice were mock-vaccinated with PBS (upper panels) or vaccinated with NSR-sHA (middle panels) twice with a 3 week interval. The vaccine inoculum and volumes are shown above each graph. Challenge with a lethal dose of influenza was performed 3 weeks after the second vaccination. The lower panels depict unvaccinated and unchallenged mice as controls for body weight. The vaccinations with the two different volumes were performed in two different studies. DPC: days post challenge.

Our study using NSR as a vaccine vector also made clear that the design of the expressed antigen can considerably influence the vaccine efficacy. Vaccination with NSR encoding the full-length, membrane anchored HA protein (NSR-HA)

resulted in complete protection of mice from lethal challenge, while vaccination with HA that encodes the soluble form of the same HA protein (NSR-sHA) did not protect any of the vaccinated mice. Additionally, vaccination with NSR-HA resulted in a significantly higher cellular immune responses than vaccination with NSR-sHA. This difference in efficacy can be explained by the results from our study on the interaction between NSR and DCs (Chapter 6 and discussed further in this chapter). Based on these findings (Chapter 6), we hypothesize that bystander dendritic cells play a central role in NSR-mediated immunity by cross-presenting antigenic peptides, acquiring antigens for cross-presentation from infected DC that had undergone apoptosis. Therefore, it can be speculated that infected cells that secrete the vectored protein are an inferior source of antigen for cross-presentation as compared to cells that retain the antigen in cell-associated from.

Towards insights into the role of infected and bystander dendritic cells in NSR-mediated immunity

In our studies, we consistently observed that NSR vaccination elicited T-cell immune responses (Chapters 3, 5 and 7) and strong Th1 polarization (Chapter 5). Recent reports showed that RVFV productively infects DCs *in vitro* [38] and that cells of the monocyte/macrophage/dendritic lineage are primary target cells *in vivo* [39]. Considering these reports and our own results, we were interested to investigate how DCs respond to infection with NSR, with the aim to better understand the molecular basis of NSR-mediated immunity.

DCs are the most potent antigen-presenting cells of the body and have a unique ability to prime naïve T-cells. They accomplish this function after maturation, a process triggered by the encounter of various pathogen associated molecular patterns (PAMPs). Replication of NSR in infected DC generates double-stranded RNA (dsRNA), known to be a powerful PAMP [40]. Therefore, infection of DCs with NSR was expected to induce DC maturation. Much to our surprise, the study described in Chapter 6 revealed that NSR-infected DCs did not fully mature as evidenced by the downregulation of CD83, the most prominent marker for DC maturation. Upregulation of CD83 is critical for the capability of DCs to prime naïve T-cells. Thus, paradoxically, NSR triggers robust T-cell immune responses, while downregulating CD83 on DCs.

Our finding that CD83 is downregulated in NSR-infected cells, combined with the detected massive apoptosis of these cells between 24 and 48 hpi led us to hypothesize that actually bystander DCs play the leading role in T-cell priming,

by cross-presentation of antigens acquired from the infected apoptotic DCs. This hypothesis is supported by the full maturation of bystander cells, as evidenced by upregulation of CD83. Curiously, we observed that at 48 hpi NSR infected cells displayed strong upregulation of CD83, in contrast to the consistent downregulation at 24 hpi. Closer investigation of these infected cells revealed the presence of two distinct populations, one expressing high levels of the GFP reporter (GFP^{high}) and one expressing low levels (GFP^{low}) (Fig. 3). These populations were discernible in cultures of cells incubated with NSR and also in cultures of cells incubated with NSR and LPS. Remarkably, the CD83 levels differed between the two populations. In GFP^{low} cells CD83 levels were comparable to those of the GFP- cells, which correspond to uninfected bystander cells, while in GFP^{high} cells CD83 levels were at least twice as low. We propose that the GFP^{high} population represents cells that are infected but not yet apoptotic, while GFP^{low} cells represent a subset of bystander DCs that have acquired GFP molecules from phagocytosed apoptotic GFP+ cells.

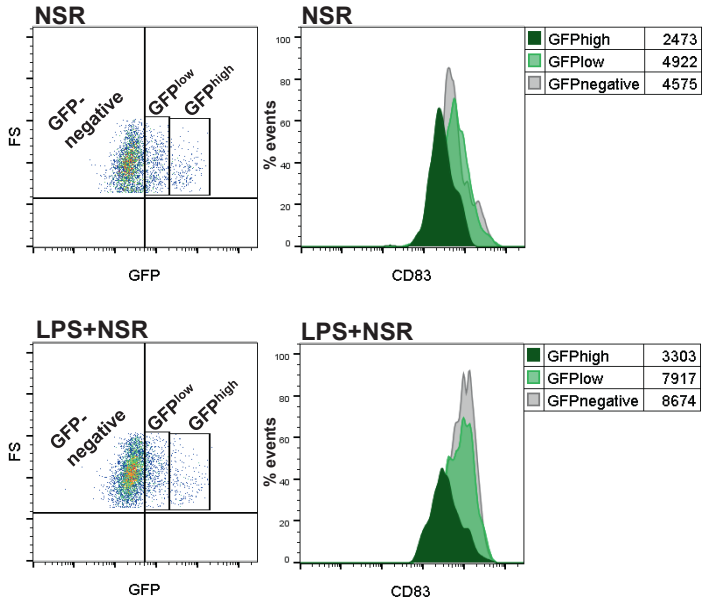


Figure 3. Correlation between GFP signal and surface expression of CD83 in DCs at 48 h after infection with NSR (upper panels) or co-stimulation with LPS and NSR (lower panels). The dot plots depict different populations, based on the strength of the GFP signal. Quadrants were set using cells from the negative control conditions. The gates for GFP^{low} and GFP^{high} populations are shown. The histograms illustrate CD83 signal of the different GFP populations. The color code and the median fluorescent intensities of each population are shown in the legends.

Regulation of CD83 expression

CD83 is the key marker for maturation of DCs [41]. Despite intensive research, however, neither a ligand, nor the signaling pathway have been identified yet. Nevertheless, CD83 has been shown to orchestrate multiple functions on immune cells [42], among which the activation of naïve $CD8^+$ T-cells by DCs [42-44], underlining its important role in immune responses.

In immature DCs, both pools of CD83 protein and of corresponding mRNA are present [45-47]. These protein and mRNA pools are readily mobilized upon cell stimulation, which explains the rapid upregulation of CD83 after LPS treatment. We and others [47] observed similar amounts of mRNA in both immature and mature DCs, which confirms the existence of mRNA pools, but also suggests that CD83 upregulation upon maturation is regulated mainly at the translational level.

In our study we were able to narrow down the underlying mechanism of the NSR-mediated downregulation to the level of translation. Recently, the AUF1 protein was shown to play an important role in regulating CD83 translation [48]. This molecule functions as a regulator of mRNAs of early response genes, such as cytokines, by decreasing their stability and increasing their decay [49-51]. With respect to CD83 mRNA, however, AUF1 does not have an effect on mRNA stability, but seems to regulate its expression by binding to a post-transcriptional regulatory element situated within the coding sequence of CD83. Levels of CD83 expression were shown to directly correlate with levels of AUF1 [48]. Therefore, AUF1 is a possible candidate that could be affected by NSR infection and it will be interesting to address in further studies its availability and function in infected cells.

NSR expresses only two viral proteins, nucleoprotein and polymerase, neither of which has a known immunomodulatory function. However, recently it was suggested that RVFV proteins other than NSs may play an immunomodulatory role in mouse macrophages [52]. Furthermore, additional mechanisms were suspected to be involved in RVFV-mediated immune evasion, aside from the well-known inhibition of type-I interferon responses by NSs [53]. Identification of an immunomodulatory function for one of the two NSR-encoded viral proteins will reveal novel insights into RVFV biology and virus-cell interactions. On the other hand, identification of cellular factors involved in the observed downregulation of CD83 may shed light onto the signaling cascade that governs the upregulation of this protein upon DC activation. In both cases, further investigation of the exact mechanism of NSR-mediated downregulation of CD83

will generate valuable information concerning DC-virus interactions and innate immune responses.

Potential role of CD83 downregulation in RVFV pathogenesis

Our finding that CD83 is downregulated in NSR-infected DCs is very intriguing. However, downregulation of CD83 is not an unusual consequence of virus infection of DCs. Several viruses, including human immunodeficiency virus type 1 (HIV-1), herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV) and varicella zoster virus (VZV) were shown to reduce CD83 cell surface levels by different mechanisms [54-58]. In all these examples, infected DCs had an impaired capability to stimulate naïve T-cells, showing that virus-mediated CD83 downregulation is a well-explored mechanism for immune evasion. Little is known about the interactions between DCs and bunyaviruses. Infections of DCs with Crimean-Congo hemorrhagic fever virus and Hantavirus were reported to result in upregulation of CD83 [59, 60], but downregulation of this protein as a result of infection with a bunyavirus has not been described thus far.

As downregulation of CD83 is observed after infection of DCs with NSR, it is very likely that also wild-type RVFV downregulates CD83 upon infection. It can be speculated that such downregulation could contribute to pathogenesis. Upon natural infection, the virus is introduced in skin tissue by a mosquito bite. Like many other arboviruses, RVFV has been shown to infect DCs [38, 61, 62] and it is therefore highly likely that some of the first cells to be infected are resident skin DCs, the Langerhans cells. As mentioned earlier, immature DCs already contain pools of both CD83 protein and corresponding mRNA, therefore CD83 is rapidly upregulated upon stimulation. If RVFV is capable of specifically blocking CD83 mRNA translation, the initially upregulated protein would be naturally recycled and degraded in infected DCs before these cells have had the chance to migrate to lymphoid tissues. Infected DCs would not reach full maturation, thereby their capability to prime T-cells would be impaired. Secretion of type-I interferons and other cytokines would also be suppressed by virtue of the action of the NSs protein [52, 63-66], thereby preventing the activation of bystander DCs. Such "incapacitated" DCs could serve as primary sites of virus replication and as transport vehicles for the virus to leave the skin tissue. As RVFV expresses also NSm which has an anti-apoptotic function [67], apoptosis of infected DCs may be delayed, thereby further favoring virus propagation. As a result of all these events, RVFV would be able to access the blood stream and reach its preferred target organs, such as the liver, resulting in explosive

replication. Meanwhile, the incapacity of infected DCs to prime T-cells would result in a delayed and suppressed cellular immune response, providing the virus with time to reach high titers in blood, essential for transmission to mosquitoes.

The hypothesis that RVFV infection of DCs results in CD83 downregulation and associated suppressed cellular immune response is in line with recently reported data by Jansen van Vuren *et al*. The authors observed a suppressed cellular immune response in spleen cells of mice inoculated with RVFV, as evidenced by downregulation of genes that are involved in B- and T-cell immunity [53]. The mechanism of downregulation of CD83 and its significance for the wild-type virus are therefore exciting topics for further research.

NSR as a vector for cancer immunotherapy

The utilization of viral vectors to deliver tumor-associated antigens (TAAs) has been recognized as a promising approach for cancer immunotherapy and has been subjected to extensive studies for over a in last decades. Viral vectors are particularly attractive for TAA delivery because they often directly infect DCs. Viral infection provides powerful "danger signals" that result in DC activation, improved co-stimulatory capacity, efficient antigen presentation and secretion of type-I IFNs and other pro-inflammatory cytokines [68-70]. These events are followed by induction of robust adaptive immune responses. Additionally, viral vectors may provide signals to break the tolerance against TAAs [71]. Viral vector-based therapies show superior performance as compared to therapies based on adjuvanted antigens [72] or plasmid-DNA encoded antigens [73-75].

Our studies with animal models convincingly show a remarkable efficacy of NSR, both as a vaccine against RVFV (Chapters 2, 3 and 4; [3]) and as a vector vaccine (Chapter 5). NSR was shown to elicit pronounced Th1 responses (Chapter 5), which is particularly desirable for cancer immunotherapy. Furthermore, both NSR and wild-type virus can efficiently infect DCs *in vitro* (Chapter 6 and [38]) and RVFV targets DCs and macrophages *in vivo* [39]. These findings prompted the evaluation of NSR as a vector vaccine for cancer immunotherapy (Chapter 7). Our study revealed that NSR elicits robust CTL responses against a TAA and is capable of clearing tumor cells that express this TAA. Direct comparison of our results with those of other studies with established vector systems in mice is difficult because of the diversity in the mouse models, types of tumors and vaccination regimens used. Nevertheless, we have identified several characteristics of NSR-mediated immunity that are

shared with established vectors and/or are known to be advantageous for cancer immunotherapy. For instance, studies with alphavirus-based vectors have demonstrated that apoptosis of infected cells is of critical importance for vector-mediated protection against melanoma and *LacZ*-expressing tumors in mice [75-77]. Additionally, in a mastocytoma model, cross-priming of DCs was suggested to play an important role in tumor clearance [78]. Our *in vitro* studies revealed that NSR-infected DCs undergo apoptosis, while bystander DCs mature and most likely acquire antigens from apoptotic DCs for cross-presentation (Chapter 6). In addition, NSR-infected DCs secrete high amounts of IFN-β, shown to be of critical importance for viral vector-mediated protection [79]. These DCs secrete also TNF and IL-6, the latter being suggested to inhibit the activity of $CD4^+CD25^+$ regulatory T-cells [80, 81]. These T-cells were shown to be important for sustaining an immunosuppressive tumor environment and their inhibition is therefore beneficial for tumor clearance [82]. Importantly, NSR-infected DCs do not secrete IL-10, which is known for its immune suppressive function and promotion of a favorable environment for tumor growth [83, 84]. The abovementioned features, together with the results from our study in mice inoculated with tumor cells, suggest that NSR can be considered a promising vector for cancer immunotherapy and warrant its further evaluation for this purpose, using humanized mouse tumor models.

A major concern about using viral vectors in cancer patients is the generation of a strong neutralizing antibody response against the vector. As cancer treatment requires multiple administrations, the problem with anti-vector immunity can present a serious drawback for a viral vector-based therapy. However, vectors that do not depend on dissemination for efficacy seem to be less sensitive to pre-existing immunity and may be used for repeated immunizations, even in the presence of neutralizing antibodies. This notion is supported by the successful repetitive use of Venezuelan equine encephalitis virus (VEEV)-based replicons in cancer patients. These replicons were able to induce immune responses against the TAA in the presence of neutralizing antibodies against the vector and regulatory T-cells [85]. Similarly, a replication-deficient adenovirus-based vector induced TAA-specific responses in mice in the presence of neutralizing antibodies against the vector [86, 87]. It can be hypothesized that non-spreading viral particles are less likely to suffer from the presence of neutralizing antibodies because they are effective after infecting their target cell only once. It is therefore plausible that repeated vaccinations with NSR, which is also non-spreading, can elicit a boost in the immunity against vectored TAAs, even in the presence of NSR-specific neutralizing antibodies.

Final conclusion

The work described in this thesis demonstrates that NSR is a powerful vaccine system for multiple applications. The NSR genome is remarkably stable, as revealed by the maintenance of NSR genomes and marker gene expression for more than 100 passages. Generation of NSR particles requires a single transfection step with one plasmid and scaling-up of the production process seems feasible. NSR-Gn proved to be a potent vaccine against RVFV and its efficacy, combined with its intrinsic safety, offer a highly valuable strategy for the prevention of RVF in ruminants. The evaluation of NSR as a vector against a respiratory infection revealed its potency for mucosal administration. Finally, NSR was shown to be efficacious in clearing tumor cells, holding promise for use in cancer immunotherapy.

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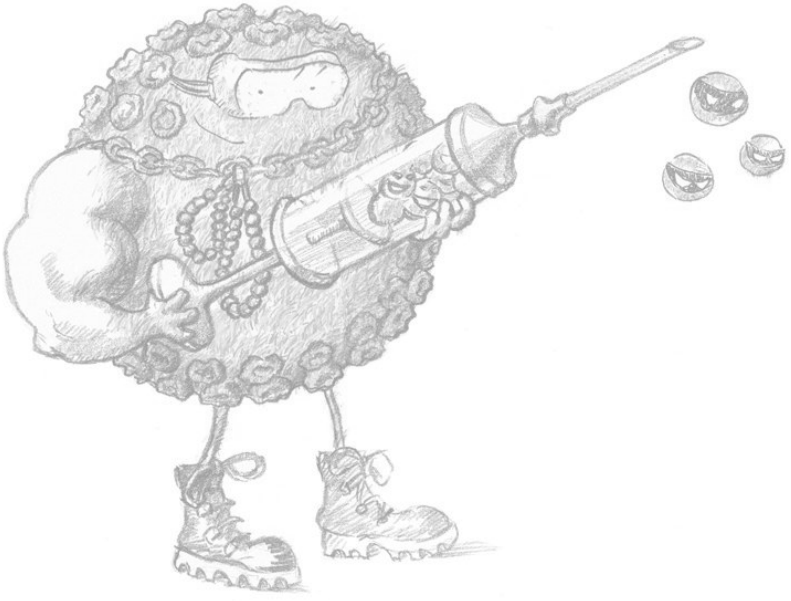
A

Appendix



Samenvatting

Rift Valley Fever virus (RVFV) behoort tot één van de grootste families van virussen, de *Bunyaviridae.* Deze virussen worden gekenmerkt door het hebben van een envelop en een negatieve-strengs RNA genoom. De familie bevat vijf genera: O*rthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus*, en *Tospovirus.* Virussen uit de eerste vier genera kunnen dieren infecteren, terwijl virussen uit het laatste genus alleen bij planten voorkomen. Bunyavirussen worden verspreid door geleedpotigen (o.a. muggen, teken, zandvliegen, tripsen), met uitzondering van hantavirussen, welke door uitwerpselen en urine van knaagdieren worden verspreid. Van de virussen die dieren kunnen besmetten, zijn er diversen die milde tot ernstige ziekte kunnen veroorzaken, bij dieren of mensen, en soms allebei.

RVFV wordt overgebracht door muggen. Het virus levert vooral schade op bij gedomesticeerde herkauwers zoals schapen, geiten, runderen, buffels en kamelen, doordat het acute ziekteverschijnselen geeft, met abortus en sterfte als mogelijke gevolgen. De ernst van de ziekteverschijnselen is sterk wisselend per diersoort, waarbij schapen het meest gevoelig zijn. RVFV kan ook mensen infecteren. Ook mensen kunnen worden besmet door een muggenbeet, maar de meeste gevallen bij mensen zijn te wijten aan direct contact met bloed of weefsels van zieke of dode dieren. Meestal zijn er slechts milde ziekteverschijnselen, met koorts, algehele zwakte, rugpijn en duizeligheid. In de meeste gevallen verdwijnen deze verschijnselen weer binnen enkele dagen. Incidenteel kunnen zich echter complicaties voordoen. Het meest voorkomend daarbij is schade aan de retina, die kan leiden tot een tijdelijke of permanente blindheid. Een klein percentage (1-2%) van de patiënten kan ernstige complicaties krijgen, zoals levernecrose, hemorragische koorts of een hersenontsteking. Patiënten die dergelijk symptomen ontwikkelen, kunnen aan de ziekte overlijden. Gelet op het zoönotische potentieel van RVFV en de grote economische schade voor de veehouderij door sterfte van dieren en door handelsbeperkingen, hebben uitbraken van RVF een grote socio-economische impact in getroffen gebieden.

Het virus werd in 1931 voor het eerst geïdentificeerd door Daubney en collega's, toen zij een uitbraak onderzochten op een boerderij bij het Naivasha-meer in de Rift Valley van Kenya. Sindsdien heeft het virus zich over het Afrikaanse continent verspreid en heeft o.a. het Arabische Schiereiland en diverse eilanden voor de kust van zuidelijk Afrika bereikt. De meest opvallende uitbraak van RVF

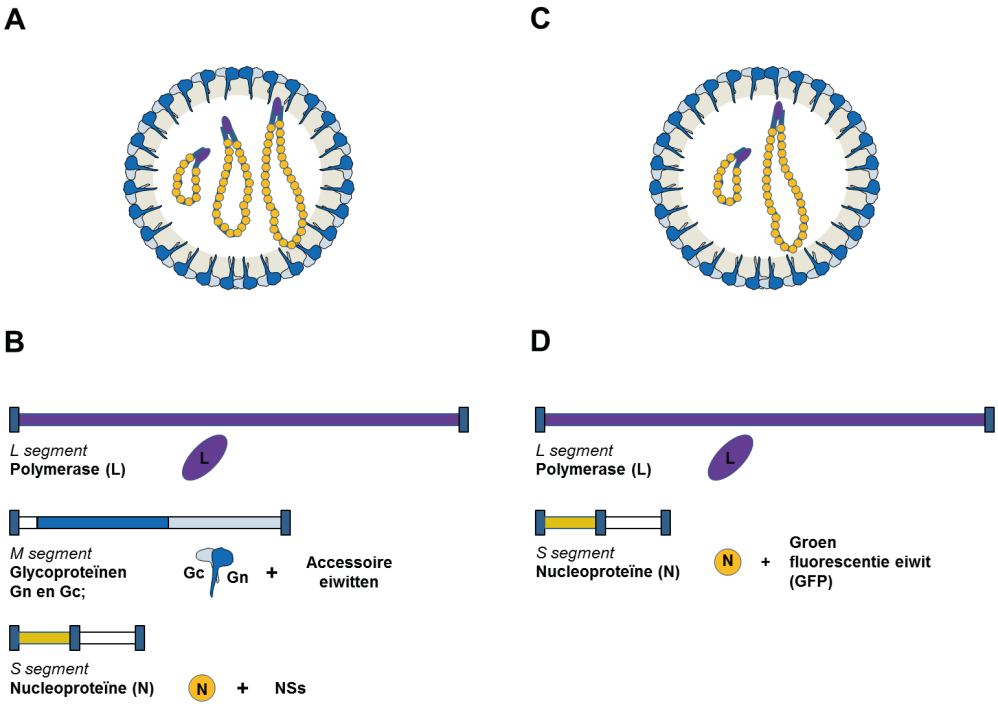
vond plaats in Kenia, in 1950-51. Naar schatting stierven hierdoor ongeveer 100.000 schapen. De grootste uitbraak bij mensen vond plaats in Egypte in 1977. Geschat wordt dat circa 200.000 mensen geïnfecteerd raakten, waarvan er 600 stierven. Hoewel het virus tot nu vooral beperkt bleef tot Afrika, is er grote zorg voor mogelijke verspreiding naar andere continenten. Muggen die als vector kunnen optreden zijn wereldwijd aanwezig.

RVF kan worden voorkomen door middel van vaccinatie. Op het moment dat het onderzoek beschreven in dit proefschrift startte, waren er in Afrika twee typen vaccin commercieel verkrijgbaar voor gebruik bij dieren. Dat betrof een levend, verzwakt vaccin (Smithburn vaccin) en een geïnactiveerd vaccin. Het levende vaccin is goedkoop te produceren en goed werkzaam na een enkele vaccinatie. Echter, het is niet geschikt voor gebruik bij drachtige dieren. Het geïnactiveerde vaccin kan daarentegen veilig worden toegediend bij alle dieren, in alle productiestadia. Nadelen van dit vaccin zijn dat het moeilijk te produceren is en meerdere vaccinaties nodig zijn voor een goede bescherming. Dit betekent ook hogere kosten. Nadat ons onderzoek was gestart, kwam er een derde vaccin beschikbaar op de Afrikaanse markt: Clone 13. Dit is een levend, verzwakt vaccin wat veilig en zeer effectief is in het beschermen van herkauwers tegen RVF. Buiten Afrika zijn er geen vaccins geregistreerd. Ook zijn er geen vaccins voor mensen beschikbaar. Het doel van het onderzoek beschreven in dit proefschrift was het ontwikkelen van een vaccin dat beschermd tegen RVF en werkzaamheid en veiligheid optimaal combineert.

Net als alle Bunyavirussen heeft RVFV een RNA-genoom, opgebouwd uit 3 aparte segmenten. Deze segmenten zijn genoemd naar hun grootte: groot (L van large), middel (M van medium) en klein (S van small) (Fig. 1A en 1B). Het grote segment codeert voor slechts één eiwit, een polymerase. Dit polymerase zorgt ervoor dat het genoom gekopieerd en vermenigvuldigd kan worden, een proces wat bekend staat als genoom replicatie. Nieuwe kopieën van het genoom zijn nodig om nieuwe virusdeeltjes te maken. Het middel segment codeert voor twee glycoproteïnen, Gn en Gc, en twee accessoire eiwitten. Gn en Gc bevinden zich aan het oppervlak van het virusdeeltje. Ze vormen als het ware de schil van het virus en zorgen ervoor dat het virus zich aan een cel kan hechten en daarin kan binnendringen. Het kleine segment codeert onder andere voor het nucleoproteïne, N. Dit eiwit omhult de genoomsegmenten en samen met het polymerase werkt om het genoom te repliceren. Daarnaast zorgen beide ervoor dat transcriptie plaatsvindt. Daarbij wordt de informatie op het virale genoom vertaald naar boodschapper-RNA (mRNA). Dit mRNA vormt een template voor het synthetiseren van (virale) eiwitten, een proces dat translatie wordt

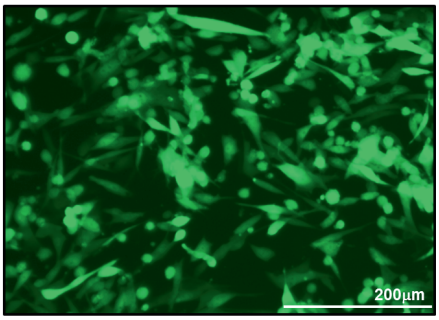
genoemd. Een tweede eiwit waar het kleine segment voor codeert is NSs, wat de immuunrespons van de gastheer verstoort.

In hoofdstuk 2 van dit proefschrift wordt de ontwikkeling van een niet-spreidend Rift Valley fever virus (NSR) beschreven. In tegenstelling tot RVFV heeft NSR maar twee genoomsegmenten: L en S (Fig. 1C en 1D). NSR deeltjes lijken op het originele virus en zijn in staat om een cel te infecteren. Ze zijn ook in staat om hun genoom te kopiëren en vermenigvuldigen. Ook transcriptie en translatie van het polymerase en nucleoproteïne is mogelijk, waardoor deze eiwitten worden geproduceerd in de geïnfecteerde cel. Echter, door het ontbreken van het M-segment wordt er geen Gn en Gc geproduceerd. Deze beide eiwitten zijn essentieel om nieuwe virusdeeltjes te vormen. In hun afwezigheid kunnen dus geen nieuwe virusdeeltjes worden geproduceerd. Op deze manier kan NRS een cel maar één keer infecteren en is dus "niet-spreidend".



Figuur 1. Schematische weergave van (A) een RVFV deeltje met (B) de drie genoomsegmenten, in vergelijking met (C) een NSR deeltje met (D) de twee genoomsegmenten. De verschillende kleuren representeren de virale structurele eiwitten (L, Gn, GC en N) en de respectievelijke genen voor deze eiwitten op de gensegmenten.

Aanvullend op het niet-spreidend zijn, ontbreekt bij NSR ook het gen dat codeert voor NSs. Dit eiwit remt normaal gesproken de celgebonden afweermechanismen en onderdrukt daarmee de immuunrespons. In afwezigheid van NSs kan de immuunrespons zich echter ongehinderd ontwikkelen, met een betere immuniteit als gevolg. Het gen voor NSs is bij NSR vervangen door een gen dat codeert voor een groen fluorescentie eiwit (GFP). In cellen die door NSR worden geïnfecteerd, wordt dus ook GFP geproduceerd. Op deze manier kan een infectie onder een fluorescentiemicroscoop worden bekeken (Fig. 2).



Figuur 2. Expressie van GFP in cellen die zijn geïnfecteerd met NSR deeltjes.

De effectiviteit van NSR als vaccine tegen RVF werd in eerste instantie onderzocht in muizen (**hoofdstuk 2** van dit proefschrift). Wij vonden daarbij dat een enkelvoudige vaccinatie met NSR muizen volledig beschermt tegen sterfte en ziekteverschijnselen na een infectie met RVFV. In een andere studie met lammeren (niet opgenomen in dit proefschrift) bleek een enkelvoudige vaccinatie ook te beschermen tegen ziekteverschijnselen na een infectie met RVFV. Echter, viraal RNA kon wel worden aangetoond in het bloed (een status bekend als viraemie), zij het in geringe hoeveelheden. Schapen zijn extra gevoelig voor RVFV en zelfs een lage viraemie kan ertoe leiden dat de vrucht in de baarmoeder geïnfecteerd wordt. Dit kan leiden tot afwijkingen aan de vrucht, of zelfs sterfte, vaak gevolgd door een abortus. Om niet alleen de volwassen dieren, maar ook hun vruchten te beschermen, is een vaccin nodig dat een viraemie volledig voorkomt. De sterke immuniteit die daarvoor nodig is, wordt ook wel een steriele immuniteit genoemd.

In **hoofdstukken 3** en **4** beschrijven we de ontwikkeling en evaluatie van een verbeterd NSR vaccin. In dit vaccin is ook het gen voor één van de oppervlakte-

eiwitten, Gn, aanwezig in het NSR genoom. Deze variant van NSR wordt NSR-Gn genoemd. Gn is het meest immunogene oppervlakte-eiwit. Een geïnfecteerde cel gaat nu ook Gn produceren, wat bijdraagt aan een betere effectiviteit van het vaccin. Met één enkele vaccinatie in lammeren kon met dit vaccin een steriele immuniteit worden opgewekt.

Behalve het gebruik van NSR als vaccine tegen RVF, waren we ook geïnteresseerd in het gebruik van NSR als vectorvaccin. Virale vectorvaccins worden daarbij ontworpen om een gen te dragen van een andere ziekteverwekker. Vaccinatie met een dergelijk vectorvaccin induceert dan een immuniteit tegen die betreffende ziekteverwekker. Vectorvaccins zijn vooral interessant als de veiligheid van levende vaccins tegen deze andere ziekteverwekkers ter discussie staat. Denk daarbij bv. aan het humaan immunodeficiëntievirus (HIV) of Ebolavirus. Bovendien zorgen vectorvaccins vaak voor een bredere en sterkere immuunrespons dan geïnactiveerde vaccins of subunitvaccins (dit zijn vaccins die op een stukje van een ziekteverwekker zijn gebaseerd).

Er is veel interesse in het ontwikkelen van vectorvaccins die via de slijmvliezen toegediend kunnen worden. Veel ziekteverwekkers dringen het lichaam binnen via de slijmvliezen, en met een goede lokale immuniteit kunnen ze ter plekke al geneutraliseerd worden. In ons onderzoek waren we dan ook speciaal geïnteresseerd in het gebruik van NSR als een toepassing via de slijmvliezen van de luchtwegen. RVFV is immers zeer besmettelijk via deze route, wat perspectieven biedt voor NSR.

In **hoofdstuk 5** wordt de ontwikkeling van NSR-HA beschreven, waarbij het gen voor hemagglutinine (HA) van influenza A virus is ingebouwd in NSR. HA is één van de twee oppervlakte-eiwitten van influenza A en een belangrijk aangrijpingspunt voor neutraliserende antistoffen. Het NSR-HA vectorvaccin werd onderzocht op zijn beschermende werking tegen influenza in muizen. In ons modelsysteem beschermde een enkelvoudige vaccinatie in de neus de muizen volledig tegen sterfte en ziekteverschijnselen nadat ze werden blootgesteld aan influenzavirus. Deze resultaten lieten zien dat NSR een veelbelovend vectorplatform is, geschikt voor toediening via de slijmvliezen van de luchtwegen.

In de laatste tientallen jaren is er ook veel onderzoek verricht naar het gebruik van virale vectoren bij de bestrijding van kanker. De oorsprong van deze interesse is gelegen in de ontdekking dat specifieke kenmerken van de immuniteit tegen virussen ook kunnen bijdragen aan een immuunrespons tegen

tumoren. Een aantal karakteristieken van de door NSR opgewekte immuniteit komen overeen met vectoren die al voor de bestrijding van kanker gebruikt worden, of zijn anderszins nuttig bij de bestrijding van kanker. Dit dreef ons ertoe om ook een experiment uit te voeren waarbij de effectiviteit van NSR in de kankertherapie werd onderzocht. De resultaten van dit onderzoek in een muismodel zijn beschreven in **hoofdstuk 7**. Ze laten zien dat NSR tumorgroei kan reduceren, of dat tumoren zelfs geheel verdwijnen. Dit suggereert dat NSR ook een veelbelovende vector is in de kankertherapie.

In onze vaccinatie-experimenten vonden we consistent een hoge effectiviteit van op NSR gebaseerde vaccins. Dit wekte onze interesse naar de moleculaire basis van de immuunrespons. We richtten het onderzoek op de interactie tussen NSR en dendritische cellen (DC). Deze cellen spelen een cruciale rol in de immuniteit omdat zij een brug vormen tussen de aspecifieke en adaptieve immuniteit. DCs zijn uitgerust met diverse receptoren voor het herkennen van veel verschillende ziekteverwekkers. Herkenning van ziekteverwekkers leidt tot maturatie van de DCs. De DCs verplaatsen zich vervolgens naar de lymfoïde organen (milt, lymfeknopen), waar ze T-cellen gericht tegen specifieke ziekteverwekkers aanzetten tot vermenigvuldiging. Dit proces is bekend als T-cel priming. Daarmee initiëren deze gematureerde DCs de ontwikkeling van een cellulaire immuunrespons. De belangrijkste marker waaraan een gematureerde DC herkend kan worden is CD83. Als CD83 niet toeneemt, is dat een teken dat de DC niet (volledig) matureert en ook geen T-cellen kan primen.

In ons onderzoek, beschreven in **hoofdstuk 6**, laten we zien dat met NSR geïnfecteerde DCs niet volledig matureren, wat gepaard gaat met een afname van CD83. Dit was een verrassende bevinding, aangezien consistent een robuuste T-cel immuunrespons werd waargenomen na vaccinatie met NSR. We vonden echter ook dat bystander DCs (die zelf niet geïnfecteerd raakten) wel een volledige maturatie ondergaan, waarbij CD83 toeneemt. Dit resultaat suggereert dat ook deze bystander DCs een belangrijke rol spelen in de immuniteitsopbouw. Het is niet gelukt het exacte mechanisme te ontrafelen van de waargenomen daling van CD83 in de geïnfecteerde cellen. We hebben wel vastgesteld dat het plaatsvindt op het niveau van eiwittranslatie.

In conclusie:

In dit proefschrift is de ontwikkeling van een krachtig en flexibel vaccinplatform beschreven. Dit platform, gebaseerd op niet-spreidend RVFV, heeft bewezen een effectief vaccin tegen RVF op te leveren. Gecombineerd met de intrinsieke

veiligheid van dit vaccin, levert dit een waardevol hulpmiddel op voor de preventie van RVF bij herkauwers. Een op NSR gebaseerd vector vaccin, toegediend via de luchtwegen, bleek zeer effectieve tegen een luchtweginfectie (influenza), waarmee ook de mogelijkheid van toediening via de slijmvliezen werd aangetoond. Ten slotte bleek NSR effectief bij het verkleinen of opruimen van tumoren, wat veelbelovend is voor het gebruik van NSR als vectorvaccin in de kankertherapie.

A

Summary

Rift Valley fever virus (RVFV) belongs to one of the largest virus families, named *Bunyaviridae*. The family comprises enveloped viruses with negative-strand RNA genomes. Five genera are included in this family: O*rthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus*, and *Tospovirus.* The viruses of the first four genera infect animals, while those of the last genus infect only plants. Bunyaviruses are transmitted by arthropods (e.g. mosquitoes, ticks, sand flies, thrips), with the exception of the hantaviruses, which are transmitted via rodent excreta. From the viruses that affect animals, several can produce mild to severe disease in humans, in animals and sometimes in both.

RVFV is transmitted by mosquitoes. The virus most commonly affects domesticated ruminants such as sheep, goats, cattle, buffalo and camels, causing acute disease, abortions and death. The severity of symptoms varies among the different animal species, of which sheep are the most susceptible. RVFV can infect humans as well. Humans can be infected via mosquito bite, although most cases are ascribed to direct contact with tissues or blood of diseased or dead animals. Most commonly, people will experience a mild illness, manifested with fever, generalized weakness, back pain, and dizziness at the onset of illness. Typically, these symptoms resolve within few days. Sometimes however, complications develop. The most common complication is retinal damage, which may result in temporary or permanent blindness. A small percentage (1-2%) of patients may develop severe complications including hepatic necrosis, hemorrhagic fever or encephalitis. Patients that develop these severe complications may succumb to the infection. Given the zoonotic potential of RVFV and the extensive financial losses for the farming industry resulting both from death of their livestock and trade restrictions, outbreaks of RVFV have major socio-economic impact in affected regions.

The virus was identified in 1931 by Daubney and co-workers after investigation of an outbreak on a farm near Lake Naivasha in the Rift Valley of Kenya in the previous year. Since then, the virus has spread throughout the African continent and has reached the Arabian Peninsula and several islands off the shore of Southern Africa. The most notable outbreak of RVF occurred in Kenya in 1950-1951, resulting in the death of an estimated 100,000 sheep. The largest human outbreak so far was recorded in Egypt in 1977, where an estimated 200,000 people were affected and about 600 people died from the infection. Although the virus is largely confined to the African continent, its spread to new continents is

of concern because mosquitos associated with transmission of the virus in endemic areas are globally prevalent.

RVF can be prevented by vaccination. Two types of vaccines were commercially available in Africa when the research described in this thesis was started, based either on a live attenuated vaccine (Smithburn vaccine) or on an inactivated vaccine. The Smithburn-based vaccine is inexpensive to produce and very efficacious after a single administration. However, it is not suitable for use in gestating animals. The vaccine based on inactivated virus, on the other hand, can be safely applied during all life stages. However, this vaccine is laborious to produce and requires repeated administrations for optimal efficacy, implying higher production and application costs. After the research described in this thesis started, a third vaccine became commercially available in Africa, named Clone 13. This live-attenuated vaccine is safe and very efficacious in protecting ruminants against RVF. There is no veterinary vaccine registered for use outside Africa and a vaccine for humans is also not available. The purpose of the study described in this thesis was to develop a RVF vaccine that optimally combines safety and efficacy.

Like all bunyaviruses, RVFV has an RNA genome divided into 3 segments named after their size: large (L), medium (M) and small (S) (Figs. 1A and B). The L segment contains the polymerase gene. The polymerase is responsible for copying and multiplication of the viral genome, a process known as genome replication. New copies of viral genome are needed for new virus particles to be produced. The M segment encodes the two structural glycoproteins, Gn and Gc and two accessory proteins. The Gn and Gc proteins form the protein shell of the virus and are responsible for cell attachment and entry. The small segment encodes the nucleoprotein N. This protein enwraps the viral genome and together with the viral polymerase functions as a viral replication machinery. In addition to replication, the polymerase and the nucleoprotein perform transcription. During this process, the information encoded by the (viral) genome is transcribed into messenger RNA (mRNA), which on turn serves as a template for synthesis of (viral) proteins, a process termed translation. The small segment encodes also another protein, called NSs, which antagonizes the host innate immune response.

In **chapter 2** of this thesis, the creation of nonspreading Rift Valley fever virus (NSR) is described. In contrast to the authentic virus, NSR has only two genome segments, L and S (Fig. 1C and D). NSR particles resemble the authentic virus and are able to infect cells. They are also capable of viral genome replication, transcription and synthesis of the polymerase and nucleoprotein. However NSR

particles lack the M segment that encodes the glycoproteins Gn and Gc. These two proteins are essential for assembly of new virus particles and in their absence progeny virions cannot be produced. Hence, NSR are only able to infect a cell once and are "nonspreading".

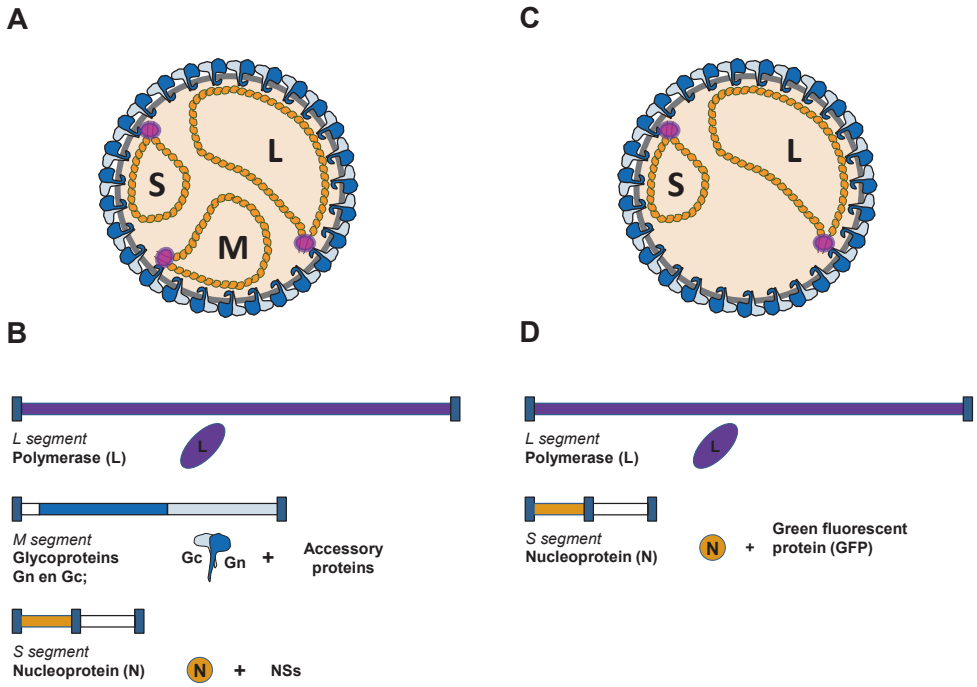


Figure 1. Schematic representation of (A) a RVFV particle with (B) the three genome segments and (C) an NSR particle with (D) the two genome segments. Color code is used to represent the viral structural proteins (L, Gn, Gc and N) and the respective genes on the genome segments.

In addition to being nonspreading, NSR lacks the gene that encodes the NSs protein. This protein suppresses the cellular defense mechanisms and interferes with the development of an immune response. In the absence of NSs, the immune response against NSR can fully develop. The gene of the NSs protein is replaced with the gene of the green fluorescent protein (GFP). In cells infected by NSR, GFP protein is produced, thereby infection can be observed under a fluorescent microscope (Fig. 2).

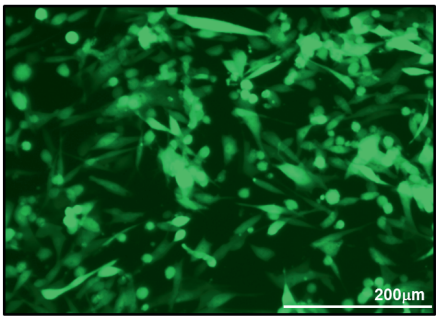


Figure 2. Expression of GFP in cells after infection with NSR particles.

The efficacy of NSR as a vaccine against RVFV was initially evaluated in mice (**Chapter 2** of this thesis). We established that a single vaccination with NSR is sufficient to fully protect mice from death and clinical signs, resulting from challenge infection with virulent RVFV. In another study (not included in this thesis), a single vaccination prevented clinical signs in lambs. However, viral RNA was present in the blood (status known as viremia) at low levels. Sheep are especially sensitive for infection. Even a low viremia in this species can result in infection of fetuses of gestating ewes, leading to fetus malformations and/or death, often followed by abortions. Therefore, to protect gestating ewes and their fetuses from disease, a vaccine should induce an immunity that completely prevents viremia. Such immunity is often called a sterile immunity.

In **Chapters 3** and **4** the development and evaluation of an improved NSR vaccine is described. In this vaccine, the gene of one of the surface glycoproteins of RVFV, Gn, was introduced in the NSR genome, creating NSR-Gn. Gn is the most immunogenic surface glycoprotein of RVFV and its production by host cells infected by NSR-Gn significantly contributed to vaccine efficacy. Accordingly, a single vaccination with NSR-Gn resulted in sterile immunity in lambs.

Apart from using NSR as a vaccine against RVFV, we were interested in testing its possible application as a vector vaccine. Viral vector vaccines are designed to carry a gene derived from a pathogen of interest. Vaccination with vector vaccines induces immunity against the pathogen of interest. Vector vaccines are especially suitable when safety of live vaccines is of concern, for example for human immunodeficiency virus or Ebola virus. In addition, vector vaccines often trigger a broader and more potent immune response than inactivated vaccines or subunit vaccines (vaccines based on parts of a pathogen).

There is much interest in developing vector vaccines specifically designed for mucosal administration. Many pathogens invade the body via mucosal surfaces and a good mucosal immunity could neutralize these pathogens already at the site of invasion. In our studies we were especially interested in using NSR as a vaccine for respiratory application, as the authentic RVFV is very infectious via the respiratory route.

In **Chapter 5** the development of NSR-HA is described that contains the gene encoding influenza A virus hemagglutinin (HA). The hemagglutinin is one of the two influenza A virus surface glycoproteins and is the target for neutralizing antibodies. The NSR-HA vector vaccine was evaluated for its protective efficacy against influenza in mice. In our model system, a single intranasal vaccination with NSR-HA completely prevented death and clinical signs following infection with influenza virus. These results show the potency of NSR as a vector platform, especially suitable for administration via the respiratory mucosa.

In the last decades, viral vectors are widely explored not only for therapeutic vaccinations against various pathogens, but also for immunotherapy against cancer. This interest originates in certain features of virus-induced immunity that might overcome the immunosuppressive environment in cancer patients, resulting in eliciting of a rigorous immune response against tumors. Some of the characteristics of NSR-elicited immunity are shared with established vectors and/or are known to be advantageous for cancer immunotherapy. Therefore we were encouraged to study the efficacy of NSR as a viral vector for this application. Our results in a mouse model, described in **Chapter 7** of this thesis, show that NSR can reduce tumor growth and even eradicate tumors, suggesting that NSR can be considered a promising vector for cancer immunotherapy.

In our vaccination experiments we consistently observed a high efficaciousness of NSR-based vaccines and we were interested to unravel the molecular basis of the NSR-induced immune response. To that end we investigated the interaction between NSR and dendritic cells (DC). These cells play a pivotal role in immunity, providing a bridge between innate and adaptive immune responses. DCs are equipped with diverse receptors for recognition of numerous pathogens. Pathogen recognition activates cellular programs that result in DC maturation and migration from the periphery (for example skin or mucosal surfaces) to lymphoid organs (spleen and lymph nodes), where DCs stimulate pathogen-specific T lymphocytes to proliferate, a process known as T-cell priming. Thereby mature DCs initiate the development of a cellular immune response. The most important marker of a mature DC is CD83. Failure to increase CD83 at the DCs surface correlates with incomplete maturation and incapability to prime T-cells.

In **Chapter 6** of this thesis, we show that NSR-infected DCs undergo incomplete maturation, associated with a decrease in CD83. This finding was surprising, given the consistently robust T-cell immune response, observed after vaccination with NSR. However we noticed that bystander DCs undergo complete maturation and upregulate CD83. Our results suggest that not only infected, but also bystander DCs play an important role in NSR-mediated immunity. Although we were unable to unravel the exact mechanism of the observed decrease of CD83 in infected cells, we established that it occurs at the level of protein translation.

In conclusion:

In this thesis, the development of a powerful and flexible vaccine platform is described. This platform, based on nonspreading RVFV, proved to be a potent vaccine against RVFV and its efficacy, combined with its intrinsic safety, offer a highly valuable tool for the prevention of RVF in ruminants. An NSR-based vector vaccine, applied intranasally, was highly effective against a respiratory infection (influenza), revealing its potency for mucosal administration. Finally, NSR was shown to be efficacious in clearing tumor cells, holding promise for use in cancer immunotherapy.

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Curriculum vitae

Nadia Oreshkova was born on the 15th of October 1976 in Sofia, Bulgaria. In 1990 she went to a high school in Sofia and she graduated cum laude in 1995. The same year she started her university education, studying Veterinary Medicine at the Forestry University in Sofia. She graduated in 2001 as a veterinarian and immediately started working in a small veterinary practice. After two years, she moved to the National Diagnostic and Research Veterinary Institute (NDRVI), Sofia, where she worked as a researcher on avian influenza and Newcastle disease viruses for five years. In 2007 she moved to the Netherlands and in 2008 she started following the Master's degree program Biomolecular Sciences at VU University Amsterdam. She graduated cum laude in 2010. During her study she followed two internships. The first one was at the VU, in the group of Dr. Joen Luirink. During this internship, she did a research on an autotransporter protein of E. coli. The second internship was at CVI, in the group headed by Prof. dr. Rob Moormann. The research carried out during this internship was expanded into a four and a half years PhD project and Nadia continued her research as a PhD student. The work in the group of Prof. dr. Moormann is described in this thesis. She finished her PhD research in April 2015. From the beginning of August 2015, Nadia continues her carrier as a post doc in the group of Prof. dr. Eric Snijder in Leiden University Medical Center.

A

List of publications

Oreshkova N, Wichgers Schreur PJ, Spel L, Vloet RPM, Moormann RJM, Boes M, Kortekaas L. *Nonspreading Rift Valley fever virus infection of human dendritic cells results in downregulation of CD83 and full maturation of bystander cells.* Submitted for publication.

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Conference contributions

Nonspreading Rift Valley fever virus vector protects mice from lethal influenza by a single intranasal vaccination. Oral presentation at the 33rd Annual Meeting of the American Society for Virology, June 21-25, Fort Collins, Colorado, USA

Vaccine efficacy of a nonspreading Rift Valley fever virus. Oral presentation at the 15th International Negative Strand Virus (NSV) Meeting, 16-21 June 2013, Granada, Spain

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Gabriela Goujgoulova, **Nadia Oreshkova**, Vladimir Savić, Elizabeth Aldous, Ruth Manvell. *Recent situation regarding Newcastle disease in Bulgaria.* Poster at the Poultry Days Symposium, Zagreb, 2007