Murine Pneumonia Virus Expressing the Fusion Glycoprotein of Human Respiratory Syncytial Virus from an Added Gene Is Highly Attenuated and Immunogenic in Rhesus Macaques

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ABSTRACT Human respiratory syncytial virus (RSV) continues to be the leading viral cause of severe acute lower respiratory tract disease in infants and children worldwide. A licensed vaccine or antiviral drug suitable for routine use remains unavailable. Like RSV, *Murine pneumonia virus* (MPV) is a member of the genus *Orthopneumovirus*, family *Pneumoviridae*. Humans are not normally exposed to MPV, and MPV is not cross-protective with RSV. We evaluated MPV as an RSV vaccine vector expressing the RSV fusion (F) glycoprotein. The RSV F open reading frame (ORF) was codon optimized, and the encoded RSV F protein was made identical to an early passage of RSV strain A2. The RSV F ORF was placed under the control of MPV transcription signals and inserted at the first (rMPV-F1), third (rMPV-F3), or fourth (rMPV-F4) gene position of a version of the MPV genome that contained a codon-pair-optimized polymerase (L) gene. The recovered viruses replicated *in vitro* as efficiently as the empty vector, with stable expression of RSV F protein. Replication and immunogenicity of rMPV-F1 and rMPV-F3 were evaluated in rhesus macaques following intranasal and intratracheal administration. Both viruses replicated at low levels in the upper and lower respiratory tracts, maintained stable RSV F expression, and induced RSV-neutralizing serum antibodies at high levels similar to those induced by wild-type RSV replicating to a 5- to 25-fold-higher titer. In conclusion, this study demonstrated that rMPV provides a highly attenuated yet immunogenic vector for the expression of RSV F protein, with potential application in RSV-naive and RSV-experienced populations.

IMPORTANCE Human respiratory syncytial virus (RSV) is an important human pathogen that lacks a licensed vaccine or antiviral drug suitable for routine use. We describe here the evaluation of recombinant murine pneumonia virus (rMPV) as a live-attenuated vector that expresses the RSV F protein, the major RSV neutralization antigen, as an experimental RSV vaccine. The rMPV-RSV-F vectors expressing RSV F from the first, third, or fourth gene position were genetically stable and were not restricted for replication *in vitro*. In contrast, the vectors exhibited highly attenuated replication in the respiratory tract of rhesus macaques, maintained stable RSV F expression, and induced RSV-neutralizing serum antibodies at high titers similar to those conferred by wild-type RSV. Given the lack of preexisting immunity to MPV in humans and the lack of cross-neutralization and cross-protection between MPV and RSV, an rMPV-vectored RSV vaccine should be immunogenic in both RSV-naive children and RSV-experienced adults.

KEYWORDS F protein, murine pneumonia virus, RSV F, fusion protein, intranasal vaccine, live-attenuated vaccine, mucosal vaccine, neutralizing antibodies, respiratory syncytial virus, vectored vaccine

Acute respiratory infections during early childhood constitute a major human health burden. Human respiratory syncytial virus (RSV) is the most important viral cause of severe acute pediatric respiratory infections worldwide. Mortality due to RSV in the postneonatal (28 days to 1 year old) population is second only to that due to malaria (1). It is estimated that RSV causes 34 million lower respiratory tract infections, 4 million hospitalizations, and 66,000 to 199,000 deaths every year in children less than 5 years of age (2, 3). Most mortality occurs in the developing world where supportive care is less available (2). Mortality is low in the developed countries, but morbidity is substantial: in the United States alone, RSV is associated with an estimated 132,000 to 172,000 hospitalizations annually in children less than 5 years old (4). There is not yet available a vaccine or an effective antiviral drug suitable for routine use.

RSV is an enveloped, single-stranded negative-sense RNA virus of the genus *Orthopneumovirus* and the family *Pneumoviridae* (5) and possesses 10 genes that encode 11 proteins. The fusion (F) and the attachment (G) surface glycoproteins are the two viral neutralization antigens and are the major protective antigens, with F playing the predominant role. RSV F is a type I transmembrane envelope glycoprotein that mediates fusion of the virion envelope with the host cell membrane during entry. RSV F-specific neutralizing antibody titers correlate with protection against RSV infection (6). The clinical use of palivizumab, an F-specific monoclonal antibody, substantially reduces hospitalization for RSV disease in high-risk infants (7). Moreover, the RSV F protein has substantial sequence and antigenic conservation between RSV strains and subgroups, in contrast to the substantial divergence of the G protein. Thus, the F protein is considered to be the most important antigen for an RSV vaccine.

RSV vaccines based on inactivated virus or protein subunits, when administered to RSV-naive infants and children, are associated with enhanced disease (8–10) upon subsequent natural RSV exposure and therefore are contraindicated for this population. In contrast, live-attenuated RSV strains and parainfluenza virus (PIV)-vectored vaccines were not associated with disease enhancement in clinical trials in this population (11–13) and thus are safe for pediatric use when suitably attenuated. A live RSV vaccine would be given by the intranasal route and thus has the additional advantage of inducing local immunity at the site of RSV infection as well as systemic immunity. The phenomenon of enhanced RSV disease has not been observed in RSV-experienced vaccinees, and therefore adults could safely be immunized with either a live or an inactivated vaccine.

Murine pneumonia virus (MPV), previously called pneumonia virus of mice (PVM), is a murine homolog of RSV and belongs to the same genus, *Orthopneumovirus*, of the family *Pneumoviridae* (5). MPV has a genome of approximately 15 kb and has essentially the same array of genes encoding the same constellation of proteins as RSV, namely (listed in 3′ to 5′ gene order), nonstructural protein 1 (NS1), nonstructural protein 2 (NS2), nucleoprotein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), attachment glycoprotein (G), fusion glycoprotein (F), M2-1 and M2-2 proteins, and polymerase protein (L). The various genes and proteins of MPV share 30 to 62% nucleotide sequence identity and 10 to 60% amino acid sequence identity with RSV (14). Certain characteristics of MPV make it an attractive vector for developing an RSV vaccine: (i) MPV replicates in the superficial epithelial cells of the respiratory tract, similar to RSV, and thus is expected to induce both systemic immunity and local immune responses in the respiratory tract, where RSV infects and replicates; (ii) MPV was shown to have highly attenuated replication in the respiratory tract of African green monkeys (AGMs) and rhesus macaques, presumably due to host range restriction (15), and therefore would be expected to be highly attenuated in the human respiratory tract; (iii) MPV was surprisingly immunogenic in primates despite the high level of restriction, which was the feature that prompted the present study; (iv) we previously reported that humans do not appear to be naturally exposed to MPV; and (v) we also reported a lack of significant antigenic reactivity between human or nonhuman primate RSV-specific serum antibodies and MPV, and a lack of cross-protection in mice between RSV and MPV (15). This lack of cross-reaction and cross-neutralization between RSV and

MPV would encourage evaluation of an MPV-vectored RSV vaccine not only in RSV-naive pediatric recipients but also in the RSV-experienced adult population and as a boost following an RSV vaccine in all age groups.

In the present study, we evaluated MPV as a vector to express the RSV F protein as a candidate RSV vaccine for intranasal immunization. Unmodified RSV F protein with a sequence corresponding to that of an early passage of the RSV strain A2 was expressed from the first (pre-NS1), third (between NS2 and N), or fourth (between N and P) gene position of the MPV genome. The vaccine candidates were characterized *in vitro* for replication, genetic stability, and protein expression and also were evaluated for replication, stability, and immunogenicity against RSV in rhesus macaques.

RESULTS

Recovery of the rMPV-RSV-F vectors expressing the RSV F protein. We previously showed that recombinant MPV strain 15 (16) is highly attenuated in rhesus macaques and African green monkeys, with only trace levels of shedding on occasional days in only some of the animals (15). Adding a supernumerary gene to a single-stranded negative-sense RNA virus can be further attenuating. In an effort to avoid overattenuation, we used as a vector a version of MPV strain 15 in which most of the L open reading frame (ORF) had been subjected to codon pair optimization (CPO). The construction of this MPV full-length cDNA bearing a CPO L gene (which for simplicity will be called rMPV here) was part of our separate study that will be described elsewhere. CPO of the L ORF was done on the premise that this might increase expression of the L protein, which in turn might increase virus replication. The effects of this modification turned out to be slight, namely, no effect on replication and a modest increase in the pathogenicity in mice (unpublished data), and so the vector is similar to recombinant wild-type (wt) MPV strain 15.

The RSV F ORF (strain A2) was codon optimized for efficient expression in humans, and the encoded protein was modified to contain the two amino acid assignments (K66E and Q101P), called HEK, that make it identical at the amino acid level to that of an early passage of RSV strain A2 (17). This ORF was engineered to be under the control of a set of MPV transcription signals and was inserted at three different gene positions of the rMPV genome (Fig. 1): (i) the first gene position upstream of the NS1 ORF, named rMPV-F1; (ii) the third gene position between the NS2 and N genes, named rMPV-F3; and (iii) the fourth gene position between the N and P genes, named rMPV-F4. RSV F transcription is driven by NS1 gene start-NS1 gene end, N gene start-NS2 gene end, and P gene start-N gene end for rMPV-F1, -F3, and -F4, respectively. All three rMPV-RSV-F viruses were readily recovered by reverse genetics. The viral genomes were sequenced by Sanger sequencing of the uncloned reverse transcription (RT)-PCR products and were found to be free of any adventitious mutations.

rMPV-RSV-F vectors maintain expression of RSV F during *in vitro* replication. Since the MPV vector retains all of its genes, the supernumerary RSV F gene is not needed for replication and thus has an increased potential for accepting mutations, including ones that might silence expression (18). Therefore, we investigated the stability of expression of RSV F protein following *in vitro* replication. Specifically, samples from viral stocks (passage 1 [P1]; see Materials and Methods) from four independent recoveries for each of the rMPV-RSV-F vectors were analyzed by a dual-staining plaque assay to detect the coexpression of RSV F and MPV proteins. This showed that, for each of the three MPV-RSV-F vectors, >99% of the viral plaques expressed RSV F protein (Fig. 2A).

rMPV-RSV-F vectors are not temperature sensitive. We previously observed with parainfluenza virus type 1 and 3 vectors that the insertion and expression of the RSV F gene could impart temperature sensitivity to the vector (19, 20). To assess possible temperature sensitivity of the MPV vectors, virus stocks were titrated at 42°C, 37°C, and 32°C. In no case was there a 100-fold or greater reduction in titer at 37°C or 42°C compared to the permissive temperature of 32°C, indicating that none of the viruses has a temperature-sensitive phenotype (data not shown).
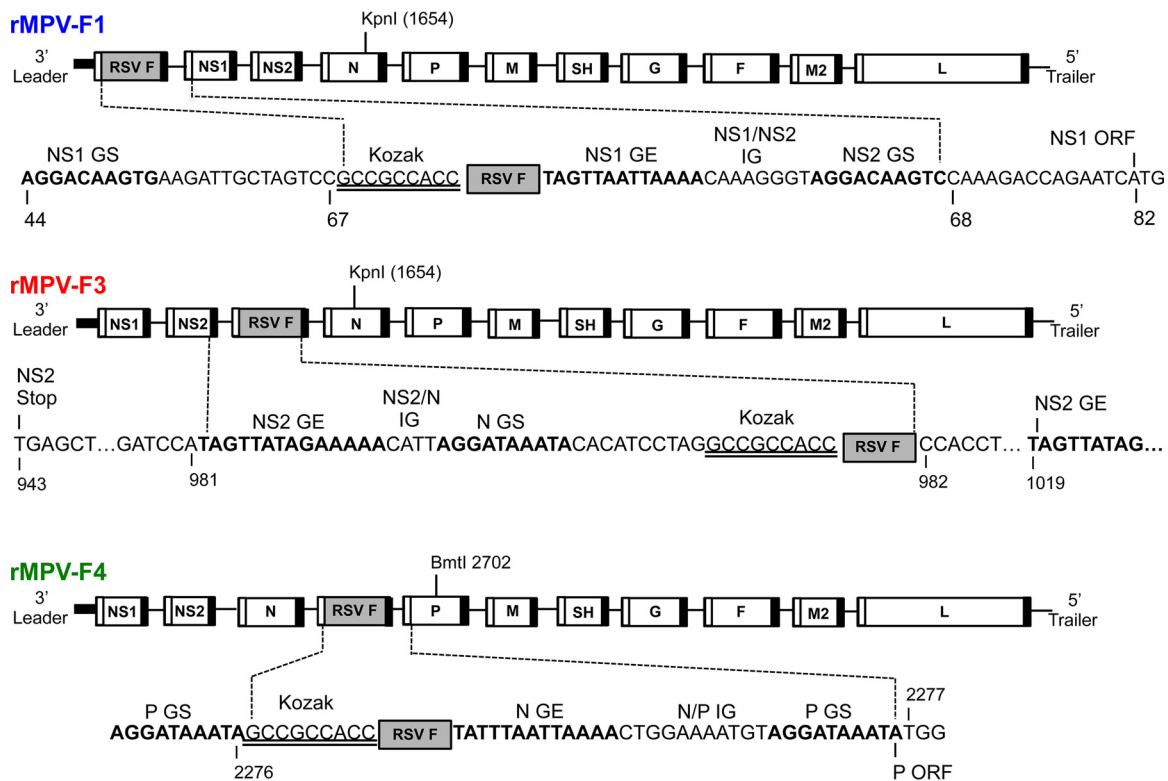
FIG 1 Diagrams of rMPV antigenomes containing the RSV F gene added as a supernumerary gene in the first (F1), third (F3), or fourth (F4) gene position. MPV genes are shown as unfilled rectangles, and the RSV F gene is shown as a shaded rectangle. The MPV gene start (GS) and gene end (GE) transcription signals are indicated by unfilled and filled bars, respectively, flanking each gene, including the supernumerary RSV F gene. The nucleotide sequence flanking the RSV F ORF is shown under each gene map, with the following features identified: RSV F ORF (represented by a shaded box), gene start (GS) and gene end (GE) transcription signals (bold), intergenic regions (IG), and the "Kozak" sequence (double underlined) placed upstream of the RSV F ORF to promote efficient translation.

Multicycle replication kinetics and cytopathology of the rMPV-RSV-F viruses *in vitro*. The kinetics of multistep replication of the rMPV-RSV-F vectors *in vitro* were evaluated in human lung epithelial A549 cells (Fig. 2B) and African green monkey kidney Vero cells (Fig. 2C). The latter cell line does not produce type I interferon (IFN) in response to virus infection (21) and is an approved cell substrate for manufacturing human vaccines. All three rMPV-RSV-F constructs and the empty rMPV vector replicated with similar kinetics and to similar final titers in each cell line. In addition, there was no difference in plaque size for any of the rMPV-RSV-F constructs compared to the empty vector in either cell line (Fig. 2A shows examples of plaques in Vero cells). Thus, the presence of the RSV F insert and the expression of the RSV F protein did not appear to reduce or increase viral replication or spread *in vitro*. Comparing the two cell lines, the peak titers were obtained much sooner (day 4) in Vero cells than in A549 cells (day 10), likely due to the lack of IFN responsiveness of Vero cells. To evaluate cytopathic effects, Vero cells were infected at a multiplicity of infection (MOI) of 10 PFU per cell with rMPV-F1, -F3, -F4, empty rMPV vector, or wt RSV. Typical syncytium formation was observed in cells infected with wt rRSV at 48 h (data not shown) and at 96 h (Fig. 3), but rMPV-F1, -F3, and -F4 infection did not produce the classical syncytia up to 96 hours postinfection (hpi) and instead formed clumps of fused cells (Fig. 3, top panel), which were absent for the empty vector, suggesting this was mediated by the RSV F protein.

Expression of RSV F and MPV proteins during *in vitro* infection. Vero cells were infected with each of the three vectors, or with empty vector, or with wt RSV at an MOI of 10 PFU per cell and incubated for 96 h. Cell lysates were prepared, subjected to SDS-PAGE under reducing and denaturing conditions, and analyzed by Western blotting using antisera and antibodies that detected the RSV F and MPV G, N, P, NS1, NS2,
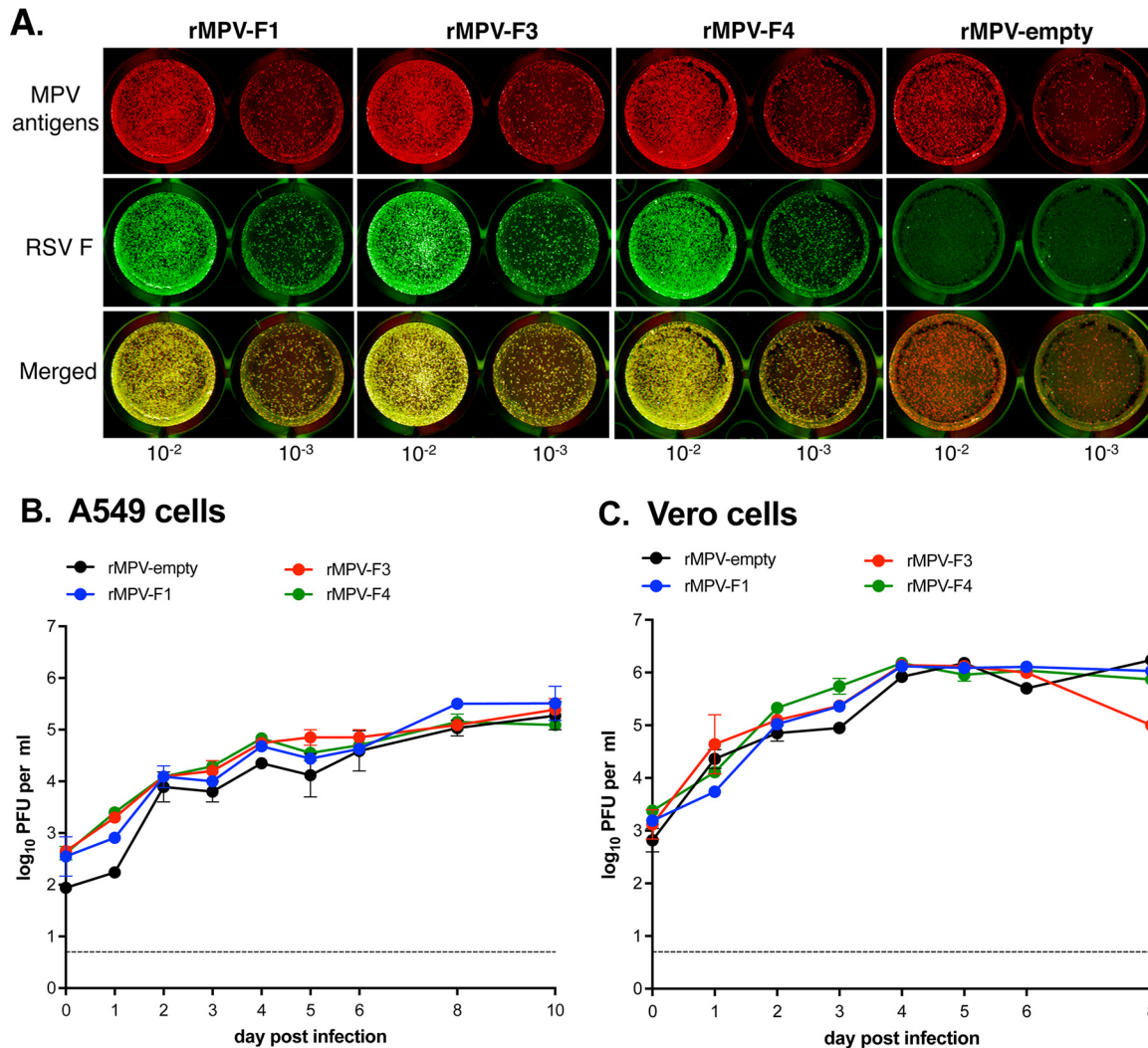
FIG 2 Stability of RSV F expression and kinetics of multicycle replication *in vitro*. (A) The stability of expression of RSV F by the rMPV vectors was determined by a dual staining plaque assay. Vero cells were inoculated with serial dilutions of P1 virus stocks and incubated for 4 days under a 0.8% methylcellulose overlay. Monolayers were fixed and probed for RSV F and MPV antigens using specific antibodies followed by the corresponding infrared dye-conjugated secondary antibodies. RSV F and MPV antigens appear green and red, respectively, and appear yellow when merged. Displayed are representative images from four independent experiments. (B, C) Multicycle growth kinetics of the rMPV-RSV-F vectors in human A549 lung epithelial cells (B) and African green monkey kidney Vero cells (C). Replicate cultures of A549 and Vero cells were infected with an MOI of 0.1 PFU per cell with the indicated viruses. At 24-h intervals, two cultures per virus in each cell line were harvested by scraping and vortexing, and clarified supernatants were prepared and flash frozen. Viral titers were subsequently determined in duplicate by plaque assay. Data are shown as mean values with the standard errors of the means, although in many cases the error bars are obscured by the symbols, given the small margin of error. The limit of detection was 0.7 $\log_{10}$ PFU per ml (dotted line).

and F proteins (Fig. 4). All three vectors efficiently expressed the RSV F protein, at levels that were somewhat less than that of wt RSV (Fig. 4A and B). There was little or no difference in the level of RSV F protein expression among the three rMPV vectors (Fig. 4A and B, lanes 1 to 3). Furthermore, the presence of the RSV F gene did not decrease the expression of any of the MPV vector proteins that were analyzed (Fig. 4A and C) with the sole exception of the MPV F protein, whose expression was reduced to very low levels in the case of all three rMPV-RSV-F vectors (Fig. 4A and C, lanes 1 to 3 versus lane 4).

rMPV-RSV-F vectors are highly restricted in rhesus macaques. In the *in vitro* studies described above, the three rMPV-RSV-F vectors had very similar properties, and rMPV-F1 and rMPV-F3 were chosen for evaluation in rhesus macaques. rMPV-F1 was chosen because its promoter-proximal position might provide increased expression *in*
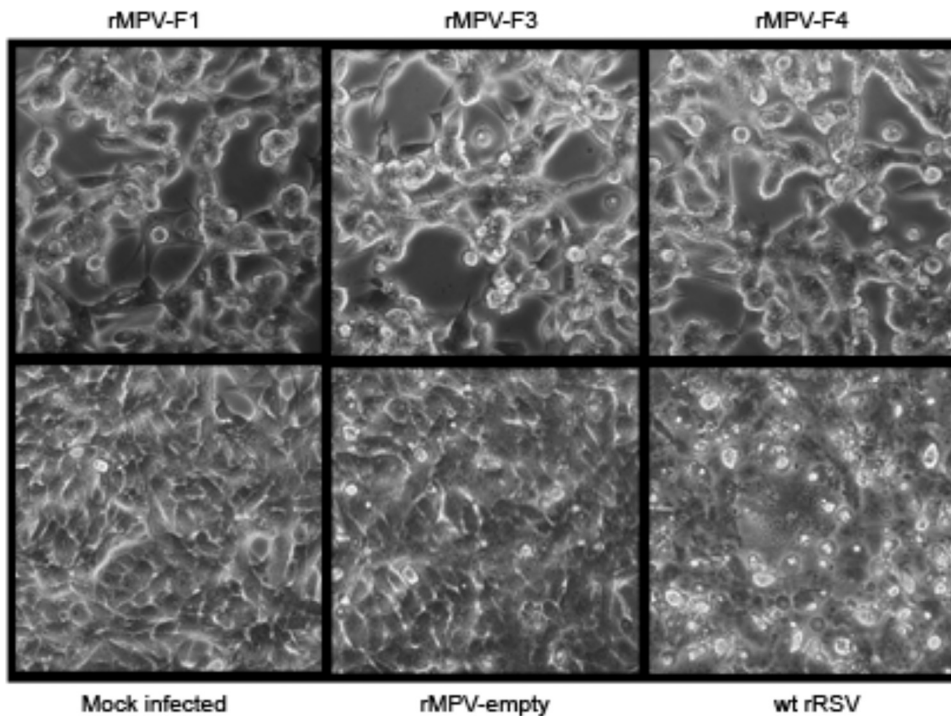
FIG 3 Cytopathic effects in Vero cells infected with rMPV-RSV-F vectors. Vero cell monolayers were infected with the indicated rMPV-RSV-F vectors, empty vector, or wt rRSV at an MOI of 10 PFU per cell or mock infected. The cultures were incubated for 96 h at 32°C and subjected to light photomicroscopy at a magnification of ×200. The images shown are representative of two independent experiments.

vivo, even though that was not evident *in vitro*; and rMPV-F3 was chosen because of its somewhat-greater expression of RSV F *in vitro*. Empty rMPV vector was not included as a control for viral replication due to an insufficient number of animals. We chose to evaluate two vectors representing two different insertion sites, rather than a single insertion site versus empty vector, because sometimes a given site has disproportionate properties *in vivo*, such as increased attenuation or instability. Monkeys were confirmed to be seronegative for RSV and MPV by separate RSV- and MPV-specific 60% plaque reduction neutralization tests ($PRNT_{60}$). The animals were inoculated by the combined intranasal and intratracheal routes with a total of 6.3 $\log_{10}$ PFU of the rMPV-RSV F vectors as described in Materials and Methods. In the interest of using a minimum number of animals, control animals immunized with wt RSV were not included in this study; instead, data from a separate study in which rhesus macaques from the same cohort were inoculated by the same route with a total of 7.3 $\log_{10}$ PFU of wt RSV A2 are shown here for comparison. None of the animals exhibited any signs of clinical illness following inoculation. Replication in the upper and lower respiratory tracts was assessed by collecting, respectively, nasopharyngeal swabs and tracheal lavage samples and determining their virus titers.

In the upper respiratory tract (Table 1), rMPV-F1 and rMPV-F3 had a mean shedding duration of 4.8 days for both vectors. One animal in each of the rMPV-F1 and rMPV-F3 groups did not have detectable replication on any of the sampling days. The mean peak virus titers were 1.6 and 1.4, $\log_{10}$ PFU/ml for rMPV-F1 and rMPV-F3, respectively (Table 1). wt RSV-inoculated animals shed virus for an average of 6.5 days with a peak titer of 2.8 $\log_{10}$ PFU/ml, which was 15- and 25-fold higher than the peak titers for the rMPV-F1 and rMPV-F3 vectors, respectively.

In the lower respiratory tract, rMPV-F1 and rMPV-F3 had mean durations of virus shedding of 3.5 and 1.8 days, respectively, and the mean peak virus titers were 1.8 and 1.7 $\log_{10}$ PFU/ml, respectively (Table 2). All animals had detectable shedding from the lower respiratory tract except for one animal (animal ID G), which was inoculated with
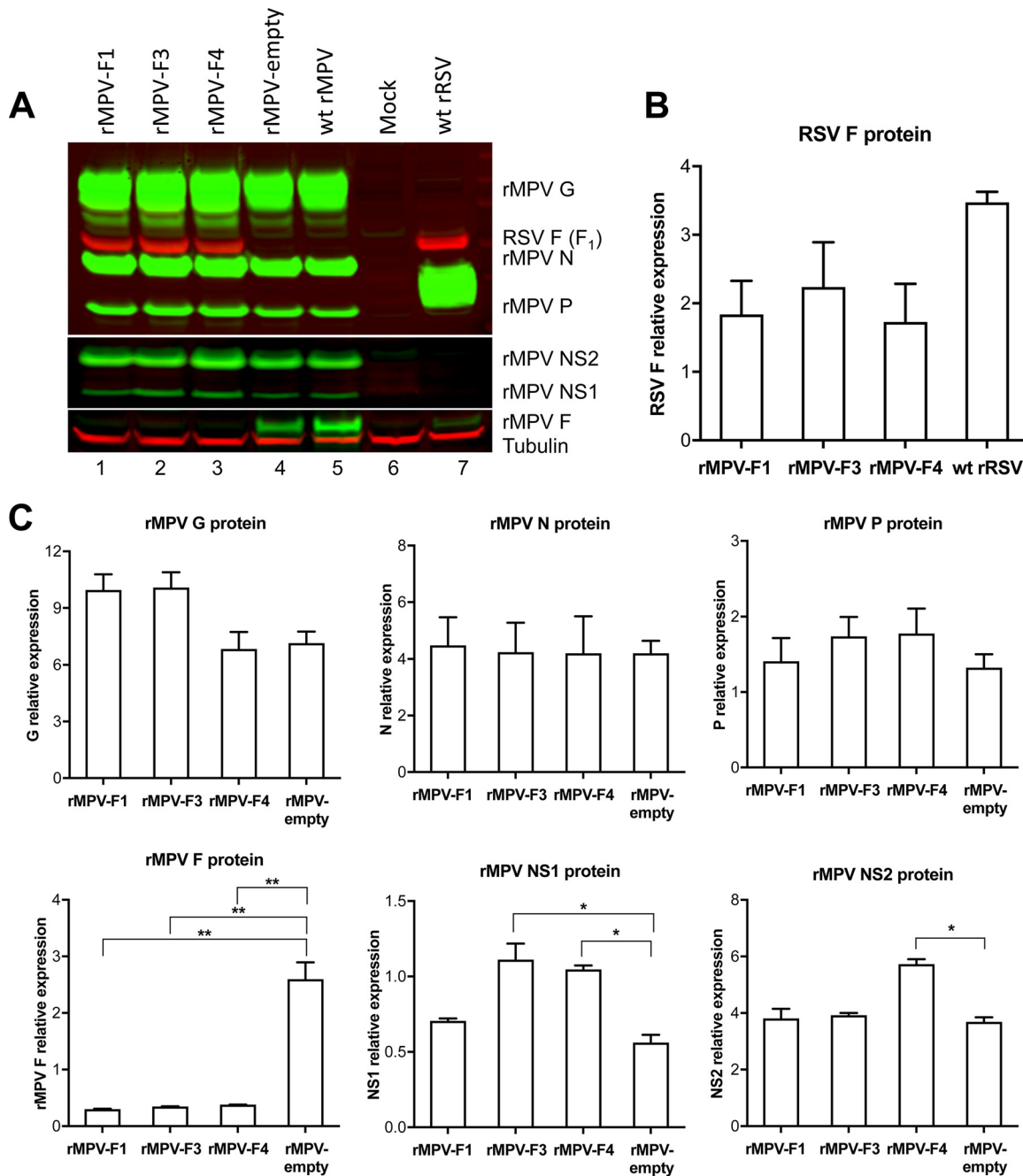
FIG 4 Evaluation of RSV F and rMPV vector protein expression in infected cells. Cell lysates were prepared at 96 hpi using infected cells from the experiment shown in Fig. 3. Denatured and reduced lysates were subjected to Western blot analysis. RSV F was detected with a mouse monoclonal antibody. The MPV G, N, and P proteins were detected with a hyperimmune serum raised against sucrose gradient-purified rMPV virions. The MPV F protein was detected with a rabbit polyclonal antiserum raised against a recombinant vaccinia virus expressing only the MPV F protein. The MPV NS1 and NS2 proteins were detected with individual rabbit hyperimmune sera each raised against a synthetic peptide derived from the respective protein. Tubulin was probed as a loading control and used to normalize each sample. The Western blot images (A) and the quantification plots of protein bands (B and C) are from the same experiment and are representative of three independent experiments. Data shown in panels B and C were analyzed by one-way analysis of variance using Dunnett's multiple-comparison test. The differences in protein expression, when significant, are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$. Standard errors are shown.

rMPV-F3 and also did not have detectable shedding from the upper respiratory tract. Animals inoculated with wt RSV had a mean shedding duration of 4 days, with the mean peak virus titer at 2.5 $\log_{10}$ PFU/ml, which was 5- to 6-fold higher than the peak titers of the vectors.

TABLE 1 Viral titers of nasopharyngeal swab samples from the upper respiratory tract of rhesus macaques inoculated with the indicated rMPV-RSV F vectors or with wt RSV[a]

Group	Animal ID(s)	Virus titer[b] ($\log_{10}$ PFU/ml) on day:										Peak virus titer	No. of days of shedding[d]
		1	2	3	4	5	6	7	8	9	10		
rMPV-F1	A	2.0	—[c]	2.2	1.6	2.6	—	—	—	—	—	2.6	5
	B	—	—	1.8	1.5	1.5	1.0	1.7	—	1.9	—	1.9	7
	C	—	—	1.7	1.7	1.5	1.0	1.6	—	1.2	—	1.7	7
	D	—	—	—	—	—	—	—	—	—	—	—	0
	A to D (mean ± SD)											1.6 ± 1.1	4.8 ± 3.3
rMPV-F3	E	—	—	—	—	1.4	—	—	—	—	—	1.4	1
	F	1.7	1.6	2.0	1.6	1.7	1.7	—	1.6	1.3	—	2.0	9
	G	—	—	—	—	—	—	—	—	—	—	—	0
	H	2.3	2.0	1.7	2.0	1.6	1.4	—	1.3	1.7	—	2.3	9
	E to H (mean ± SD)											1.4 ± 1.0	4.8 ± 4.9
wt RSV	I	0.7	—	0.7	1.8	0.7	1.0	1.0	0.7	—	—	1.8	8
	J	—	2.4	3.6	3.3	1.9	2.2	2.2	—	—	—	3.6	6
	K	—	2.6	2.7	3.4	1.6	1.2	1.2	—	—	—	3.4	6
	L	—	—	0.7	1.9	2.0	1.0	2.2	1.3	—	—	2.2	6
	I to L (mean ± SD)											2.8 ± 0.9	6.5 ± 1.0

[a]Rhesus macaques were inoculated by the combined intranasal and intratracheal routes with 6.0 $\log_{10}$ PFU per site with rMPV vectors. In a separate study, animals from the same cohort as those used for rMPV vectors were inoculated with 7.0 $\log_{10}$ PFU per site of recombinant wt RSV. The total dose per animal was 6.3 $\log_{10}$ PFU of rMPV vector or 7.3 $\log_{10}$ PFU of wt RSV. Nasopharyngeal swabs were collected on days 0 to 10, 12, 14, 21, and 28. After day 10, samples had no detectable virus (results not shown).
[b]The lower limit of detection was 0.7 $\log_{10}$ PFU/ml.
[c]—, no detectable virus.
[d]Time period spanning the first day to the last day on which virus was detected, including negative days (if any) in between.

The nasopharyngeal swabs and tracheal lavage samples from rMPV-F1- and rMPV-F3-inoculated rhesus macaques also were analyzed by the fluorescent dual-staining plaque assay to determine the stability of RSV F expression during *in vivo* replication. The majority of the samples showed 100% of the rMPV plaques expressing RSV F antigen (Table 3), indicating a substantial level of stability of expression for the RSV F protein.

TABLE 2 Viral titers of tracheal lavage samples from the lower respiratory tract of rhesus macaques inoculated with the indicated rMPV-RSV F vectors or with wt RSV[a]

Group	Animal ID(s)	Virus titer ($\log_{10}$ PFU/ml) on day:				Peak virus titer	No. of days of shedding[c]
		2	4	6	8		
rMPV-F1	A	1.7	—[b]	1.7	—	1.7	5
	B	1.0	1.5	—	—	1.5	3
	C	1.0	—	2.0	—	2.0	5
	D	2.1	—	—	—	2.1	1
	A to D (mean ± SD)					1.8 ± 0.3	3.5 ± 1.9
rMPV-F3	E	1.5	—	2.0	—	2.0	5
	F	2.3	—	—	—	2.3	1
	G	—	—	—	—	—	0
	H	2.6	—	—	—	2.6	1
	E to H (mean ± SD)					1.7 ± 1.2	1.8 ± 2.2
wt RSV	I	3.2	2.5	—	—	3.2	3
	J	2.2	2.1	2.7	—	2.7	5
	K	—	—	—	—	—	0
	L	4.1	3.0	2.6	1.5	4.1	7
	I to L (mean ± SD)					2.5 ± 1.8	4.0 ± 3.6

[a]From the experiment described in Table 1, tracheal lavages were performed on days 2, 4, 6, 8, 10, 12, 14, 21, and 28; after day 8, samples had no detectable virus (results not shown). Virus titers were determined by plaque assay as described for Table 1.
[b]—, no detectable virus. The lower limit of detection was 0.7 $\log_{10}$ PFU per ml.
[c]Time period spanning the first day to the last day on which virus was detected, including negative days (if any) in between.

TABLE 3 Percentage of plaques expressing RSV F in samples collected from the upper and lower respiratory tracts of rhesus macaques inoculated with rMPV vectors[a]

Sample type and virus vector	Monkey ID	% PFU expressing RSV F on day:								
		1	2	3	4	5	6	7	8	9
Nasopharyngeal swabs										
rMPV-F1	A	—	—	100	50	80	—	—	—	—
	B	—	—	100	67	100	100	100	—	80
	C	—	—	100	100	100	100	100	—	50
	D	—	—	—	—	—	—	—	—	—
rMPV-F3	E	—	—	—	—	100	—	—	—	—
	F	100	83	100	100	100	100	—	67	80
	G	—	—	—	—	—	—	—	—	—
	H	75	100	100	100	100	—	—	100	—
Tracheal lavage fluid										
rMPV-F1	A	nc	—	nc	—	nc	75	nc	—	nc
	B	nc	—	nc	100	nc	—	nc	—	nc
	C	nc	100	nc	—	nc	—	nc	—	nc
	D	nc	100	nc	—	nc	—	nc	—	nc
rMPV-F3	E	nc	100	nc	—	nc	100	nc	—	nc
	F	nc	100	nc	—	nc	—	nc	—	nc
	G	nc	—	nc	—	nc	—	nc	—	nc
	H	nc	94	nc	—	nc	—	nc	—	nc

[a]The nasopharyngeal swabs and tracheal lavage samples collected on the indicated days after intranasal immunization were analyzed by fluorescent dual-staining plaque assay on Vero cells to determine the percentage of viral plaques coexpressing RSV F and MPV antigens during *in vivo* virus replication. These percent values were determined from approximately 100 plaques per sample. —, no detectable virus; nc, no sample was collected.

Immunization conferred strong virus-neutralizing serum antibody responses against RSV and MPV. In the rhesus macaque experiment described above, serum samples were collected at 14, 21, and 28 days postimmunization and analyzed by RSV- and MPV-specific $PRNT_{60}$ assays to determine titers of serum RSV- and MPV-neutralizing antibodies. None of the sera collected on day 0 prior to immunization had detectable virus-neutralizing activity against RSV or MPV (Tables 4 and 5), confirming the animals were seronegative.

At day 14, RSV- and MPV-specific neutralizing activity was detected in sera from monkeys immunized with rMPV-F1 and rMPV-F3. These titers generally did not change

TABLE 4 RSV 60% plaque reduction neutralization titers ($PRNT_{60}$) of serum samples from rhesus macaques immunized with the rMPV-RSV-F vectors or with wt RSV

Group	Animal ID(s)	RSV PRNT[a] (log_2 60% titer) on day:			
		0	14	21	28
rMPV-F1	A	<3.3	8.2	9.8	9.6
	B	<3.3	5.4	6.3	7.3
	C	<3.3	7.1	8.4	8.6
	D	<3.3	6.9	7.9	7.4
	(A to D) mean $\pm$ SD	<3.3 $\pm$ 0.0	6.9 $\pm$ 1.2	8.1 $\pm$ 1.4	8.2 $\pm$ 1.1
rMPV-F3	E	<3.3	5.5	6.2	7.0
	F	<3.3	5.0	8.3	8.2
	G	<3.3	7.6	8.0	7.8
	H	<3.3	7.4	8.7	8.3
	E to H (mean $\pm$ SD)	<3.3 $\pm$ 0.0	6.4 $\pm$ 1.3	7.8 $\pm$ 1.1	7.8 $\pm$ 0.6
wt RSV	I	<3.3	7.8	7.9	7.4
	J	<3.3	7.8	8.7	8.3
	K	<3.3	7.7	8.9	9.1
	L	<3.3	8.1	8.7	8.8
	I to L (mean $\pm$ SD)	<3.3 $\pm$ 0.0	7.9 $\pm$ 0.2	8.6 $\pm$ 0.4	8.4 $\pm$ 0.7

[a]The lower limit of detection of the RSV $PRNT_{60}$ assay was 3.3 log_2 60% titer. An RSV serum sample with a log_2 $PRNT_{60}$ value of $\geq$5.3 was considered positive.

TABLE 5 MPV 60% plaque reduction neutralization titers ($PRNT_{60}$) of serum samples from rhesus macaques immunized with the rMPV-RSV F vectors[a]

Group	Animal ID(s)	MPV PRNT ($\log_2$ 60% titer) on day:			
		0	14	21	28
rMPV-F1	A	<3.3	13.7	13.8	13.7
	B	<3.3	10.9	11.0	10.4
	C	<3.3	10.7	11.7	12.5
	D	<3.3	8.8	8.8	8.6
	A to E (mean ± SD)	<3.3 ± 0.0	11.0 ± 2.0	11.3 ± 2.1	11.3 ± 2.3
rMPV-F3	E	<3.3	11.6	11.2	11.5
	F	<3.3	10.4	10.2	11.8
	G	<3.3	9.5	9.6	10.5
	H	<3.3	9.5	11.5	11.7
	E to H (mean ± SD)	<3.3 ± 0.0	10.3 ± 1.0	10.6 ± 0.9	11.4 ± 0.6

[a]The lower limit of detection of the ($PRNT_{60}$) assay was 3.3 $\log_2$ 60% titer. An MPV serum sample with a $\log_2$ $PRNT_{60}$ value of ≥5.3 was considered positive.

significantly over the next 2 weeks, as indicated by analysis of sera from days 21 and 28 (Tables 4 and 5). On days 14, 21, and 28 postimmunization, the mean RSV $\log_2$ $PRNT_{60}$ values were 6.9, 8.1, and 8.2, respectively, for rMPV-F1 and 6.4, 7.8, and 7.8, respectively, for rMPV-F3. The respective mean MPV $\log_2$ $PRNT_{60}$ values on these days were 11.0, 11.3, and 11.3 for rMPV-F1 and 10.3, 10.6, and 11.4 for rMPV-F3. Mean RSV- and MPV-specific $\log_2$ $PRNT_{60}$ values induced by rMPV-F1 and rMPV-F3 were similar to each other on days 14, 21, and 28 postimmunization. Rhesus macaques from the same cohort as those used for rMPV-F1 and -F3 were immunized in a separate study by the same route with a 10-fold-higher dose (7.3 $\log_{10}$ PFU) of wt RSV A2, and the serum RSV $PRNT_{60}$ values determined side by side with rMPV-F1 and -F3 sera are shown in Table 4. wt RSV induced serum RSV-neutralizing titers at 7.9, 8.6, and 8.4 $\log_2$ $PRNT_{60}$ at days 14, 21, and 28 postimmunization, respectively, which were similar to those conferred by rMPV-F1 and -F3 at those time points.

DISCUSSION

RSV is the most important viral respiratory pathogen of infancy and also has a substantial impact in children and adults, especially the elderly. The high worldwide pediatric morbidity and substantial mortality (particularly in the developing world) associated with RSV emphasize a continuing urgent need for a pediatric RSV vaccine in particular. In addition, the occurrence of substantial RSV disease in older adults with waning RSV immunity indicates the need for a booster RSV vaccine. Thus, a vaccine that could be used in RSV-naive (children) and RSV-experienced (adults) recipients would be highly desirable. A number of different vaccine strategies are presently being pursued, including a live-attenuated RSV and live viral vectors for pediatric use and subunit vaccines for the elderly as well as for pregnant women to increase maternal antibody immunity (reviewed in reference 22), but no candidate has yet been licensed.

In the present study, a codon-optimized RSV F ORF was engineered to be under the control of MPV transcription signals and inserted as an added gene into three different locations in the rMPV genome. The MPV genome that was used in this study contained an L ORF of which the downstream 67% had been subjected to CPO, as part of another study, but this had no effect on replication in mice (our unpublished data). In future work, it would be of interest to compare this CPO version versus wt MPV for replication in rhesus macaques.

The three rMPV-RSV-F vectors and the empty vector were readily recovered by reverse genetics and replicated efficiently *in vitro* with little evidence of instability of expression of the RSV F protein. Interestingly, insertion of RSV F at any of the three locations yielded essentially the same level of expression of the RSV F insert. This was unexpected because single-stranded negative-sense RNA viruses characteristically exhibit a polar gradient in which gene expression decreases with increasing distance from

the single promoter at the 3′ end of the genome (23). For example, in a previous study using a chimeric bovine/human parainfluenza virus type 3 (PIV3) vector, the relative levels of expression of RSV F from gene positions 2 and 3 were 42 to 50% and 9 to 12%, respectively, that of gene position one (19). In another study using two different attenuated HPIV1 vectors, a strong gradient of decreasing expression of RSV F was observed with one attenuated HPIV1 vector but not another (20). In addition to the lack of difference in the level of expression of the RSV F protein, the three rMPV-RSV-F vectors also were very similar with regard to *in vitro* growth kinetics and final viral yield.

Insertion of the RSV F gene into the rMPV vector did not decrease the expression of the MPV G, N, P, NS1, and NS2 proteins, which is another indication that the vector generally was not inhibited by the presence of the added RSV F gene. However, the expression of one vector protein, namely, MPV F, was reduced to very low levels in all three constructs. The reason why MPV F alone was strongly reduced remains unclear. In previous studies with PIV vectors, the addition of the RSV F insert often reduced the expression of one or more downstream vector genes, particularly the one or two vector genes immediately downstream of the insert (19, 20). A likely explanation is that, due to the polar gradient of transcription, the added RSV F gene decreased the amount of polymerase that reached downstream vector genes. Of the six MPV proteins that were monitored in the present study, three (P, G, and F) were from genes located downstream of the RSV F insert in all three vectors, one additional gene (encoding N) was downstream of the RSV F insert in rMPV-F3, and all six genes were downstream of the RSV F insert in rMPV-F1. Why only the MPV F protein, among all of these downstream genes, was reduced remains unknown. Thus, the factors that affect the expression of a foreign insert and the vector genes in vectors based on single-stranded negative-sense RNA viruses are more complex than the simple model of a polar gradient of transcription.

In previous studies, we investigated a number of strategies to increase the immunogenicity of the RSV F protein expressed from PIV vectors (19, 24–26). Two factors in particular yielded increases in the quantity and quality of RSV-neutralizing serum antibodies, namely, (i) the use of mutations that stabilize RSV F protein in the prefusion (pre-F) conformation and (ii) modification of the RSV F protein to be efficiently packaged in the vector virions. The pre-F conformation is a metastable form in which the newly synthesized RSV F protein appears. The pre-F conformation contains a number of unique, highly effective neutralization epitopes (27–31). When the pre-F protein contacts a target host cell membrane, it is readily triggered to undergo a massive irreversible conformational change that drives fusion between the virion and host cell membranes and also results in the loss of major pre-F-specific epitopes. Pre-F also can readily be triggered prematurely, which also results in the loss of these major epitopes. However, this conformational change can be inhibited by stabilizing this optimal antigenic form through structure-based mutations (30). In addition, RSV F can be packaged efficiently into the vector particle by replacing its transmembrane (TM) and cytoplasmic tail (CT) domains with those of the vector F protein. Both of these strategies are available for evaluation with the rMPV-RSV-F vectors. However, we chose to evaluate unmodified RSV F in the present study because, in previous studies with PIV vectors the optimized forms were very immunogenic and may have approached or reached the limit of the responsiveness of the animals. Therefore, our goal was to first examine whether MPV could be used as a vector and to identify the optimal RSV F insertion site. We subsequently will modify the RSV F insert to increase the quantity and quality of induced RSV-neutralizing antibodies.

As noted, the three rMPV-RSV-F vectors were nearly indistinguishable with regard to *in vitro* replication and protein expression. The two vectors that were chosen for evaluation in rhesus macaques, rMPV-F1 and rMPV-F3, also were nearly indistinguishable *in vivo*. Both constructs were very highly restricted for replication but induced high titers of RSV-neutralizing serum antibodies. The titers that were detected in monkeys immunized by either of the two constructs were not greatly different from each other. The responses were strong by day 14 and did not increase significantly by days 21 and

28 postvaccination. One animal did not have detectable shedding in the upper tract (animal D), and another did not have detectable shedding in either the upper or lower tract (animal G). However, there was no evident decrease in RSV-neutralizing serum antibody titers. This suggests that an undetectable low level of replication produced sufficient RSV F protein to induce high titers of RSV-neutralizing serum antibodies. It is also important to note that although the rMPV vectors were administered at a 10-fold-lower dose and replicated 5- to 25-fold less efficiently than wt RSV, they did confer RSV-neutralizing antibodies at high titers similar to those of wt RSV. Thus, we anticipate that due to their desirably attenuated but highly immunogenic phenotype, rMPV-based RSV vaccines may be considered for clinical evaluation.

The high level of attenuation of MPV in primates observed here and in a previous study (15) reflects host range restriction, specifically, inefficient replication due to the incompatibility of the murine virus for the primate system. MPV is a murine homolog of RSV, comparable to bovine PIV3 (BPIV3) as the bovine homolog of HPIV3. BPIV3 has previously been shown to be highly attenuated in nonhuman primates as well as in adult and pediatric volunteers, including young infants, and previously was evaluated as an experimental "Jennerian" HPIV3 vaccine (32). BPIV3 also was used as an attenuated backbone to construct the chimeric B/HPIV3 virus, in which the F and HN genes were replaced by their counterparts from HPIV3, and which has been evaluated as an HPIV3 vaccine (33), as well as a vector to express the RSV F protein (13, 19, 24–26). The percent amino acid sequence identities between the N, P, M, F, and L proteins of BPIV3 versus HPIV3 are 85, 59, 90, 79, and 89%, respectively (34), compared to 60, 33, 42, 43, and 53%, respectively, for the corresponding proteins between MPV and RSV (14). Thus, MPV and RSV are substantially more divergent from each other than are BPIV3 and HPIV3 from each other. Since host range restriction generally is thought to be the cumulative effect of many amino acid (and perhaps nucleotide) sequence differences in multiple genes (35), the high percentage of sequence differences between MPV and RSV suggests that the attenuation phenotype of MPV in humans should be very stable, as appears to be the case for BPIV3-based vaccines. However, this remains to be investigated. It will also be of interest to evaluate MPV vectors in African green monkeys, in which the MPV-neutralizing antibody titers induced by wt MPV are nearly twice those seen in rhesus macaques (15).

A theoretical concern of expressing the RSV F protein from an rMPV vector is that the RSV F protein might enable the murine virus to infect and replicate more efficiently, and in particular to acquire an increased tropism for human cells. However, there was no evidence of this *in vitro*: there was no increase in replication kinetics or viral yield in human or nonhuman primate cells or in plaque size. Additionally, *in vivo* the immunized animals were healthy throughout the study and clinical signs were absent. Possible effects of expression of RSV F on replication were not evaluated in rhesus macaques because the empty vector was not included due to an insufficient number of animals. However, the rMPV-RSV-F vectors were highly attenuated, and thus any possible effect would have been modest. In any event, possible effects of RSV F in enhancing replication or changing tropism should not be an issue with vectors expressing stabilized versions of pre-F protein, because these stabilized forms will be largely nonfunctional.

HPIV1 and B/HPIV3 vectors expressing RSV F also are being developed in our laboratory as vectored RSV vaccines (19, 20, 24–26). They have the advantage of conferring bivalent protection against the respective HPIV and RSV. However, they would be anticipated to be strongly restricted in adults by the preexisting immunity acquired by previous natural exposures to the respective HPIV and thus likely could be used only in HPIV-naive infants and children and not in adults. We have previously shown a lack of MPV-neutralizing activity in human sera and also a lack of MPV cross-neutralization by RSV-specific antibodies, as well as a lack of cross-protection between RSV and MPV in mice (15). Therefore, we anticipate that an rMPV vector would not be inhibited by immunity to common human viruses, including RSV, with the caveat that expression of RSV F by this vector might confer some susceptibility to restriction by RSV-specific immunity. Since the rMPV vectors appear to be highly

attenuated, it is anticipated that they will be well tolerated as a primary immunization in all age groups. The rMPV-RSV-F vectors also provide the potential for boosting RSV responses in subjects who previously received an RSV vaccine, with the caveat of possible restriction by RSV-specific immunity of the vector expressing RSV F.

In summary, the vaccine candidates developed here show promising immunogenicity, which could be significantly augmented by including the more immunogenic forms of RSV F such as stabilized pre-F (30) and the next generations of single-chain pre-F (36) and by including the packaging signals to enable RSV F incorporation into the MPV virus particles as previously described for PIV-vectored RSV vaccines (24, 25).

MATERIALS AND METHODS

Cells and viruses. Vero cells (African green monkey kidney cells, CCL-81; ATCC, Manassas, VA) were maintained in Opti-Pro medium supplemented with 4 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA) and 10% fetal bovine serum (FBS; HyClone, Logan, UT). Human lung epithelial A549 (CCL-185; ATCC, Manassas, VA) cells were maintained in F-12K medium (ATCC) supplemented with 4 mM L-glutamine. BHK BSR T7/5 cells are BHK-21 (baby hamster kidney 21) cells (37) that were maintained in Glasgow's minimum essential medium (MEM) (Thermo Fisher Scientific) supplemented with 3% FBS. Geneticin (Thermo Fisher) was included in the medium for every other passage to ensure selection of T7 RNA polymerase-expressing cells.

Recombinant (r) wt RSV strain A2 was used as a control and has been previously described (16, 38). All *in vitro* tissue culture experiments were done at 37°C unless otherwise noted. rMPV and the rMPV-RSV-F vectors were propagated on Vero cells by infecting at an MOI of 0.1 PFU per cell. Virus stocks were harvested about 2 weeks postinfection, when cytopathic effects disrupted the monolayer. rMPV titers were determined by plaque assay on Vero cells under a 0.8% methylcellulose overlay as described previously (15). Plaques were visualized by immunostaining with rabbit hyperimmune serum raised against sucrose-gradient-purified MPV followed by a horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (KPL, Gaithersburg, MD). Bound secondary antibodies were detected by incubation with a peroxidase substrate (KPL). Each sample was tested in duplicate, and the average is reported.

Construction of the rMPV viral vectors expressing RSV F protein from an added gene. The rMPV-RSV-F vectors were constructed using a previously described reverse genetics system (16). The rMPV vector backbone used for expressing RSV F was derived from MPV (previously called PVM) strain 15 cloned in a pBluescript plasmid vector (pBS) that contained the two introduced restriction sites (AgeI and BstBI) that were absent in the parent strain 15 as described previously (16) and also contained a partially modified L ORF. The downstream 67% of the L ORF was codon pair optimized (CPO) to contain synonymous changes that increased the content of codon pairs associated with efficient expression in humans while preserving both the overall codon usage and the encoded amino acid sequence (39, 40). The RSV F ORF from strain A2 was codon optimized for human codon usage (GenScript, Piscataway, NJ) to obtain greater protein expression, as previously reported (25). RSV F also carried the two previously described HEK amino acid assignments of 66E and 101P (17) that make the encoded F protein amino acid sequence identical to that of an early passage of wt strain A2 and the clinical isolates. We designed three MPV vector constructs in which the RSV F insert was placed in the first gene position, upstream of the NS1 gene (rMPV-F1), or in the third gene position, between the NS2 and N genes (rMPV-F3), or in the fourth gene position, between the N and P genes (rMPV-F4) (Fig. 1). The RSV F inserts were designed so that the RSV F ORF was flanked by MPV gene start (GS) and gene end (GE) transcription signals (16) to enable transcription of RSV F as a separate mRNA. The Kozak consensus sequence GCCGCCACC was placed immediately upstream of the RSV F AUG start codon to provide efficient context for translation initiation as described previously (41). The RSV F inserts were synthesized commercially (Genscript) as long genome segments designed for insertion into the rMPV antigenomic plasmid using the XmaI restriction site in the plasmid (pBS) sequence upstream of the leader region (not shown) and the downstream KpnI site (rMPV-F1 and -F3) or the KpnI and BmtI sites (rMPV-F4) as indicated (Fig. 1). The final construct rMPV-F1 contains 1,768 additional nucleotides, placed immediately after nucleotide position 67 (upstream of NS1); rMPV-F3 contains 1,775 additional nucleotides, placed immediately after nucleotide position 981 (between NS2 and N); and rMPV-F4 contains 1,771 additional nucleotides, placed immediately after nucleotide position 2276 (between N and P) of the MPV genome.

The rMPV vectors were recovered from cDNA as previously described (16) in BHK BSR-T7/5 cells that constitutively express the T7 RNA polymerase (37). The cells were transfected with the MPV antigenome plasmid and support plasmids expressing MPV N, P, M2-1, and L proteins; 24 h later, the cells were scraped and vortexed, and the cell suspension was cocultured with a Vero cell monolayer for approximately 2 weeks to create P1 viral stocks (16). We confirmed that the P1 virus stocks did not contain any adventitious mutations introduced during recovery: specifically, viral RNA was isolated and Sanger sequence analysis of the complete viral genomes was performed using uncloned overlapping RT-PCR fragments. Control RT-PCRs lacking reverse transcriptase did not yield an amplified product, confirming that the PCR products were amplified from the viral RNA and not from cDNA used for virus rescue. Titers of virus stocks were determined by plaque assay and immunostaining as described above.

Fluorescent dual-staining plaque assay quantifying the rMPV plaques coexpressing RSV F and vector antigens. To evaluate the stability of expression of RSV F protein following *in vitro* replication, four independent viral recoveries of each rMPV-RSV-F construct were analyzed by a dual-staining plaque assay to determine the stability of RSV F expression. The plaque assay was set up as previously described

(15), and cells were fixed using 80% methanol at day 4 postinfection (p.i.). To identify MPV proteins, a rabbit anti-rMPV polyclonal primary antibody described above was used at 1:5,000 and detected by a goat anti-rabbit 680 LT antibody (Li-Cor, Lincoln, NE). RSV F was probed with a mixture of three RSV F-specific mouse monoclonal antibodies (1129, 1243, and 1269) each diluted at 1:200, followed by a goat anti-mouse 800 CW secondary antibody (Li-Cor). Both secondary antibodies were used at a 1:800 dilution. Plates were scanned on an Odyssey infrared imager (Li-Cor), and the images were analyzed to determine the percentage of plaques coexpressing RSV F and rMPV antigens. Plaque images were pseudocolored to appear red and green for MPV and RSV F antigens, respectively. On merging the two channels, rMPV plaques coexpressing RSV F would appear yellow while those with loss of RSV F expression would appear red. To evaluate the stability of expression of RSV F protein following *in vivo* replication, nasopharyngeal swab and tracheal lavage samples from the rhesus study described below were analyzed by plaque assay followed by fixation and dual-stain plaque assay as described above.

Multicycle growth kinetics. To evaluate multicycle replication kinetics, replicate monolayer cultures of Vero and A549 cells in T25 flasks were inoculated with each virus at an MOI of 0.1 PFU per cell and incubated at 32°C. The virus inoculum was adsorbed for 3 h, after which monolayers were washed twice and replenished with fresh medium. On days 1 through 6 and days 8 and 10 (A549) or on days 1 through 6 and day 8 (Vero), two cell monolayers per virus and per cell type were scraped into the supernatant, vortexed, clarified by centrifugation, and frozen. Viral titers were determined by plaque assay on Vero cells.

Analysis of expression of RSV F and MPV vector proteins. Vero cell monolayers in 12-well plates were infected at an MOI of 10 PFU per cell with the rMPV-RSV-F vectors, empty vector, wt rMPV, or wt RSV or were mock infected. At 96 h p.i., images of infected cells were acquired and cell lysates were prepared to examine viral protein expression by Western blotting. Cells were washed twice with 1× phosphate-buffered saline (PBS) and lysed with 200 μl of cell lysis buffer containing 1× NuPAGE LDS sample buffer (Thermo Fisher Scientific) and 1× cOmplete Ultra protease inhibitor (Roche, Basel, Switzerland) in protease-free water. Each lysate was spun through a QIAshredder spin column (Qiagen, Valencia, CA) and flash frozen on dry ice. A 90-μl volume of each lysate was combined with 10 μl of 10× reducing agent (Thermo Fisher Scientific) and denatured and reduced at 70°C for 10 min. Twenty-five microliters was loaded per lane followed by SDS-PAGE and Western blotting. rMPV G, N, and P proteins were detected with hyperimmune serum raised against sucrose-gradient-purified MPV virions as previously described (15). The MPV F protein was detected with a rabbit polyclonal antiserum raised against a recombinant vaccinia virus expressing the MPV F protein (15). NS1 and NS2 were detected with rabbit hyperimmune serum raised individually against the synthetic peptides TNFDRSDLET and SDSEESGDEA derived from the corresponding proteins, respectively. RSV F was probed with monoclonal antibodies as described previously (19). Tubulin was detected as a loading control using mouse monoclonal antitubulin antibody (catalog number A-11126; Thermo Fisher Scientific). Primary antibodies were detected with the corresponding species-specific secondary antibodies conjugated with an infrared dye (Li-Cor). Membranes were scanned on an Odyssey infrared imaging system. Band signal values were obtained and background corrected using ImageStudio Lite, version 5.2.5 (Li-Cor).

Evaluation of selected rMPV-RSV-F viral vectors in rhesus macaques. The NIH National Institute of Allergy and Infectious Diseases Animal Care and Use Committee approved the nonhuman primate experiment described in this study, which was performed in accordance with the recommendations from the *Guide for the Care and Use of Laboratory Animals*. Eight young adult rhesus macaques (*Macaca mulatta*) were pretested to confirm that they were seronegative for both RSV and MPV by separate $PRNT_{60}$ assays (described in "$PRNT_{60}$ assays" below). Two groups of four monkeys each were inoculated through the combined intranasal and intratracheal routes with 1.0 ml of inoculum per site containing 10^6 PFU of rMPV-F1 or -F3 diluted in L-15 medium (Thermo Fisher Scientific). In a separate study, four rhesus macaques from the same cohort as those used for rMPV vectors were inoculated with 7.0 $\log_{10}$ PFU per site of recombinant wt RSV strain A2. All four animals were prescreened to be seronegative for RSV. Clinical observations were made daily. Nasopharyngeal (NP) swabs were collected on days 0 to 10, 12, 14, 21, and 28. Tracheal lavage (TL) samples were collected postinfection on days 2, 4, 6, 8, 10, 12, 14, 21, and 28. The NP and TL samples were analyzed by plaque assay as described above to quantify viral shedding. To assess the immunogenicity, sera were collected on days 0, 14, 21, and 28 postimmunization and were analyzed for RSV- and MPV-neutralizing antibodies by $PRNT_{60}$.

$PRNT_{60}$ assays. Serum samples were analyzed for RSV- and MPV-neutralizing antibody titers by performing $PRNT_{60}$ assays on Vero cells as previously described (15, 42) using RSV-GFP (where GFP is green fluorescent protein) and MPV-GFP, respectively. Sera from all 12 animals immunized with rMPV-F1, -F3, or wt RSV were analyzed side by side for RSV-neutralizing antibody levels in the same experiment. Briefly, prior to use, serum samples were incubated for 30 min at 56°C to inactivate the serum complement. Serial dilutions of serum were then mixed with an equal volume of diluted RSV-GFP or MPV-GFP and incubated at 37°C for 30 min. The RSV neutralization assay was performed in the presence of 10% guinea pig complement (Lonza, Walkersville, MD) (43). Complement was excluded from the MPV neutralization assay, as it inactivates the virus (15). At day 6 postinfection, images of the RSV-GFP and MPV-GFP plaques were obtained by scanning on a Typhoon imager (GE Healthcare) and $PRNT_{60}$ was calculated. Each sample was tested in duplicate, and the average values are reported as $\log_2 PRNT_{60}$.

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