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Research article

Identification of a murine cell line that distinguishes virulent from attenuated isolates of the morbillivirus *Peste des Petits Ruminants*, a promising tool for virulence studies

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ABSTRACT

Comprehensive pathogenesis studies on *Peste des Petits Ruminants* virus (PPRV) have been delayed so far by the absence of a small animal model reproducing the disease or an *in vitro* biological system revealing virulence differences. In this study, a mouse 10T1/2 cell line has been identified as presenting different susceptibility to virulent and attenuated PPRV strains. As evidenced by immunofluorescence test and RT-PCR, both virulent and attenuated PPR viruses penetrated and initiated the replication cycle in 10T1/2 cells, independently of the presence of the SLAM goat receptor. However, only virulent strains successfully completed their replication cycle while the vaccine strains did not. Since 10T1/2 cells are interferon-producing cells, the role of the type I interferon (type I IFN) response on this differentiated replication between virulent and attenuated strains was verified by stimulation or repression. Modulation of the type I IFN response did not improve the replication of the vaccine strains, indicating that other cell factor(s) not yet established may hinder the replication of attenuated PPRV in 10T1/2. This 10T1/2 cell line can be proposed as a new *in vitro* tool for PPRV-host interaction and virulence studies.

1. Introduction

Peste des Petits Ruminants (PPR) is an acute and highly contagious disease affecting both domestic and wild small ruminants with high mortality rates (Banyard et al., 2010). PPR is responsible for great economical losses in endemic regions of Africa, Middle and Near-East and Asia (Kaukarbayevich, 2009; Munir et al., 2013). The disease reached Europe in June 2018 with an outbreak in Bulgaria. In recent years, multiple outbreaks have been recorded in Turkey, Georgia and the Maghreb region posing a threat to other countries in Europe. PPR has been identified as the next target of a global eradication campaign launched in 2015 by the World Organization for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO) [38].

The disease´s causative agent is the *Peste des Petits Ruminants* virus (PPRV), a negative-sense single-stranded RNA virus that belongs with the Measles virus (MV), Canine Distemper Virus (CDV) and Rinderpest virus (RPV) to the genus *Morbillivirus* in the *Paramyxoviridae* family (Gibbs et al., 1979; ICTV, 2016). PPRV genome is approximately 16 kb in length and encodes six essential structural proteins: nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin (H) protein, large RNA-dependent polymerase (L) protein, and two non-structural proteins (C and V), arranged in the following order: 3'-N-P/C/V-M-F-H-L-5' (Bailey et al., 2005).

Currently, the signaling lymphocyte activation molecule (SLAM, also called CD150) is recognized as the main PPRV receptor, interacting with the H protein of the virus (Sato et al., 2012). However, before the SLAM was recognized, the CD46 molecule had been widely studied as the main receptor, mainly because of its presence in Vero cells, an African green monkey derived cell line commonly used for PPRV

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isolation (Hamdy et al., 1976; Lefèvre and Diallo, 1990). Nectin-4 has also been described as an alternative receptor used by PPRV (Birch et al., 2013; Fakri et al., 2016). In addition, the tyrosine kinase receptor (ALK) was included as a receptor candidate to PPRV infection *in vitro* (Chaudhary et al., 2015).

For successful *in vitro* replication of PPRV, most of the studies focus on the use of the Vero cells (Balamurugan et al., 2010; Diallo et al., 1989; Kumar et al., 2016). In addition to these, the marmoset B95a cells (Sen et al., 2014) have also been used to isolate PPRV. The isolation of wild-type PPRV in non-ruminant cells can take several blind passages before the observation of a cytopathic effect (Saliki et al., 1994). The time needed for PPRV isolation has been shortened through the use of modified cell lines, mainly Vero cells expressing morbillivirus receptors. VeroDogSLAM or also CHS-20, a monkey CV1 cell, are modified cell lines that express dog or goat SLAM, respectively (Adombi et al., 2011; Sato et al., 2012; Seki et al., 2003). In addition to these, VeroNectin-4, a Nectin-4 expressing cell line, has been used successfully for PPRV isolation (Fakri et al., 2016).

Besides the queries on the main receptor for PPRV entry, both *in vitro* and *in vivo*, many gaps persist in the elucidation of the effects of the innate immune response on PPRV replication. PPRV replication was shown to be reduced in Vero cells pre-treated with human type I interferon alpha (IFNα) (Dhanasekaran et al., 2014). However, PPRV can inhibit the type I interferon (type I IFN) pathway through the expression of the V and C protein (Ma et al., 2015; Sanz Bernardo et al., 2017), and more recently described, also through the expression of N protein (Zhu et al., 2019).

Notwithstanding the remaining questions about PPRV pathogenesis and permissiveness *in vitro,* there is a lack of a small animal model for PPRV *in vivo* studies. This condition has delayed the progress in the understanding of virus-host interactions and the validation of new vaccines and therapeutic tools against PPR.

Aiming to mimic the appropriated conditions for the development of a future mice model to PPRV infections, we first looked for mouse cell lines, in which we introduced the goat SLAM receptor to promote PPRV infection. Unexpectedly, this approach ended up with the identification of a mouse fibroblastic embryonic cell line C3H/10T1/2 (10T1/2) with selective permissiveness to different PPRV strains, in the presence or absence of the goat SLAM receptor.

Our results indicate that the attenuated PPRV strains analyzed here display a defective replication in 10T1/2 cells, while virulent strains successfully completed their life cycle, resulting in a productive infection. These results seem to be independent of goat SLAM receptor expression and the innate antiviral type I IFN response of the cells. Interestingly, a vaccine measles virus strain (MV) showed the same behavior than the vaccine PPRV. Since PPRV attenuated vaccine strains can enter the cells but cannot replicate in the 10T1/2 cell line, we propose these cells as an alternative tool for PPRV-host interaction and virulence studies. In addition, further analyses are needed to evaluate the interest of these cells for other morbilliviruses.

2. Materials and Methods

2.1. Cells and viruses

ETM52 cells, an ovine embryonic cells, obtained from Dr. Pierre RUSSO, ANSES Sophia Antipolis (Thabti et al., 2002), and Vero cells, an African green monkey kidney cells, (ATCC CCL-81, USA) were cultured in Minimum Essential Medium (MEM). C3H/10T1/2 (10T1/2) cells, a mouse fibroblastic embryonic cell, (ATCC CCL-226, USA) were cultured in Basal Medium Eagle (BME). All mediums were supplemented with 2 mM L-Glutamine and 10% fetal bovine serum. All mediums and reagents are from Gibco Life Technologies, USA. Cells were cultured at 37 °C and 5% CO_2.

The PPRV Nigeria 75/1 wild and vaccine strains (accession no. X74443), and India 94/1 virulent strain were obtained from a collection available in our laboratory, while the vaccine strain Sungri 96 (accession no. KF727981) was obtained from MSD Animal Health (The Netherlands). The two first strains belong to the lineage II while the two others are clustered into lineage IV (Kerur et al., 2008). The vaccine strain Schwarz measles vaccine (MV-Schwarz) was obtained from Dr Frédéric Tangy, Pasteur Institute (France).

Viral stocks were prepared by collecting infected Vero cells with the supernatants when approximately 80% of the cells displayed cytopathic effect (CPE). Cells were frozen/thawed three times before collection. Finally, viral titers were determined using the Reed–Muench method (Reed and Muench, 1938) and expressed as $TCID_{50}$/mL. Virus stocks were aliquoted and stored at - 80 °C.

2.2. Virus infection and cell assays

All cell assays were performed using 24h-seeded cells. All viral infections were performed at multiplicity of infection (MOI) of 0.1. After 1 hour of virus adsorption, the inoculum was discarded and replaced with an adequate volume of complete medium with 5% of fetal bovine serum.

2.2.1. Kinetic study of PPRV strains in 10T1/2

10T1/2 and Vero cells were seeded in 96-wells plates (2×10^4 cells per well). Vero cells were included as positive control to PPRV infection. Cells were infected with wild and vaccine Nigeria 75/1 strains, India 94/1 and Sungri 96 strains. After 8 hours, 24 hours and 8 days post-infection (pi), cells were fixated with acetone as described later and subsequently analyzed for N-PPRV expression by immunofluorescence assay (see paragraph 2.3.).

2.2.2. Viral entry assay

To evaluate the susceptibility of 10T1/2 cells to PPRV strains infection, after 1 hour of virus adsorption, cells were washed with acid-glycine buffer 0.6 M at pH 2.5 (Sigma Aldrich, France) for 2 seconds and again with PBS. Cells were then fixated and subsequently analyzed for N-PPRV expression by immunofluorescence assay (see paragraph 2.3.).

2.2.3. Infection with other morbilliviruses and cell lines

10T1/2 cells were infected with MV-Schwarz. The presence of cytopathic effect (CPE) was analyzed during 8 days pi by microscopic observation. The permissiveness of ETM52 cells to PPRV infection was tested using the same conditions of infection and N-PPRV immunofluorescence analysis. Cells were fixated and analyzed 72 hours pi.

2.2.4. Goat SLAM receptor, Poly I:C and RNA interference transfections

Transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. 10T1/2 and Vero cells were seeded in 24-wells plates (6×10^4 per well) and were transfected using 0.6 µg of pCHS-GO1, an expression vector containing goat SLAM coding sequence that was obtained from Dr. Adama Diallo (FAO/AIEA, Vienna, Austria). At 24 hours post-transfection, cells were infected with wild-type and vaccine Nigeria 75/1 strains.

The differences in cellular susceptibility to PPRV regarding the endogenous type I IFN response were also determined. In 96-well plates, 10T1/2 cells were transfected with either the IFN inducer Poly I:C (200 ng/ well, Sigma Aldrich, France) or a mouse siRNA targeting the IFN-α/βRα (100 pmol/ well, Santa Cruz Biotech, USA), stimulating or blocking the type I IFN response respectively. A mock-transfected control was included in the experiment. At 24 hours post-transfection, cells were infected with either wild-type or vaccine Nigeria 75/1 strains. Eight days after PPRV infection, virus replication was evaluated by the presence of N-PPRV through immunofluorescence assay and the expression of IFNα was obtained through a quantitative RT-PCR (RT-qPCR) (see paragraph 2.3 and 2.4.3).

2.3. Immunofluorescence assay

Cells were washed 3 times with PBS 1X and fixated with cold 80% acetone for 30 min at – 20 °C. They were washed twice with PBS and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, France) for 10 min at room temperature. After one wash with PBS 1X, cells were blocked in 0.2% gelatin solution for 20 min. Monolayers were immunostained with N-TRITC polyclonal antibody specific for PPRV nucleoprotein produced in our laboratory, at a dilution of 1:100 for 30 min at room temperature. Cells were washed twice with 0.2% gelatin solution and once with PBS 1 × . Finally, cells were observed under a fluorescence microscope (Olympus IX70, Japan).

2.4. RNA extraction and mRNA analyses by RT-PCR

All RNA extractions were performed using RNeasy Mini kit (Qiagen, France) according to the manufacturer's instructions. RNA samples were qualified using a NanoDrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) with an expected A260:A280 ratio higher than 2. Genomic DNA (gDNA) contamination was removed with TURBO DNA-free™ Kit (Thermo Fisher Scientific, France) according to the manufacturer's instructions and the absence of gDNA amplification was confirmed by control amplification without reverse transcription reaction.

2.4.1. Expression of goat SLAM and nucleoprotein-PPRV genes by RT-qPCR

At 48 hours pi, total RNA was extracted from cells cultured in 24-well plates. PolyA + mRNA was purified using the Oligotex mRNA Mini Kit (Qiagen, UK).

Goat SLAM RT-qPCR assays were performed using Brilliant II SYBR® Green QRT-PCR Master Mix Kit, 1-Step (Agilent, USA). Reactions were performed in a final volume of 25 µL following the manufacturer's conditions with 2 µL of purified mRNA and 100 nM of final concentration of each primer (Table 1). Reactions were run on an Mx3000 P instrument (Agilent, USA). The samples were tested in duplicate (from extraction to amplification) using the following RT-qPCR 1-step program: 50 °C for 30 minutes; 95 °C for 10 minutes; and 40 cycles of 94 °C for 30 seconds, 56 °C for 1 minute, and 72 °C for 30 seconds. A final dissociation step was included in all reactions.

Nucleoprotein-PPRV (N-PPRV) RT-PCR assay was performed as previously described (Kwiatek et al., 2010) (see Table 1).

2.4.2. Expression of matrix protein and hemagglutinin-PPRV genes by RT-PCR

Using the same mRNA previously described, reverse transcription was performed with SuperScript III First-Strand Synthesis SuperMix (Invitrogen, USA), according to the manufacturer's instructions. Then, each sample was analyzed with conventional PCR. All reactions were performed in a final volume of 25 µL containing: 2.5 µL of 10X PCR buffer, 1 µL of dNTP 10µM, 1 U of Taq DNA polymerase, 2 µL of cDNA and 400 nM of each primer (Table 1). The cycling program was as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute; and a final step at 72 °C for 5 minutes. The PCR products attempted were 352 bp and 312 bp to matrix protein and hemagglutinin PCR, respectively, and were analyzed by electrophoresis in 1.5% agarose gel.

2.4.3. Expression of IFNα in 10T1/2 cells by RT-qPCR

At 8 hours pi, total RNA was extracted from cells cultured in 24-well plates. Aiming to access the quantification of IFNα expression, 84 nanograms of each RNA sample was reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, USA) according to the manufacturer's instructions.

Quantitative PCR reactions were performed using the Brilliant II SYBR Green QPCR Master Mix (Agilent, USA) in a final volume of 25 µL with 2 µL of cDNA and 600 nM of each primer (Table 1). The cycling program was as follows: 95 °C for 10 min; 40 cycles of 95 °C for 30 seconds, 60 °C for 1 minute, and 72 °C for 30 seconds. All reactions were performed in duplicate and included a final dissociation step.

Relative expression was normalized with β-actin gene and was calculated with the comparative Ct method (ΔΔCt method) using the following equation: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Differences in gene expression were expressed as fold-changes (Log($2^{-\Delta\Delta Ct}$).

2.4.4. Expression of CD46, Nectin-4, ALK and SLAM receptors by RT-PCR

Total RNA extracted from brains, lungs and blood of a NMRI (Naval Medical Research Institute) mouse were used as positive control for gene expression. RNA from the solid fresh tissues and blood was extracted with RNeasy mini Kit following the "Purification of Total RNA from Animal Tissues" protocol including the DNase treatment step. RNA was reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, USA) according to the manufacturer's instructions.

PCR assays for mouse ALK receptor were performed following the

Table 1
Primer sequences and references for PCR experiments.

Target gene	Primer names	Primer sequence (5'-3')	Tm (°C)	Amplicon size (bp)	Reference
Goat SLAM	SLAM-BC0273F	F- CAAAGGATCCCTTTCCTGGAG	56	249	(Adombi et al., 2011)
	SLAM-BC0273R	R- CATGGACTCGATTCTGAGGAG			
Nucleoprotein	NPPRf	F- GAGTCTAGTCAAAACCCTCGTGAG	55	351	(Kwiatek et al., 2010)
	NPPRr	R- TCTCCCTCCTCCTGGTCCTC			
Matrix protein	MP54	F- AGGAGCAAGGGCAAC	55	352	Diallo and Minet, 1994 (unpublished)
	MP47	R- AGTCCTGCCGTGCGCCTCACC			
Hemagglutinin protein	HP46	F- CGCACAAAGGGAAAGGATCA	55	312	Diallo and Minet,1994 (unpublished)
	HP47	R- CTGATGCCGACTTCATCACC			
IFNα	IFNαf	F- CTGCTGGCTGTGAGGACATA	60	115	(Wang et al., 2013)
	IFNαr	R- AGGAAGAGAGGGCTCTCCAG			
β-actin	βact1	F- ACCAACTGGGACGACATGGAGA	55	455	(Minet et al., 2009)
	βact2	R- AGCTGGTCCTGCTCGAAGTC			
ALK	Mouse ALKF	F- TTCTCCTTGTGAGCTCCGCA	58	296	(Lu et al., 2015)
	Mouse ALKR	R- TCCACTGATGGTAAGGTAGC			
CD46	CD46F	F- ATGCCTGTGAACTACCACGG	60	275	*In house*
	CD46R	R- TTTAGCTCGAGCACCCCATG			
Mouse SLAM	Mouse SLAMF	F- CAAAGGATCCCTTTCCTGGAG	55	720	*In house*
	Mouse SLAMR	R- CATGGACTCGATTCTGAGGAG			
Nectin-4	Nectin4 CDSF	F- GGATTCTCAGGTCACTGTGG	61	399 mouse	*In house*
	Nectin4 CDSF	R- TTCATGGCCTGTTTGATGCC		477 monkey	

[1]Abbreviations: F, forward; R, reverse; bp, base pairs; Tm, melting temperature.

protocol described by Lu et al. (2015) (Lu et al., 2015). PCR assays for mouse CD46, SLAM receptor and mouse and monkey nectin-4 were developed in this study. Primers were designed with Primer3 Software 2.4.0 (Untergasser et al., 2012) on the following sequences mouse CD46 (accession number NM_010778.4), mouse SLAM receptor (NM_013730.4), mouse nectin-4 receptor (NM_001122680) and monkey nectin-4 receptor (XM_001117709). All reactions were performed in a final volume of 25 µL containing: 2.5 µL of 10X PCR buffer, 1 µL of dNTP 10µM, 1 U of Taq DNA polymerase, 2 µL of cDNA, 400 nM of each primer. The cycling conditions were as follows: 94 °C for 5 min; 35 cycles of amplification at 94 °C for 45 seconds, primer-specific annealing temperature for 45 seconds and 72 °C for 1 minute; and a final extension of 72 °C for 5 minutes. The PCR products were analyzed by electrophoresis in 1.5% agarose gel. Amplicons from control samples and 10T1/2 cells were sequenced by Beckman Coulter Genomics (Takeley, United Kingdom) through Sanger methodology and their nucleotide sequence analyzed using the software Geneious version 6.1 (http://www.geneious.com) (Kearse et al., 2012). The primer sequences, annealing temperatures and amplicon sizes are listed in Table 1.

3. Results

3.1. PPRV strains show different replication level in 10T1/2 cell line

The 10T1/2 permissiveness to PPRV virulent and attenuated strains was evaluated by an immunofluorescence assay against the viral nucleoprotein (N-PPRV) at different time points post-infection. A steady and rapid increase in virus production was observed over time in the cells inoculated with the two virulent strains, wild-type Nigeria 75/1 and India 94/1. In contrast, cells infected with the attenuated strains, vaccine Nigeria 75/1 and Sungri 96 of the corresponding lineages II and IV, respectively, showed a stable low level of N-PPRV expression at all time points (Fig. 1A).

Since the entry of virulent PPRV strains in the mouse 10T1/2 cells was unexpectedly easy, we further looked at the expression of PPRV cell receptors. RT-PCRs were performed to assess expression of SLAM, CD46, ALK-tyrosine kinase and Nectin-4 mouse receptors. CD46 and ALK but not Nectin-4 or SLAM receptors are expressed in 10T1/2 cells (Figure S1,2). However, the same profile is found in Vero cells, into which virulent and attenuated PPRV strains replicate efficiently (Figure S2) (Chaudhary et al., 2015; Lecouturier et al., 1996; Noyce et al., 2011). We further checked whether trans-expression of the goat SLAM PPRV-receptor in 10T1/2 could improve the replication of attenuated PPRV strains. 10T1/2 cells were transfected with a plasmid expressing the goat SLAM receptor. The expression of goat SLAM receptor was confirmed 24 hours post-transfection by a RT-qPCR targeting its messenger RNA (mRNA); however, no modification in the cell permissiveness to PPRV was observed (Table S1).

To identify the stage of the virus replication cycle where PPRV vaccines are blocked in 10T1/2 cells, we first examined the ability of these strains to enter in these cells (classically defined as susceptibility). Infected cells were washed with PBS 1X and then with an acidic buffer solution to remove any bound, but non-internalized virus. Both wild-type and attenuated PPRV strains were able to enter 10T1/2 cells, as shown by the immunofluorescence results (Fig. 1A).

Additionally, conventional RT-PCR was performed to detect mRNA of H and M proteins at 24 hours post infection (pi). The results showed an equal expression of PPRV transcripts in attenuated and virulent Nigeria 75/1 PPRV strains at early stages of the infection, suggesting that the blockage of the attenuated strain occurs later in the virus replication cycle (Figure S3).

As an additional control for virus infection, all PPRV strains were inoculated in Vero cells and immunofluorescence for N-PPRV detection was performed at 1 hour and 8 days pi in this cell line. The replication cycle of PPRV strains occurred as expected (Fig. 1B, C).

3.2. Replication-defective of attenuated PPRV is not reproduced in ETM52 cells

In order to assure that the differences observed on the interaction between the PPRV strains and 10T1/2 cell line are specific events linked to the mouse cell, a cell line from a PPRV host species was included in the tests. ETM52, a sheep embryonic cell line, were inoculated with both PPRV attenuated strains and the wild-type India 94/1 strain and, 72 hours pi, the N-PPRV expression was analyzed by immunofluorescence. The ETM52 cells were found equally susceptible to attenuated and virulent PPRV strains, as demonstrated by the high number of N-PPRV positive cells 72 hours pi (Fig. 1D).

3.3. Type I IFN response does not interfere with the PPRV replication cycle

Type I IFNs are known to inhibit morbillivirus replication and, conversely, wild-type morbilliviruses may down-regulate the IFN response through the expression of C, V and W proteins (Avia et al., 2016; Ma et al., 2015; Sanz Bernardo et al., 2017; Sparrer et al., 2012). On the other hand, some vaccine strains seem to lose their capacity to down-regulate the IFN response while still being controlled by type I IFNs. This lost property would result from mutations in the non-structural genes (Chinnakannan et al., 2013; Sparrer et al., 2012). Accordingly, we investigated whether type I IFN responses could be responsible for the control of vaccine strains replication in 10T1/2 cells. Untreated and type I IFN-induced or -suppressed cells were infected with vaccine and wild-type PPRV strains.

A RT-qPCR to IFNα mRNA was applied to estimate IFN expression in these experiments 8 hours after PPRV infection (Fig. 2). Expression of IFNα mRNA was not detected in any other conditions than those involving cells transfected with Poly I:C. Infection by vaccine and wild-type PPRV strains do not induce IFNα response. In contrast, both attenuated and virulent PPRV strains inhibit the IFN response induced by Poly I:C, the virulent strain being more active, with 78% of reduction, compared to 39% with the attenuated strain, probably because the former does a complete replication cycle whereas the latter does not. A short interfering RNA (siRNA) against to IFN-α/βRα was introduced in 10T1/2 cells to block type I IFN expression in response to PPRV infection. However, as no PPRV strain was able to induce mRNA IFNα in this cell line, the siRNA action could not be observed.

PPRV replication was determined by the detection of N-PPRV expression by the immunofluorescence assay at 8 days pi. Whatever the cell treatment, no differences in the virus replication were seen (Fig. 3). The vaccine strains were never able to replicate in 10T1/2 cells, at least for the first 8 days pi, in presence or absence of IFNα. In contrast, the wild type strains showed efficient replication under all cell treatments, including cells pretreated with Poly I:C (Fig. 2).

3.4. Attenuated MV exhibit the same behavior of PPRV vaccine strains in 10T1/2 cells

A vaccine strain of measles virus (MV-Schwarz) was tested in parallel with PPRV on 10T1/2 cells. 10T1/2 cells infected with MV-Schwarz were observed for 8 days pi and presence of CPE was never detected (data not shown).

4. Discussion

Peste des Petits Ruminants is the cause of huge socio-economic problems in endemic countries. Following the rinderpest example, a global PPR control and eradication strategy started in 2015, aiming at the eradication of PPR by 2030 (FAO, 2015). All experts' efforts are now focused in bridging gaps on the actual knowledge on PPRV pathogenesis and control, to achieve the aim of this global campaign. In order to understand the virus-host interactions that determine pathogenesis, it is desirable to generate a small animal model for the disease

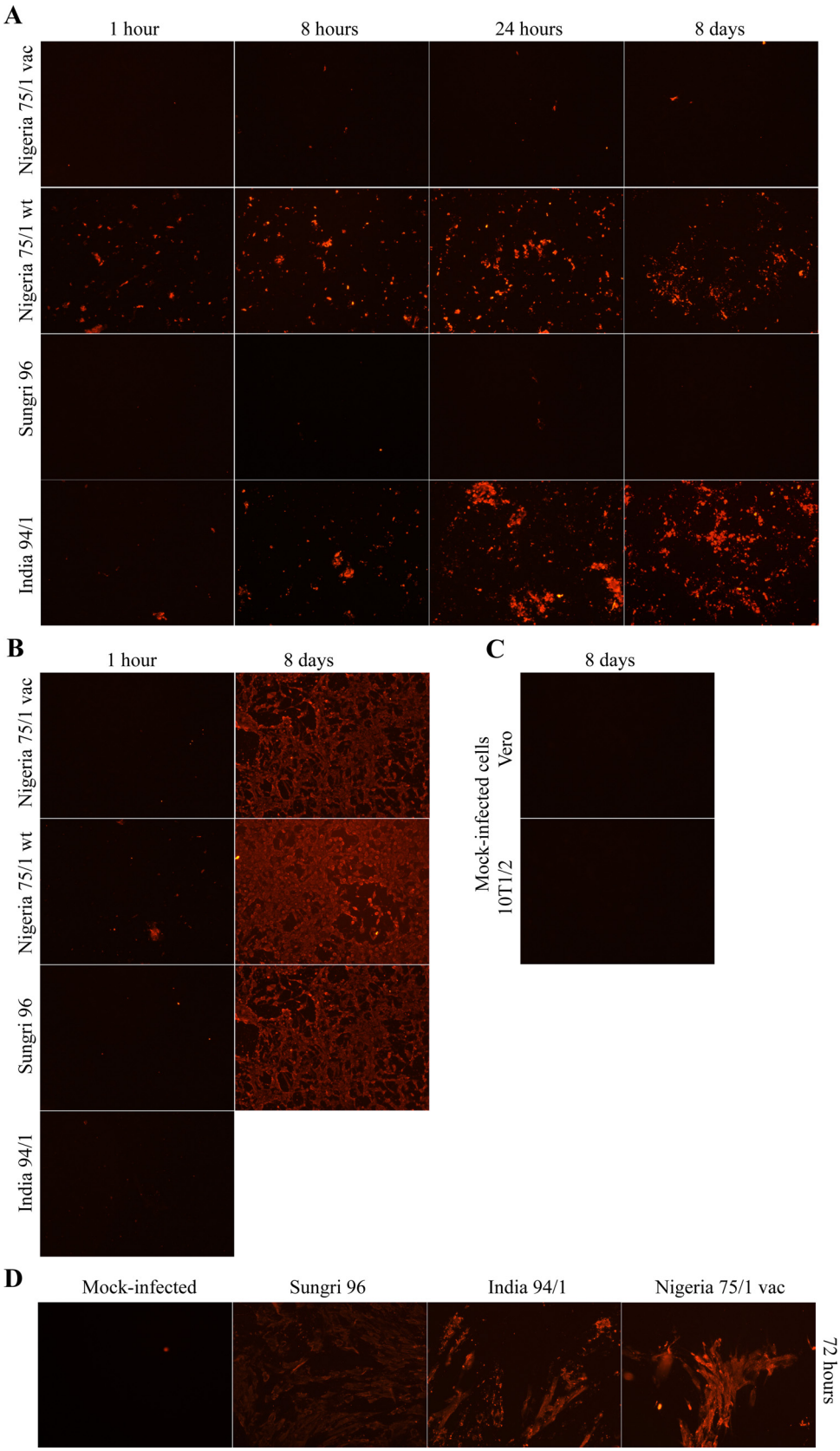

Fig. 1. Expression of N-PPRV in 10T1/2, Vero and ETM52 cell lines. **(A)** Immunofluorescence to N-PPRV showing the infection kinetics of the different PPRV strains at four time points pi in 10T1/2 cells **(B)** and in two time points pi in Vero cells. At 8 days pi, the India 94/1 strain completely destroyed the Vero cell monolayer (figure not included). **(C)** Mock-infected 10T1/2 and Vero cells were used as negative controls **(D)** and the ovine embryonic cell line ETM52 was used as an additional embryonic cell control. Image magnification 10 × .

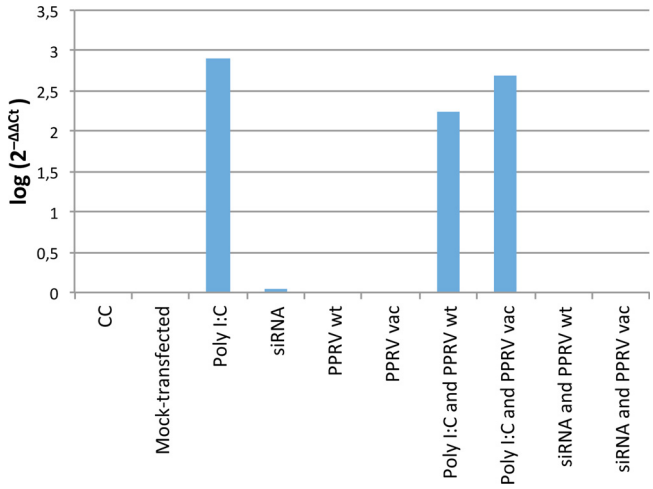

Fig. 2. Relative expression of IFNα mRNA in different conditions of infection with of 10T1/2 with the vaccine or wild-type Nigeria 75/1 strains. Upregulation of IFNα mRNA was observed whenever the cells were transfected with Poly I:C. Nigeria 75/1 wt and vac strains downregulated IFNα mRNA expression 8 hours pi. CC: cell control (not treated or infected); wt: wild type; vac: vaccine.

or an *in vitro* biological system. With the objective to develop a future transgenic mouse model to study PPRV pathogenesis, we first started to study the permissiveness of a mouse embryonic 10T1/2 cell line to PPRV infection in order to determine whether the constitutive expression of a caprine receptor in these cells and later on in mice, would be a prerequisite to allow PPRV entry.

In a first experiment including wild-type Nigeria 75/1 and India 94/1 PPRV strains, which are usually more difficult to propagate in cell culture (Vero, B95a cells) compared to attenuated strains, an intense replication in 10T1/2 cells was observed at the outset, leading to the development of extensive CPE throughout the monolayers. Even if these cells express the murine CD46 and ALK receptors, we were not expecting an efficient interaction of these murine receptors with wild-type PPRV strains. Indeed, optimal *in vitro* adaptation of wild-type morbilliviruses usually requires several passages in cell culture, often resulting in attenuation of the strains for the host species (Ishii et al., 1986; Lee et al., 2016). This is how the attenuation of the Nigeria 75/1 vaccine strain was achieved in Vero cells, which is a monkey cell line (Diallo et al., 1989). Another surprising finding was that the replication of the attenuated Nigeria 75/1 and Sungri 96 PPRV strains was not observed in 10T1/2 cells. Even in the presence of a goat SLAM receptor in these cells, the attenuated PPRV strains were not able to replicate, suggesting that the inhibition was not resulting from the absence of a PPRV receptor. Furthermore, after removal of non-internalized viruses shortly after adsorption, entry of the vaccine strains was confirmed by immunofluorescence. These results agree with a previous study, which showed that 2 minutes after adsorption, the nucleocapsids from paramyxovirus can be already found in the cell cytoplasm (Singh and Helenius, 1992). In 10T1/2 cells, both wild-type and vaccine strains expressed intracytoplasmic mRNA of N, M and H PPRV proteins, showing that vaccine strains can enter the cells and start transcription. It suggests that the blockage occurs later in the virus replication cycle. These observations highlight important differences in virus-host interaction between attenuated and wild PPRV strains.

Mechanisms used by the host to control the morbillivirus life cycle, or even by the virus to escape from the host immune defenses, are in constant study and many questions remain unsolved. Chinnakannan

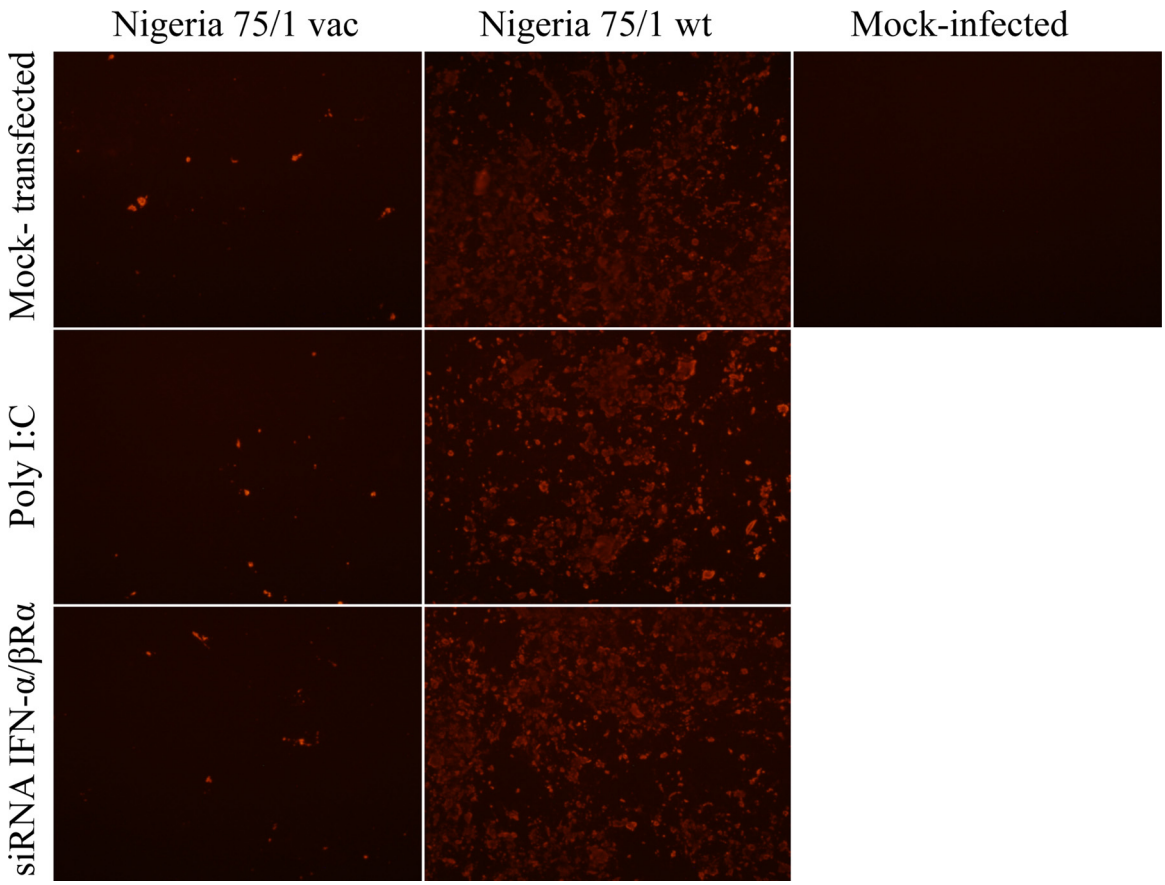

Fig. 3. Nigeria 75/1 wild-type and vaccine strains replication in untreated, type I IFN activated (transfected with Poly I:C) or type I IFN suppressed (transfected with siRNA against IFNAR) 10T1/2 cells. The immunofluorescence to N-PPRV was performed 8 days pi. Image magnification 10 × .

et al., for example, have found different abilities of the morbillivirus species to block IFN from a same cell line (Chinnakannan et al., 2013). Dhanasekaran et al focused on the observation of innate antiviral immune responses caused by the same PPRV strain in different species of goats and buffaloes and found that high type I IFN levels in buffaloes may afford reduced virus replication (Dhanasekaran et al., 2014). Other studies have shown differences in IFN susceptibility and IFN blocking ability of morbilliviruses, such as the canine morbillivirus (CDV) being susceptible to IFN response (Carvalho et al., 2014), while PPRV actively blocking the induction of IFN (Chinnakannan et al., 2014; Zhu et al., 2019).

Referring to the studies previously quoted, we investigated the role of type I IFN response. Our hypothesis was that attenuated strains might be more susceptible to the IFN response of 10T1/2 cells or less likely to control this response compared to virulent strains. However, the results indicated that both wild and attenuated Nigeria 75/1 PPRV could down-regulate the synthesis of type I IFN mRNA, the wild-type strain reducing about 3 times more the synthesis of IFNα transcripts compared to the vaccine strain. These findings reinforce the results observed by others, which confirmed the interference of morbilliviruses with the type I IFN signaling pathways (Ma et al., 2015; Sanz Bernardo et al., 2017; Stewart et al., 2014). In addition, IFNα expression level in 10T1/2 cells is not affected by the infection with either a wild or vaccine PPRV strains and vaccine PPRV strains replication is not increased when IFNα expression is inhibited. These results suggest that the specific behavior of those vaccine strains in 10T1/2 cells is due to another mechanism.

All together, our results indicate that neither absence of specific morbillivirus cellular receptor or type I IFN are responsible for the inhibition of attenuated virus in 10T1/2 cells. Transcriptomic profile of cells infected with attenuated and virulent PPRV will be a useful tool in order to raise alternative hypotheses on the origin of the different replication capacity displayed by these virus strains. Alternatively, differences in the genome sequence between wild-type and attenuated virus could provide important information about viral proteins that are determining virus attenuation and, thus, a possible key player involved in the viral-host interaction (Billing et al., 2014; Chen et al., 2015). An analysis of these mutations between vaccine and wild-type PPRV Nigeria 75/1 strains was carried out by our group and we were able to identify putative mutations associated with virus attenuation (Eloiflin et al., 2019). We intend now to deep our study and verify if these mutations are involved in the attenuated versus virulent behavior of PPRV in 10T1/2 cells.

This study demonstrated for the first time differences in the interaction between vaccine and wild-type PPRV strains in 10T1/2 cells. Furthermore, this peculiarity for PPRV could be shared by other morbilliviruses as shown here for attenuated measles virus. This cell line is a useful tool to study viral and cellular factors involved in virulence of morbilliviruses.

Accession numbers

Nigeria 75/1 vaccine strain: X74443; Sungri 96 vaccine strain: KF727981 (Siddappa et al., 2014), wild-type PPRV Nigeria 75/1 strain: Genbank submission in progress.

CRediT authorship contribution statement

Juliana Comerlato: Conceptualization, Investigation, Writing - original draft. **Emmanuel Albina:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Carinne Puech:** Investigation, Writing - review & editing. **Ana C. Franco:** Project administration, Supervision, Writing - review & editing. **Cécile Minet:** Methodology, Writing - review & editing. **Roger-Junior Eloiflin:** Methodology, Writing - review & editing. **Valérie Rodrigues:** Methodology, Writing - review & editing. **Renata Servan de Almeida:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2020.198035.

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