Recombinant measles virus incorporating heterologous viral membrane proteins for use as vaccines

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Recombinant measles virus (rMV) vectors expressing heterologous viral membrane protein antigens are potentially useful as vaccines. Genes encoding the mumps virus haemagglutinin-neuraminidase (MuV-HN), the influenza virus haemagglutinin (Flu-HA) or the respiratory syncytial virus fusion (RSV-F) proteins were inserted into the genome of a live attenuated vaccine strain of measles virus. Additionally, in this case rMV with the MuV-HN or the influenza HA inserts, chimeric constructs were created that harboured the measles virus native haemagglutinin or fusion protein cytoplasmic domains. In all three cases, sucrose-gradient purified preparations of rMV were found to have incorporated the heterologous viral membrane protein on the viral membrane. The possible utility of rMV expressing RSV-F (rMV.RSV-F) as a vaccine was tested in a cotton rat challenge model. Vaccination with rMV.RSV-F efficiently induced neutralizing antibodies against RSV and protected animals from infection with RSV in the lungs.

INTRODUCTION

Measles virus (MV) harbours a negative-strand RNA genome that is packaged inside a pleomorphic lipid envelope in which the integral membrane proteins haemagglutinin (H) and fusion (F) proteins are embedded. The matrix (M) protein is located internally to the membrane and is thought to interact with the cytoplasmic domains of the membrane proteins. The 15.9 kb genomic RNA is wound into a helical structure in association with the viral nucleoprotein (N), phosphoprotein (P) and large protein (L). The vaccine strains in use today, such as Edmonston–Zagreb, Schwarz or AIK-C are based on isolates that harbour attenuating mutations ensuring the safety of the live virus vaccine when administered to infants.

Vaccination against measles has proven to be one of the most successful public health measures to have been universally adopted. Following the development of a viral rescue system from cloned DNA (Radecke et al., 1995), insertion of foreign sequences into the measles genome to create vectors has been shown to be practicable (Spielhofer et al., 1998). Such vectors offer the possibility of the creation of novel vaccines based on a platform that is already in widespread use and found to be safe and efficacious in humans (Ramsauer et al., 2015). Similarly to a measles vaccine, such a vectored vaccine would be expected to replicate and express encoded genes, including the foreign transgene(s), following vaccination. This may be advantageous where an effective T-cell response is considered to be a desirable component of a protective immune response against the targeted infectious agent. When a recombinant measles virus (rMV) expressing the hepatitis B surface antigen protein was tested in mice, only the replicative form, and not the UV-inactivated virus, was found to result in the production of antibodies against the transgene (Singh et al., 1999). This suggested that at least in some instances, rMV vectors need to replicate in order to elicit a strong immune response to encoded transgenes. However, an rMV vector displaying transgene antigens on the surface may be expected to elicit protective antibodies even in the absence of viral replication. This has been demonstrated using an UV-inactivated chimeric MV vector where the native H and F proteins had been replaced with the vesicular stomatitis virus (VSV) G protein, and that was shown to protect mice against a lethal VSV challenge (Spielhofer et al., 1998).

Recombinant measles vectors may be considered for the construction of paediatric vaccines for other
paramyxoviruses such as mumps virus (Wang et al., 2001) or respiratory syncytial virus (RSV) (Mok et al., 2012; Sawada et al., 2011; Yamaji & Nakayama, 2014). The attenuated mumps virus is already included in the paediatric cocktail vaccine together with measles and rubella viruses (MMR). In principle, an rMV vector capable of expressing mumps proteins is an attractive proposition because it would result in immunization against both diseases. Another attractive feature of using an rMV vector as a paediatric vaccine would be to add functionality to the existing proven measles vaccine by adding antigenic components of disease agents such as RSV against which vaccines are not available. However, rMV vectors have previously been found to be genetically unstable when harbouring genes from other related paramyxoviruses, such as the mumps virus fusion protein, whereas unrelated proteins, like the simian immunodeficiency virus (SIV) envelope protein, was found to be stable (Wang et al., 2001). It was therefore suggested that rMV vectors expressing additional proteins from closely related paramyxoviruses such as mumps virus are intrinsically unstable, as opposed to chimeric vectors where the native homologues are deleted (Mourez et al., 2011; Spielhofer et al., 1998).

One possible approach towards enhancing the stability of the expression of foreign viral envelope proteins in rMV vectors without deleting the native H and F proteins, i.e. to retain the measles vaccine characteristics, is to incorporate the cytoplasmic domains of MV transmembrane proteins in the exogenous protein in order to facilitate interactions with the MV M protein. Here we report on the construction, physical characterization and stability of rMV vectors expressing envelope proteins from the related paramyxoviruses mumps and RSV, as well as the unrelated (orthomyxovirus) influenza virus haemagglutinin (Flu-HA) protein, using either the full-length native sequence and, in case of mumps virus haemagglutinin-neuraminidase (MuV-HN) and influenza HA, chimeric constructs harbouring measles virus H or F cytoplasmic domains, respectively. We further report on the vaccine efficacy of rMV expressing the respiratory syncytial virus fusion (rMV.RSV-F) protein in a cotton rat challenge model.

RESULTS

Recombinant MV vectors based on the Berna measles vaccine strain (Edmonston Zagreb 19 strain, MVEZb) harbouring foreign transgenes were created by transfection of cDNA plasmids as previously described (Cantarella et al., 2009; Liniger et al., 2009). This strain differs by 38 nucleotides from the canonical Edmonston Zagreb (MVEZ) strain (Zuniga et al., 2013) that has also been used to construct measles vectors (Rennick et al., 2015). Two versions of each of the foreign viral envelope proteins were cloned into the MV plasmid: one where the native viral protein sequence was used, and another, where the cytoplasmic domain of the MV H protein or the MV F protein was used, as appropriate, i.e. depending on whether the insert was a type I or a type II transmembrane protein, as detailed below. These constructs were inserted into the MV genome as shown in Fig. 1.

Recombinant mumps virus expressing mumps virus haemagglutinin-neuraminidase

The MuV-HN is a type II transmembrane protein. In order to create a chimeric version of this protein (sequence derived from strain Jeryl Lynn), the N-terminal 54 residues (comprising its cytoplasmic and transmembrane domains) were replaced by the homologous N-terminal 59 residues of the MV H protein, i.e. the C-terminal 528 residue fragment of Mu-HN was fused at its N terminus to the first 59 residues of the MV H protein.

MV recombinants harbouring the full-length or the chimeric Mu-HN protein (rMV.Mu-HN or rMV.Mu-HN.ecto, respectively) were rescued and amplified and passaged as described in Methods. Both viruses could be rescued and amplified to titres that were a little lower than those obtained with MV not harbouring an insert (MVEZ). To analyse the stability of the transgene, rMV clones were sequencially passaged as described in Methods, viral RNA was isolated from culture supernatants, and the transgene region was reverse transcribed and amplified by PCR (RT-PCR). The RT-PCR product was directly sequenced looking for secondary nucleotide peaks that may point towards the emergence of mutations. Two clones, each of rMV.Mu-HN and rMV.Mu-HN.ecto, were tested in this manner at passage numbers 3, 6 and 10, respectively. Mutations were detected in both rMV.Mu-HN clones at passage 6. In one clone, a sub-population could be detected where a single base change resulted in residue 324 to be changed from a glycine to a tryptophan. This residue is located in the third canonical sialidase propeller domain; the significance if any of this mutation has not been ascertained. In the other rMV.Mu-HN clone, a single base change resulted in a stop codon being introduced in place of a lysine codon at residue 74, i.e. most of the extra-cytoplasmic domain was truncated. Thus, although the rescued rMV.Mu-HN expressed the Mu-HN transgene for at least six passages, the emergence of mutant sub-populations was detected by genome RT-PCR. The proportion of the mutant recombinants appeared to increase with successive passages: in case of the mutant where the stop codon was found, it comprised the majority of the preparation by passage 10. For studies on this recombinant virus (described below), the passage 4 stock was used, where any mutation was below the level detectable by Sanger sequencing. In contrast to rMV.Mu-HN, in case of rMV.Mu-HN.ecto (where the cytoplasmic and transmembrane domains were derived from the MV H protein) mutations were not detected in the either of clones for at least the 10 passages that were tested.

In order to determine whether the transgenes Mu-HN and Mu-HN.ecto were expressed and incorporated in the recombinant virus particles, the amplified recombinant viruses were purified on a 5–60% sucrose gradient.
Fractions were collected from the bottom of the tube and aliquots of each fraction were assayed for neuraminidase activity deriving from the presence of the Mu-HN protein. The infectious titre in each fraction was also determined. Unexpectedly, peaks corresponding to the neuraminidase activity and the viral TCID$_{50}$ were found to be overlapping, but not concordant (Fig. 2a). This observation suggests the possibility of the presence of a population of particles of lower density containing the Mu-HN protein and exhibiting neuraminidase activity, but with reduced infectivity, i.e. similar to virus-like particles (VLPs). As this was the case for both Mu-HN and Mu-HN.ecto constructs, i.e. that with the native cytoplasmic domain of Mu-HN as well as the chimeric version harbouring the cytoplasmic domain from the MV H protein, the putative VLP-like particle formation is not dependent on MV-specific interactions between the MV matrix protein and the Mu-HN chimeric protein. It has been reported that expression of the Mu-HN protein alone does not result in VLP formation (Li et al., 2009). We also determined that transfection of cells with expression plasmids for the mumps HN proteins, either alone or in combination with the measles M protein, did not result in detectable VLP that could be banded on a sucrose gradient (data not shown); so the nature of the particles with high neuraminidase activity but low infectivity could not be determined.

Since the immunogenicity of an rMV vaccine may be expected to be dependent on antigen dosage, it was of interest to compare the amount of Mu-HN and Mu-HN.ecto proteins that were present in the rMV preparation compared to a stock of a vaccine strain of mumps virus. To do this, the neuraminidase activity in the rMV expressing the MuV-HN proteins was found to be 30–50% lower than the mumps virus stock (Fig. 2b). Neuraminidase activity was also measured in sucrose-gradient purified rMV.Mu-HN.ecto virus, where it was also found to be comparable to mumps virus (data not shown).
Recombinant measles virus expressing influenza virus haemagglutinin

As described above for the MuV-HN protein, two versions of the influenza virus haemagglutinin (Flu-HA) protein (a type I transmembrane protein) were cloned into the MV plasmid (Fig. 1); the full length protein from the A/Brisbane/59/2007 (H1) strain (Flu-HA), as well as a chimeric protein containing the N-terminal 561 aa residues (i.e. the extra-cytoplasmic and transmembrane domains) of Flu-HA fused at its C terminus to the C-terminal 35 residues of the MV F protein that constitute its cytoplasmic domain (Flu-HA.ecto).

Recombinant MV expressing either the full-length influenza HA protein (rMV.Flu-HA) or the chimeric protein (rMV.Flu-HA.ecto) could be rescued, propagated and purified on sucrose density gradients. In order to determine whether the haemagglutinin that was present in the purified rMV preparations was folded into its native conformation as might be expected if it were expressed on the viral membrane, two anti-influenza haemagglutinin mAbs that specifically bind to native conformational epitopes were used. Sucrose-gradient purified rMV preparations of rMV.Flu-HA or rMV.Flu-HA.ecto were coated onto ELISA plates and probed with the human mAbs CR6261 (Throsby et al., 2008) or CR9020 (Brandenburg et al., 2013). The antibody CR6261 binds to the stem region of the trimeric influenza haemagglutinin protein; CR9020 binds to the head domain of H1 subtype of haemagglutinin. Binding of both CR6261 and CR9020 to the purified rMV preparation could be demonstrated (Fig. 3b).

Recombinant measles virus expressing respiratory syncytial virus-fusion protein

As described above for the MuV-HN and the influenza HA proteins, two versions of the RSV-F protein (a type I
RSV-F antibody CR9503 was found to specifically bind to purified rMV.RSV-F, but not to other rMV (Fig. 3b). The monoclonal anti-RSV-F antibody CR9503 was used as a negative control, which was found to only bind rMV.RSV-F, rMV.RSV, rMV expressing RSV-F.

The incorporation of transgene RSV-F protein into rMV. RSV-F particles was tested by ELISA. Sucrose-gradient purified rMV, RSV-F was coated on to ELISA plates (as described above for rMV.Flu-HA). The monoclonal anti-RSV-F antibody CR9503 was found to specifically bind to rMV.RSV-F, but not to other rMV (Fig. 3b).

The presence of the RSV-F protein in purified rMV.RSV-F was also tested by Western blotting (Fig. 4a). A preparation of rMV.RSV-F was fractionated on a sucrose gradient and aliquots of each fraction probed for the presence of RSV-F (upper) or measles N (lower) proteins by Western blotting as indicated. The purified virus was found to harbour both proteins.

Because expression of recombinant RSV-F protein results in secretion of F into the supernatant, we wanted to confirm that the detected F protein present in the purified rMV. RSV-F preparation was not due to the presence of secreted RSV-F protein aggregates or micelles being co-purified with the rMV preparation. In order to do this, we tested whether the measles virus protein N would be pulled down if the purified rMV.RSV-F preparation were subjected to immunoprecipitation with an anti-RSV-F mAb. Accordingly, rMV.RSV-F virus or purified measles viruses (as a negative control) or purified RSV (as a positive control) were immunoprecipitated with the human anti-RSV-F mAb CR9503. The eluted virus was then subjected to Western blotting, probing with anti-RSV-F (Fig. 4b, upper, the same antibody used for immunoprecipitation) or an anti-measles virus N protein antibody (Fig. 4b, lower). The presence of RSV-F on measles N containing particles was observed, thereby indicating that at least a proportion of the purified rMV. RSV-F harboured rMV decorated with RSV-F protein. Comparison of the intensity of the measles N protein band in the starting material (Fig. 4b, lower gel, rightmost lane) with the intensity of the band in the immunoprecipitated material (Fig. 4b, lower gel, leftmost lane) indicates that only a small proportion of the measles N-containing particles incorporated RSV-F.

The morphology of the rMV.RSV-F particles was also examined by immunoelectron microscopy (Fig. 4c). Immunogold particles (10 nm) labelled with anti-RSV-F or anti-measles virus H, were used as probes. Pleomorphic viral particles with a morphology that is consistent with measles virus could be stained with either antibody, strongly suggesting that rMV.RSV-F contained RSV-F on the viral membrane.

The immune response to the rMV.RSV-F vaccine would be expected to be related the amount of RSV-F protein that is present in the inoculum, as well as adequate expression of the transgene protein during replication following vaccination. A semi-quantitative estimation of the RSV-F protein present in the inoculum, as well as adequate expression of the transgene protein during replication following vaccination. A semi-quantitative estimation of the RSV-F protein that was present in the rMV.RSV-F preparation in comparison to that present in wild-type RSV was carried out by Western blotting (Fig. 5). The amount of RSV-F protein present in rMV.RSV-F (purified and concentrated by ultrafiltration) was found to be 5–10-fold lower than that was present on RSV on a per infectious unit basis.

**Immuneogenicity and efficacy of rMV.RSV-F in cotton rats**

Female cotton rats (6–8 weeks old) were randomly allocated into experimental groups (eight animals per group) as shown in Table 1. The cotton rats were immunized with 10⁵ or 10⁶ TCID₅₀ of rMV.RSV-F administered intra-muscularly as described in Methods. Control groups received either diluent

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**Fig. 3.** (a) Recombinant measles virus with the inserted the influenza HA sequence (upper) or the chimeric HA sequence (lower) were fractionated on a sucrose gradient and aliquots of each fraction probed for the presence of HA or measles N proteins as indicated. (b) Binding of anti-HA conformation-specific antibodies CR6261 and CR9020 to purified rMV expressing HA. The anti-RSV-F antibody CR9503 was found to only bind rMV.RSV-F. rMV.RSV, rMV expressing RSV-F.

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buffer, empty measles vector (10⁶ TCID₅₀) delivered intramuscularly or RSV delivered intranasally [A2 subtype, 10⁴ plaque-forming units (p.f.u.)] to simulate immunity from a natural exposure to RSV. Twenty-eight days later, two of the experimental groups were administered the booster dose of rMV.RSV-F at the same dose as the priming dose (Table 1). On day 49, all the animals were challenged with a dose of RSV-A2 (10⁵ p.f.u.) that has previously been determined to be highly pathogenic in this model (Boukhvalova et al., 2009; Prince et al., 1978, 1979). All the animals were terminally bled 5 days later (day 54) and lung and nasal turbinate tissues harvested for analyses as described in Methods.

The titres of the antibodies elicited by immunization with rMV.RSV-F that were capable of neutralizing infectious RSV are shown in Fig. 6a. A clear dose effect in the elicited antibody titre was evident; at the 28-day time-point, animals that received the higher dose (10⁶ TCID₅₀) of the rMV.RSV-F vaccine had on average approximately three-fold higher anti-RSV neutralization titres than animals that had received the lower dose of vaccine. However, the neutralization titres declined to similar levels at the 49-day time-point in animals in both dosage groups. In both cases it was possible to arrest this decline with a second dose of the rMV.RSV-F vector, i.e. the titres on day 28 were not significantly different from the titres on day 49 in the groups that received either of the boosting doses. This failure to boost the response beyond the neutralization titre obtained after priming was possibly due to an antibody response to the measles vector (Fig. 7) which may have compromised the effectiveness of the boosting dose, leading to only a minor restimulation of the primary response. It is also

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**Fig. 4.** (a) RSV-F was fractionated on a sucrose gradient and aliquots of each fraction probed for the presence of RSV-F (upper) or measles N (lower) proteins by Western blotting. The measured sucrose percentage of the fractions (22–36 %) is shown. The majority of the RSV-F protein is in the form of the furin-mediated cleavage product F₁. (The approximately 20 kDa band corresponds to an uncharacterized RSV-F degradation product.) (b) Co-immunoprecipitation of the measles virus N protein with an anti-RSV-F antibody. Purified rMV.RSV-F was immunoprecipitated using the human anti-RSV-F antibody CR9503. Measles virus (MV) and RSV were used as negative and positive controls, respectively. The immunoprecipitated products were eluted, electrophoresed and probed for the presence of RSV-F (upper) or measles N (lower) proteins as indicated. An aliquot of rMV.RSV-F starting material (not immunoprecipitated) was electrophoresed on the rightmost lane. The arrows indicate the position of the uncleaved RSV-F protein - F₀, and furin-cleaved F₁. (c) Detection of RSV-F protein on the surface of purified rMV.RSV-F by immunoelectron microscopy. Purified MV (left, upper and lower) and rMV.RSV-F (right, upper and lower) were examined by electron microscopy after immunogold-labelling as described in Methods. The colloidal gold particles were labelled with an anti-measles virus H (top row) or anti-RSV-F (bottom row).

**Fig. 5.** Semi-quantitative estimation of RSV-F present in purified rMV.RSV-F compared to RSV. The indicated amounts of each virus were electrophoresed and RSV-F was detected by Western blotting. The indicated amounts of purified soluble recombinant RSV-F protein were electrophoresed on the same gel to serve as standards. (Some degradation of the RSV-F protein in the rMV. RSV-F preparation is observed; the recombinant RSV-F preparation contains some aggregates.)
Table 1. Cotton rats were divided into treatment groups as shown, eight animals per group

Four groups of cotton rats were immunized with recombinant measles virus expressing the RSV-F protein (rMV.RSV-F) given intra-muscularly (i.m.) using the doses indicated. Control groups received empty measles vector i.m. or were immunized with a low dose of RSV given intra-nasally (i.n.). Two of the groups were boosted 28 days later using the indicated dose of rMV.RSV-F. The animals were challenged i.n. with RSV A2 (10^5,p.f.u.) on day 49 of the experiment and sacrificed on day 54.

<table>
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<th>Groups</th>
<th>Route</th>
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<th>Dose per rat</th>
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<tr>
<td>Prime only</td>
<td>i.m.</td>
<td>rMV.RSV-F</td>
<td>10^5 TCID&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>Prime and boost</td>
<td>i.m.</td>
<td>rMV.RSV-F</td>
<td>10^5 TCID&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>Negative control</td>
<td>i.m.</td>
<td>rMV.RSV-F</td>
<td>10^5 TCID&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>Positive control</td>
<td>i.n.</td>
<td>RSV (A2 strain)</td>
<td>10^8 p.f.u.</td>
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interesting to note that the neutralizing antibody titre against RSV elicited by wild-type RSV given intra-nasally did not decline between days 28 and 49, unlike that seen with the rMV.RSV-F vaccine.

The effectiveness of the vaccine was determined by measuring the amount of live RSV that could be recovered from the lungs and from the nasopharyngeal tissues of the sacrificed animals (Fig. 6b). It was found that although sham-vaccinated animals (i.e. animals that had been administered the empty measles vector) had readily detectable live RSV that could be recovered from the lungs, none of the vaccinated animals harboured RSV in the lungs (Fig. 6b, upper). The titre of RSV recovered from the upper respiratory tract was also significantly lower in all the vaccinated groups when compared with the sham-vaccinated group (Fig. 6b, lower). There was no significant difference (P<0.05) between the vaccinated groups with respect to the titres of RSV recovered from the nasal tissues. Thus, unlike in the lung, vaccination with rMV.RSV-F did not result in total resolution of the RSV infection in the upper respiratory tract following challenge. This was in marked contrast to the animals that had been immunized by low-dose RSV given intra-nasally (i.e. simulating a natural infection) in which case RSV could not be recovered from either the lungs or the nose. Thus, vaccination with rMV.RSV-F resulted in full protection from RSV in the lung and partial protection in the nose.

Since one of the possible added benefits of using recombinant measles as a paediatric vaccine vector is to retain its ability to protect against measles, we measured the anti-measles antibody titres that were elicited by the rMV.RSV-F (Fig. 7). The data shown are from sera obtained 28 days after immunization. We also analysed sera obtained at the 49-day time-point; these titres were not significantly different from the titres at the earlier (28-day) time-point (data not shown).

As expected, the recombinant measles vaccine stimulated an anti-measles antibody response. However, when compared with animals that received the control ‘empty’ measles vector, the animals that received the vector expressing RSV-F had antibody titres that were on average about three-fold lower. This difference in the magnitude of the anti-measles response may be due to a reduced ‘fitness’ of the RSV-F expressing vector in its ability to replicate in the host. Another possible reason for the diminished response is a reduction in the amount of displayed measles antigens (e.g. F or H) due to displacement by RSV-F.

DISCUSSION

We have tested whether rMV vectors harbouring either of three different integral membrane proteins from other viruses incorporate these antigens into progeny particles. Because of previous reports that heterologous viral envelope proteins are poorly incorporated into rMV unless a MV-specific cytoplasmic domain is engineered into the transgene (Guerbois et al., 2009; Spielhofer et al., 1998), two versions of each of the three heterologous proteins were tested: in case of MuV-HN, the chimeric protein harboured the cytoplasmic domain of the MV H protein; in case of the influenza virus HA and the RSV-F proteins, the MV F protein cytoplasmic domain was used. We were not successful in rescuing the chimeric version of the RSV-F construct. However, in the case of all three transgenes, the native full-length versions were found to be incorporated into particles that could be banded on sucrose gradients; the Mu-HN, ecto and the Flu-HA.ecto constructs were also found on particles that could be purified on sucrose gradients. Interestingly, the sucrose fractions where most of these particles banded differed from the fractions where the maximum TCID<sub>50</sub> titres were obtained (e.g. see Fig. 2a); the possibility exists that the incorporation of the heterologous envelope is accompanied by a reduction in infectivity. Alternatively, there is the formal possibility that the heterologous transgenes were incorporated into VLPs, as was previously found when the human immunodeficiency virus gag and envelope proteins were expressed simultaneously (Guerbois et al., 2009). In either case, the rMV preparation could be useful as an effective live attenuated vaccine.
It has been shown that the incorporation into rMV of an additional closely related component, e.g. the mumps virus F protein (i.e. from within the same subfamily Paramyxovirinae) resulted in genetic instability, whereas unrelated components from SIV were better tolerated (Wang et al., 2001). Wang et al. (2001) did not detect instability for the MuV-HN transgene by monitoring expression levels until 10 passages. However, we could detect mutations arising within the Mu-HN transgene sequence within a few passages using population sequencing after genome amplification. In case of the chimeric Mu-HN.ecto insert, where the cytoplasmic and transmembrane domains were derived from measles virus H protein, genetic stability was improved at least to the point where no mutations were detected after 10 passages. In contrast to the native MuV-HN transgene, rMV harbouring the full-length RSV-F protein was stable for at least 10 generations. It is possible that RSV (belonging to the subfamily Pneumovirinae) is sufficiently different from MV protein sequences so that its presence does not result in instability.

It has been suggested that the impaired yields of rMV expressing Mu-HN renders it impractical for use as a vaccine (Wang et al., 2001). We also observed reduced yields for both MuV-HN inserts. The titres of the Mu-HN constructs – rMV-Mu-HN as well as rMV-Mu-HN.ecto – ranged from $10^5$ to $10^6$ TCID$_{50}$ units ml$^{-1}$, contrasted with titres of greater than $10^7$ TCID$_{50}$ that were obtained with the empty vector in the absence of an insert. This reduction in yield is probably not attributable to the fact that the transgene belongs to a related paramyxovirus, because rMV expressing GFP also resulted in titres that were similar to those obtained with the Mu-HN transgenes (data not shown). However, we have been able to concentrate large-scale (500 ml) preparations of rMV expressing Mu-HN using tangential flow filtration that has resulted in infectious titres suitable for animal experimentation (data not shown).
A previous study has reported protection from RSV challenge in cotton rats using recombinant measles vector (based on the temperature-sensitive AIK-C strain) genetically modified to express the full-length RSV-F (Sawada et al., 2011). Similarly to the study reported here, the full-length F protein was inserted between the P and M genes of a measles virus genome. A single vaccination dose (10^{6} TCID_{50} per cotton rat) corresponding to the higher dose used in this study was used, and at this dose the anti-RSV neutralization titres in the sera of cotton rats were similar to those reported here. However, unlike the purified rMV, RSV-F used in this study, RSV-F protein was not detected on purified rMV in the AIK-C-based vector preparation used to inoculate cotton rats. In contrast, we have shown using co-immunoprecipitation and by electron microscopy that the rMV.RSV-F used in this study did harbour the RSV-F protein on the membrane surface. The reason for this difference is not clear, although it is plausible that incorporation of RSV-F into either rMV particles or into RSV-F VLPs occurs in a stochastic manner, resulting in an rMV population that harbours little or no RSV-F protein.

The second more recent study (Mok et al., 2012) also demonstrated protection from RSV challenge using rMV-F expressing RSV-F in the cotton rat model. The RSV-F molecule that was cloned was C-terminally truncated (i.e. missing the transmembrane and cytoplasmic domains) to create a secreted molecule that would not be expected to be incorporated into virus particles, and in this respect was different from the constructs used by Sawada et al. (2011) or that described here. Two different insertion locations in the measles genome were tested: one where the insertion site was between the P and M genes [as was done by Sawada et al. (2011) as well as the cloning strategy used here], and another where the insertion site was proximal to the N gene, resulting in 10-fold higher expression of the RSV-F protein. Paradoxically, in spite of the higher expression levels, it was reported that the latter construct was inferior to the other construct in eliciting both neutralizing antibody and T-cell responses; this was attributed to the greater degree of attenuation in the vector with the higher expression of RSV-F.

Chimeric measles constructs where the native F and H proteins are replaced by heterologous viral components such as from VSV have been shown to acquire the targeting conferred by the donor molecule (Spielhofer et al., 1998). Proteins such as RSV-F and Flu-HA can similarly be expected to bind to their cognate cellular targets, and thereby influence the tropism of the recombinant MV described here. The effects of any such change in tropism in vivo with respect to safety and immunogenicity need to be evaluated.

Because live attenuated measles virus is an established paediatric vaccine, a recombinant vector based on the same virus at low dose is likely to be safe and can be considered for use in infants who are older than 6 months, the earliest that the measles vaccine has been shown to be effective (McLean et al., 2013). The rMV.RSV-F vaccine described in this study successfully protected cotton rats from an RSV challenge as discerned by the absence of recoverable RSV from the lungs of challenged animals, i.e. a reduction of at least 4 logs compared with control animals (Fig. 6b, upper). However, the elicited immune response was inadequate with respect to elimination of RSV from the nasopharynx, in contrast to the immunity elicited by an intra-nasal exposure to low-dose RSV (Fig. 6b, lower). This was in spite of the fact that rMV.RSV-F vaccinated animals frequently had higher anti-RSV antibody titres than the animals exposed to wild-type RSV (Fig. 6a). It is also possible that secreted IgA is an important component of a protective response, and the intra-muscularly administered rMV.RSV-F vaccine is less efficient in this respect. A similar result, i.e. effective protection from challenge in the lung but persistent RSV in the upper respiratory tract, was also observed in cotton rats vaccinated intra-muscularly with a single dose of up to 10^{11} viral particles of a recombinant replication-defective adenovirus vaccine (Kim et al., 2014; Widjojoatmodjo et al., 2015). Interestingly, a single dose of the same vaccine administered intra-nasally resulted in a more robust protection, further supporting the hypothesis that local immunity is an important determinant of protection that future vaccine strategies should consider. However, it is clear that RSV infection can be prevented in the presence of sufficient quantities of circulating anti-RSV-F antibodies as shown by passive immunization studies (Prince et al., 1985) as well as by the successful use of palivizumab in the clinic (Storch, 1998).
METHODS

Cell culture. Vero cells were maintained in Eagle’s minimal essential medium (MEM; Life Technologies, Waltham, MA, USA) supplemented with 5% FBS. MRC5 cells were cultured in Basal medium Eagle (BME; Life Technologies) supplemented with 10% FBS and 4 mM l-glutamine (Life Technologies). Suspension PER.C6® cells were maintained in PERMEXCIS™ medium (Lonza, Atlanta, GA, USA) supplemented with 4 mM l-glutamine, shaking at 100 r.p.m. The helper cell line 293® (Life Technologies) supplemented with 10% FBS and 1.2 mg ml⁻¹ Geneticin® (Life Technologies). All cell lines were incubated at 37°C with 5% CO₂.

Cloning, rescue and passaging of recombinant measles virus vectors. The measles vector backbone was based on the Borna vaccine Edmonston Zagreb 19 strain (MVEZb; GenBank accession number HV542005.1), which constituted the measles component of the Trivirax vaccine (Schwarzer et al., 2005.1), which constituted the measles component of the Trivirax vaccine (Schwarzer & Naim, 2005; Welliver et al., 2007). Additionally, in case of RSV-F, expression of the protein in situ following vaccination with a vector should result in the presentation of both pre- and post-fusion conformations to the immune system allowing for a broad antibody response that recapitulates natural infection. Also, T-cell responses against viral proteins likely play an important role in the elimination of infection and an effective CD8 T-cell response to RSV is an important component of a natural cure (Welliver et al., 2007). Genetic vaccines may have an advantage over recombinant protein vaccines or inactivated virus vaccines by favoring the elicitation of a Th1 response against a highly-expressed protein.

Purification of recombinant measles virus. For sucrose cushion purification, 25 ml of clarified virus harvest was underlaid with 5 ml of 20% sucrose in NaCl-Tris-EDTA (NTE) buffer [100 mM NaCl, 1 mM Tris and 10 mM EDTA (pH 7.8)] followed by 5 ml of 60% sucrose in NTE. Then, ultracentrifugation was performed at 60,000 g for 2.5 h at 4°C using a Beckman SW32 rotor. For the preparation of a linear gradient, a gradient mixer was used to prepare a gradient ranging from 5 to 60% sucrose using 13 ml of 60% sucrose and 7 ml of 5% sucrose. Then, 16 ml of the clarified virus was layered carefully on the top of the gradient. After centrifugation for 2 h at 175,000 g at 4°C, the bottom of the tube was punctured, fractions were collected and stored at −80°C.

Neuraminidase activity assay. Neuraminidase activity was measured using the NA-XTD Influenza Neuraminidase Assay Kit (Applied Biosciences, Foster City, CA, USA) using the reagents supplied in the kit and following the manufacturer's protocols. Sucrose-purified rMV, MuHN was initially diluted 10-fold, and fivefold further serial dilutions were measured in triplicate.

Immunoprecipitation and Western blotting of viral particles. Sucrose-purified rMV.RSV-F and the control viruses MVEZ and RSV (A2 strain) were immunoprecipitated using Dynabeads® M-280 Tosyl-lactivated (Invitrogen, Waltham, MA, USA), according to the manufacturer’s protocol. The Dynabeads were coupled to an anti-RSV-F mAb, CR9503 (Wu et al., 2007) and 1 mg of antibody-coupled Dynabeads were incubated with either MVEZ or rMV.RSV-F (2.8×10⁶ TCID₅₀ ml⁻¹) or RSV A2 (9.4×10⁸ p.f.u.) for 1 h at 37°C. Then samples were rinsed five times with PBS and the bound virus was directly eluted into SDS sample loading buffer by incubating at 70°C for 10 min. Finally, eluted samples were electrophoresed on a 4–12% gradient polyacrylamide gel, transferred to a PVDF membrane and probed with a mouse monoclonal anti-MV N protein antibody (Merck Millipore, Bedford, MA, USA) or with CR9503.

Detection of expressed proteins on recombinant measles virus by ELISA. Maxisorp™ ELISA plates (Nunc) were coated overnight at 4°C with 100 µl of sucrose purified viruses diluted 10-fold in PBS. Next, the wells were washed with 0.1% Tween 20 in PBS (v/v) and subsequently blocked for 1 h at room temperature with blocking buffer [1% (v/v) BSA fraction V and 0.1% (v/v) Tween 20 in PBS]. Human monoclonals anti-RSV-F CR9503 (1.4 µg ml⁻¹), anti-influenza HA CR6261 (5 µg ml⁻¹) (Throsby et al., 2008) or CR9020 (5 µg ml⁻¹) (Brandenburg et al., 2013) in blocking buffer was added to the wells and incubated for 1 h at room temperature. After washing, the bound primary antibodies were detected and quantified using HRP-conjugated mouse-anti-human IgG.

Immunolabelling and electron microscopy. Sucrose-purified virus was incubated with either mouse monoclonal anti-MV H protein (Millipore) or human anti-RSV-F (CR9503) in blocking buffer [2% (v/v) BSA fraction V, 0.1% (v/v) Tween 20 in PBS] for 1 h at room temperature. Virus was coated onto carbon-coated Formvar grids and washed with blocking buffer. Subsequently, goat anti-mouse or goat anti-human colloidal gold (Aurion, Wageningen, The Netherlands)-conjugated antibodies were applied to the grids for 1 h at room temperature. Then the grids were washed and fixed with 1.5% glutaraldehyde for 5 min. Finally, negative staining was done by applying phosphotungstic acid to the protein grid.
the grids. The grids were analysed at 80 kV using a Tecnai 12B Twin (FEI) transmission electron microscope equipped with an Eagle 4K HS camera.

**Cotton rat immunization with rMV.RSV-F and challenge with respiratory syncytial virus (A2 subtype).** The studies were carried out at Sigmovir Biosystems (Rockville, MD, USA). The animal study protocol was approved by Sigmovir's Institutional Animal Care and Use Committee. Female inbred cotton rats (Sigmodi), between 6 and 8 weeks of age, were used for the study. Blood samples were obtained by retro-orbital puncture on the day of immunization, and thereafter on days 28 and 49. Immunizations with rMV.RSV-F were carried out using a total volume of 400 µl divided into two 200 µl portions administered intra-muscularly to each quadriceps (see Table 1). For intra-nasal immunization with RSV, and for intra-nasal RSV challenge, the animals were anesthetized with isoflurane and the virus dose (or sham) in 100 µl was instilled over the nares; breathing through the nose allows the fluid to be aspirated into the nasopharynx and subsequently into the more distal parts of the respiratory tract. In all cases, aliquots of each diluted RSV dose taken before and after the dosing procedure were kept aside and titrated to confirm the intended dose.

**Respiratory syncytial virus titration in tissue samples.** To titrate RSV in lung and nasal turbinate tissues, tissues obtained at day 54 were homogenized and the homogenates were clarified by centrifugation at 2000 r.p.m. (Jouan M4 rotor) at 2–8 °C for 10 min. The homogenization medium consisted of Hanks balanced salt solution (Lonza) supplemented with 0.218 M sucrose, 4.4 mM glutamate, 3.8 mM KH$_2$PO$_4$ and 7.2 mM K$_2$HPO$_4$. Confluent HEP-2 monolayers were infected in duplicates with serial dilutions (1:1, 1:10 and 1:100 in EMEM) of the homogenates in 24 well plates. After a 1 h incubation at 37 °C in 5% CO$_2$, the inoculum was removed and the wells were overlaid with 0.75% methylcellulose medium. After 4 days of incubation, the overlay was removed and the cells were fixed with 0.107% crystal violet stain for 2–4 h and then rinsed and air dried. Plaques were counted and the corresponding dilutions and volume of the inoculum were considered to calculate virus titres expressed as p.f.u. per g of tissue.

**Respiratory syncytial virus and measles viruses serum neutralization titre assay.** Serum samples were heat-inactivated and fourfold serial dilutions (starting at 1/10) were incubated with 25–50 p.f.u. of RSV A (Long strain) at 25 °C for 1 h. The residual RSV remaining after neutralization was measured by inoculating the HEP-2 cells as described above. The neutralization titre recorded was the calculated serum dilution that resulted in a 60% reduction of plaques compared with the control incubation with no serum. Serum samples were also tested for neutralizing antibodies against measles using the plaque reduction neutralization assay (Albrecht et al., 1981). The Spearman and Kärber formula was utilized to calculate the 50% neutralizing end-point titres.

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