Veterinary Microbiology 231 (2019) 139–146

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

In vitro characterization of PRRSV isolates with different in vivo virulence using monocyte-derived macrophages

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ARTICLE INFO

Keywords:
PRRSV
Virulence
Monocyte-derived macrophages
Interferon

ABSTRACT

The recent emergence of highly pathogenic porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) strains has caused severe economic losses. The biological elements defining virulence and pathogenicity are still unclear. *In vitro* characteristics using natural target cells of PRRSV provide important information to understand the basis of virulence at the cellular level, and provide a mean to reduce animal experimentations to achieve this goal. Here, we compared PRRSV strains from two geographically different regions, with varying *in vivo* characteristics, in terms of their interactions with monocyte-derived macrophages (MDMs). The strains included Lena and BOR59 from Belarus, and ILI6 from Russia, as well as PR11 and PR40, both from Italy. As a reference, we used a cell culture-adapted version of Lelystad, LVP. MDMs were pre-treated with IFNγ, IL-4 or IFNβ, in order to understand responses in polarized and antiviral MDMs. In general, independent of the geographical origin, the strains with high virulence infected a higher percentage of MDMs and replicated to higher titers. These virulence-dependent differences were most pronounced when the MDMs had been treated with IFNβ. Differentiation between intermediate and low virulent PRRSV was difficult, due to variations between different experiments, but LVP differed clearly from all field strains. IFNα and IL-10 were not detected in any experiment, but PR40 induced TNF and IL-1β. Taken together, these results validate the MDM model to understand pathogenicity factors of PRRSV and confirm the importance of the escape from type I and II IFN-mediated effects for PRRSV virulence.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failure in sows and respiratory disease in growers and finishing pigs. PRRSV has a high prevalence in intensive swine farms and a huge economic impact on the pig industry worldwide (Nathues et al., 2017; Nelsen et al., 1999).

PRRSV, a member of the *Arteriviridae*, is a positive-sense, single-stranded RNA virus with a genome of an approximate size of 15 kb, organized into eleven open reading frames (ORFs). The occurrence of insertions and deletions in the genome regions coding for the structural and non-structural proteins (nsps) has been well described for PRRSV. The two genotypes, type 1 (European) and type 2 (North American), have been reclassified as two separate species, PRRSV-1 and PRRSV-2, respectively (Adams et al., 2016; Nelsen et al., 1999). PRRSV-1 is further divided into at least three genetic subtypes (Stadejek et al., 2008, 2013).

Beginning in 1996, PRRSV isolates causing overt and severe clinical signs either in sows or in weaners-growers have emerged in North America, Asia and, more recently, in Europe (Karniychuk et al., 2010; Mengeling et al., 1998; Morgan et al., 2013; Tong et al., 2007). Some of these strains were defined as “highly pathogenic”, due to their increased *in vivo* virulence, but the criteria for this classification are not well defined.

PRRSV has a limited tropism for macrophages (MØ) (Van Breedam et al., 2010). MΦ are highly plastic cells that take on distinct phenotypes, functional properties and cytokine production profiles, due to the influence of cytokines in their local microenvironment (Mosser and

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https://doi.org/10.1016/j.vetmic.2019.03.008
Received 23 October 2018; Received in revised form 7 March 2019; Accepted 10 March 2019
0378-1135/

Edwards, 2008). Recently, a model has been introduced using interferon (IFN)-γ, interleukin 4 (IL-4) and IFNβ-treated monocyte-derived MΦ (MDMs) to characterize the potential virulence of PRRSV *in vitro* (Garcia-Nicolas et al., 2014). IFNγ plays a critical role in priming MØ for pro-inflammatory responses. Following activation, IFNγ-primed MØ become more resistant to infection, increase their antigen presentation, and efficiently produce pro-inflammatory cytokines (Mosser and Edwards, 2008). On the other hand, IL-4 triggers MØ to promote tissue repair and homeostasis, while also making them more susceptible to intracellular pathogens (Garcia-Nicolas et al., 2014; Gordon, 2003; Mosser and Edwards, 2008). Type I IFNs, such as IFNβ, induce a strong antiviral state in MØ and link the innate to the adaptive immune response (McNab et al., 2015).

Several studies have shown that PRRSV is able to decrease the innate and adaptive immune responses. The character and intensity of these effects varies, depending on the PRRSV isolate. For example, it may differently regulate the expression of cytokines (Gimeno et al., 2011). How PRRSV influences the cytokine production is not fully known. However, it has been established that IFNα, IL-1β, IL-10 and TNF play a role in the development of the immune response during the PRRSV infection (Fan et al., 2016). IFNα stimulates a variety of cellular functions, such as antiviral activity in target cells, innate cell activation and adhesion molecule induction. Suppression of type I IFN synthesis has been observed in PRRSV infected cells and was found to be mediated by several viral proteins (Wang and Zhang, 2014). IL-10 is crucial in controlling the extent of inflammatory responses. Several studies have shown that IL-10 may play a role in the down-regulation of the immune response in PRRSV infection (Darwich et al., 2010; Gimeno et al., 2011). IL-1β is one of the classical pro-inflammatory cytokines. Thus, it is able to induce activation of neutrophils and MΦ during inflammation. It has been reported that low IL-1β and IL-1α-gene expression occurs in the early phase of PRRSV infection (Darwich et al., 2010), in particular after infection with virulent PRRSV (Amarilla et al., 2015; Weesendorp et al., 2014). Finally, TNF is a cytokine secreted during the early phase of innate immune responses and plays a central role in inflammatory responses.

In order to verify the hypothesis that isolates with different *in vivo* behaviour may also show different *in vitro* features, and to get a better understanding of circulating PRRSV-1 strains, the aim of this study was to evaluate the *in vitro* characteristics of several subtype 1 and subtype 2 PRRSV-1 strains, by assessing their infection ability and the induction of secretion of IFNα, IL-1β, IL-10 and TNF in MDMs. As reference strains for comparison, we employed the highly pathogenic subtype 3 PRRSV-1 strain Lena, and a cell culture-adapted strain of the low virulent subtype 1 PRRSV-1 strain Lelystad (LVP), both previously characterized in such MΦ cultures (Garcia-Nicolas et al., 2014).

2. Materials and methods

2.1. Monocyte-derived macrophages (MDMs)

MDMs were generated from blood monocytes as previously described (Garcia-Nicolas et al., 2014). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from blood of specific pathogen-free (SPF) pigs (Swiss **White** Landrace) from 5 to 10 months old, using Ficoll-paque density centrifugation (Ficoll-Paque Plus, GE Healthcare, Chicago, IL, USA). The cells were enriched by positive selection for CD172a (monoclonal antibody (mAb), clone 74-22-15A, hybridomas kindly provided by Prof. A. Saalmüller, Veterinary University of Vienna, Austria) using the magnetic cell sorting system (MACS) with LS columns (Miltenyi Biotec GmbH, Germany). Cells were then seeded onto 48-well culture plates at a density of 5×10^5 cells/ml in Dulbecco's modified Eagle's medium without phenol red (DMEM, Gibco, 21063-029, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany) and recombinant porcine colony-stimulating factor 1 (rpCSF-1; 20 U/ml) (Sautter et al., 2018) and cultured at 39 °C with 5% CO_2 for 72 h for differentiation to MDMs.

MDMs were then stimulated for further 24 h with one of the following treatment regimens: IFNγ (10 ng/ml, R&D Systems, UK), IL-4 (100 U/ml) (Carrasco et al., 2001), IFNβ (100 U/ml) (Husser et al., 2011) and untreated, to obtain "IFNγ MØ", "IL-4 MØ", "IFNβ MØ" and untreated "MØ", respectively.

2.2. Viruses

Six different PRRSV-1 isolates were analysed (two prototype/reference strains and four field strains). These isolates belong to different genetic subtypes and were selected based on the different *in vivo* virulence patterns. LVP was chosen as the prototype strain of a classical European PRRSV-1 subtype 1, and represented a Lelystad virus adapted to MARC-145 by 28 passages. The working stock was propagated once on MDMs to have a similar background as the other viruses. Lena strain (5^{th} passage on MDMs, virus kindly provided by Prof. Hans Nauwynck, Ghent University, Belgium), isolated in Eastern Europe, was used as prototype strain of a highly pathogenic PRRSV-1. It belongs to the subtype 3 (Karniychuk et al., 2010) and differs from the Lelystad prototype virus at both a genetic and antigenic level. Moreover, two Italian field isolates belonging to the subtype 1, Parma11/2014 (PR11) and Parma40/2014 (PR40), both passaged four times on MDMs, were included in the study. PR11 was isolated from a grower during a classical PRRSV outbreak. PR40 is a PRRSV strain with enhanced virulence, isolated from a weaner during an uncommon and severe PRRSV outbreak (Canelli et al., 2017). Finally, two Eastern European PRRSV-1 field strains, belonging to subtype 2, and passaged four times on MDMs, were also tested: 17008/ILI 6 (ILI6) and 9/9A-BOR59 (BOR59). BOR59 is a highly virulent strain isolated in Belarus in 2009 from the lung of a

Table 1
Viruses used, divided by species, subtypes, virulence, origin and cell culture passages.

Virus	Species	Subtype	Virulence[a]	Origin	Passage
Lelystad (LVP)[b]	PRRSV-1	1	Low[b]	The Netherlands	29
Lena	PRRSV-1	3	High[b,c]	Belarus	5
PR11/2014	PRRSV-1	1	Low[d]	Italy	4
PR40/2014	PRRSV-1	1	High[d]	Italy	4
17008/ILI6	PRRSV-1	2	Intermediate[e]	Russia	4
9/9A-BOR59	PRRSV-1	2	High[e]	Belarus	4

[a] Virulence levels are considered preliminary as not all strains were not compared in parallel.
[b] MARC145 cell-adapted, virulence refers to Weesendorp et al. (2013).
[c] Karniychuk et al. (2010).
[d] Canelli et al. (2017).
[e] Stadejek et al. (2017).

pig that died with respiratory symptoms; Russian ILI6 was isolated in 2009 from the lung of a weaner pig and appeared to have intermediate virulence (Stadejek et al., 2017). The different isolates were divided into a "highly virulent" or "not highly virulent" group, based on the *in vivo* pathogenicity (Canelli et al., 2017; Karniychuk et al., 2010; Stadejek et al., 2017). Considering only the *in vivo* features reported in these studies, a possible classification of strains is presented in Table 1.

2.3. Virus propagation

All strains were propagated *in vitro* on MDMs cultured at 1×10^6 cells/ml in T150 flasks. Three-day-cultured MDMs were infected with a MOI of 0.1 $TCID_{50}$/cell. When the cytopathic effect (CPE) had reached 50%, the cells were lysed by freezing at −70 °C overnight. The thawed lysate was clarified by 2000 × g centrifugation at 4 °C for 20 min, and stored at −70 °C.

The titer of the different viruses was determined in quintuplicate serial dilutions on 3-day-old MDMs, plated as 1×10^6 cells/ml in 96-well plates. After 48 h of incubation, the virus was inactivated with 80% acetone for 10 min at room temperature. Then, an immunoperoxidase monolayer assay (IPMA) was performed to detect PRRSV N protein by using the mAb SR30-A (RTI, Brookings, SD, USA). The titre was calculated with the Reed and Muench method, and expressed as 50% tissue culture infective dose per ml ($TCID_{50}$/ml), then converted in MOI.

2.4. PRRSV infection of MDMs

MDMs were infected with a MOI of 0.1. Purified cell-lysate of MDMs served as a mock-control. After virus adsorption of 1 h at 39 °C and 5% CO_2, MDMs were washed two times with 37 °C PBS with calcium and magnesium (DPBS (1x), Gibco, 14040-019, Thermo Fisher Scientific), and cultured at 39 °C with fresh culture medium which consisted of DMEM with FBS and rpCSF1, without the cytokines used for pre-treatment. 16 h post-infection, the isolate infectivity was assessed by flow cytometry of harvested cells and virus productivity by titration of supernatants.

2.5. Flow cytometry

The PRRSV N protein expression was evaluated by flow cytometry in order to assess the percentage of infected MDMs. For PRRSV nucleocapsid expression analysis, MDMs were harvested by 20 min incubation at room temperature (RT) with Accutase (Innovative Cell Technologies, San Diego, CA, USA), washed with 1 ml cold PBS/5xEDTA and fixed with 4% paraformaldehyde (PFA; Polysciences, Warrington, PA, USA) dissolved in PBS for 10 min at RT, washed again and permeabilized with 0.1% saponin (Applichem, Darmstadt, Germany) in PBS. The mAb SR30-A was diluted in PBS containing 0.3% of saponin (w/v) and added for 20 min to the cells on ice; after washing with PBS containing 0.1% saponin (w/v), cells were incubated with the secondary antibody, Alexa Fluor 647, (A21240, Molecular Probes) diluted in 0.3% saponin (w/v) in PBS, for 15 min on ice. After a final wash, the cells were resuspended in cell wash (BD Biosciences) for acquisition with the FACS Canto (Becton Dickinson, Eysins, Switzerland). After doublet exclusion, electronic gating based on the forward/side scatter plots was applied to exclude cells with low FSC, representing mostly dead cells and debris (using the FlowJo V.7.2.6 software, Tree stars Inc., Ashland, OR, USA).

2.6. Cytokine measurement

Cytokine production was evaluated after harvesting the culture supernatants at 16 h post infection (hpi). IFNα production was determined by using an in-house ELISA (Guzylack-Piriou et al., 2004), with a detection limit of 30 pg/ml; IL-1β, IL-10 and TNF production was measured using commercial kits (R&D Systems, UK), with a detection limit of 60 pg/ml.

2.7. Statistical analysis

All experiments were performed in triplicate and repeated five times with cells from different blood donor pigs (biological replicates). Statistical analysis was performed by using GraphPad Prism V.7 software (GraphPad Software, San Diego, California, USA) and FlowJo V.9.6.

Virological data were analyzed with a one-way ANOVA test, followed by the Bonferroni's multiple comparisons test. Cytokine data employed a two-way ANOVA with the parameters being virus strain and blood donor, and using Dunnett's correction for multiple comparisons. A p value of < 0.05 was considered as statistically significant.

3. Results

3.1. Reduced infectivity of low virulence PRRSV strains in IFN-treated MDM

The different infectivity on MDMs in terms of nucleocapsid expression was determined at 16 hpi. A representative staining is shown in Supplementary Fig. 1. In untreated MDMs, the highest levels of infectivity were found for the highly virulent strains Lena, BOR59, PR40 as well as for the ILI6 PRRSV, classified as intermediate in virulence (Fig. 1A). In IFNγ MDMs, Lena and BOR59 showed the highest infectivity, followed by ILI6, PR40 and PR11 strains. LVP induced the lowest levels of nucleocapsid expression, which was statistically different from all other strains (Fig. 1B). In IL-4 MDMs, the levels of infection and the differences between strains were similar to untreated MDMs (Fig. 1C). In IFNβ MDMs, the highest levels of infectivity were found for the highly virulent strains Lena, PR40 and BOR59, followed by ILI6 and PR11. IFNβ almost completely suppressed nucleocapsid expression of LVP (Fig. 1D). Of note, within the subtype 1 PRRSV-1 strains, PR40 infectivity was higher than that of PR11 in all MDM-conditions ($p < 0.05$), except with IFNγ MDMs.

We next performed a deeper analysis on how the viruses compared to each other, taking the *in vivo* virulence into consideration. Therefore, the data seen in Fig. 1 were divided into two groups and their relative infectivity compared to a reference strain was analyzed. The highly virulent strains PR40, BOR59 and Lena (subtypes 1, 2 and 3, respectively) were compared amongst each other, using the well-described Lena as the reference highly pathogenic PRRSV strain (Fig. 2). The same data analysis was also performed among the less virulent strains (PR11, ILI6, and LVP) in comparison to LVP, chosen as a low virulent reference strain.

BOR59 was statistically as infective as Lena, but not with IFNβ-primed MDMs. PR40 had reduced infectivity for MØs under all conditions when compared to Lena, and also when compared to BOR59 for IFNγ and IL-4-primed MØs.

When the less virulent isolates (LVP, PR11, and ILI6) were analyzed, ILI6 was the most efficient at infecting the different MØ conditions (Fig. 3). The Italian strain PR11 was statistically less able than ILI6 to infect any of the treated MØ, and also performed worse than LVP on untreated and IL-4-primed MØ ($p < 0.05$). Curiously though, PR11 had a higher efficiency than LVP in infecting the MDMs pre-treated with either of the IFNs, and LVP infected IL-4 MØ as successfully as ILI6.

3.2. Reduced replication of low virulence PRRSV strains in IFN-treated MDM

Virus replication was determined by measuring virus in the supernatant of infected MDMs. As shown in Fig. 4A and C, no virulence-dependent differences in viral titres were found when untreated MDMs

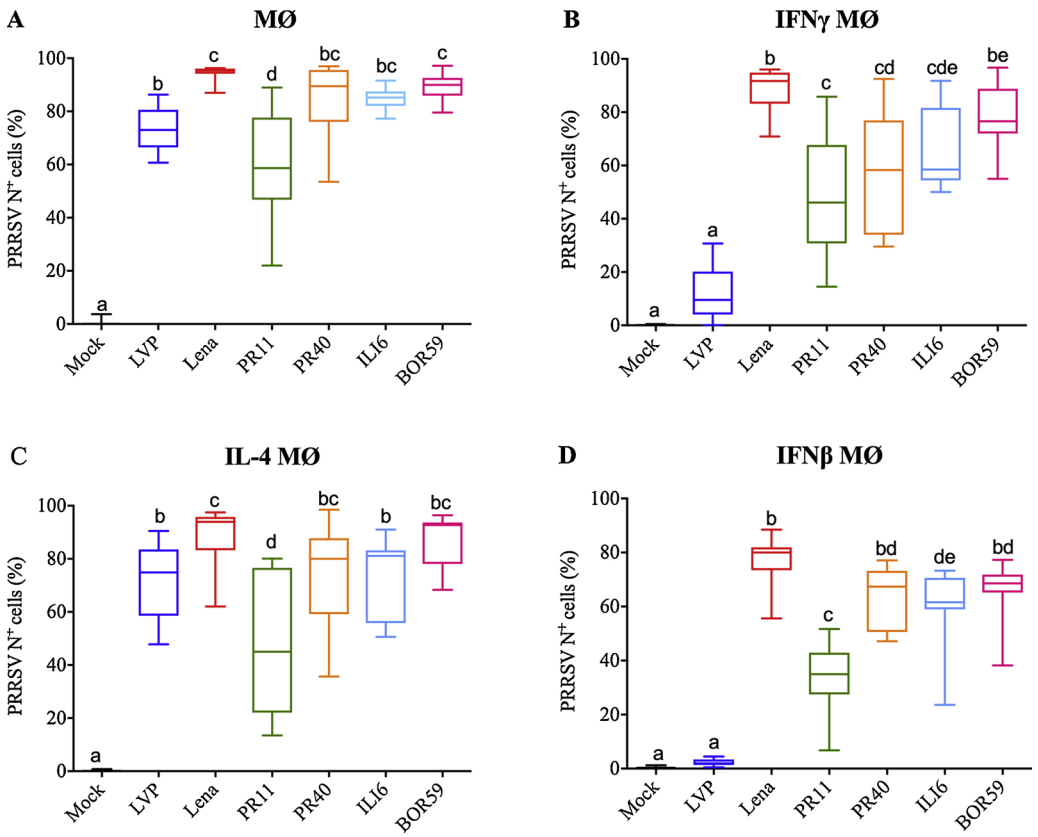

Fig. 1. Infectivity of different PRRSV strains. Percentage of PRRSV nucleocapsid (N) expression measured by flow cytometry. Untreated MØ (A), IFNγ MØ (B), IL-4 MØ (C) and IFNβ MØ (D) were infected at MOI 0.1 with: LVP, Lena, PR11, PR40, ILI6, BOR59 or Mock-treated for 16 h. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells; data from triplicate culture of five independent experiments. All box plots marked with different letters are significantly different from each other ($p < 0.05$). This applies only to a particular MDM pre-treatment.

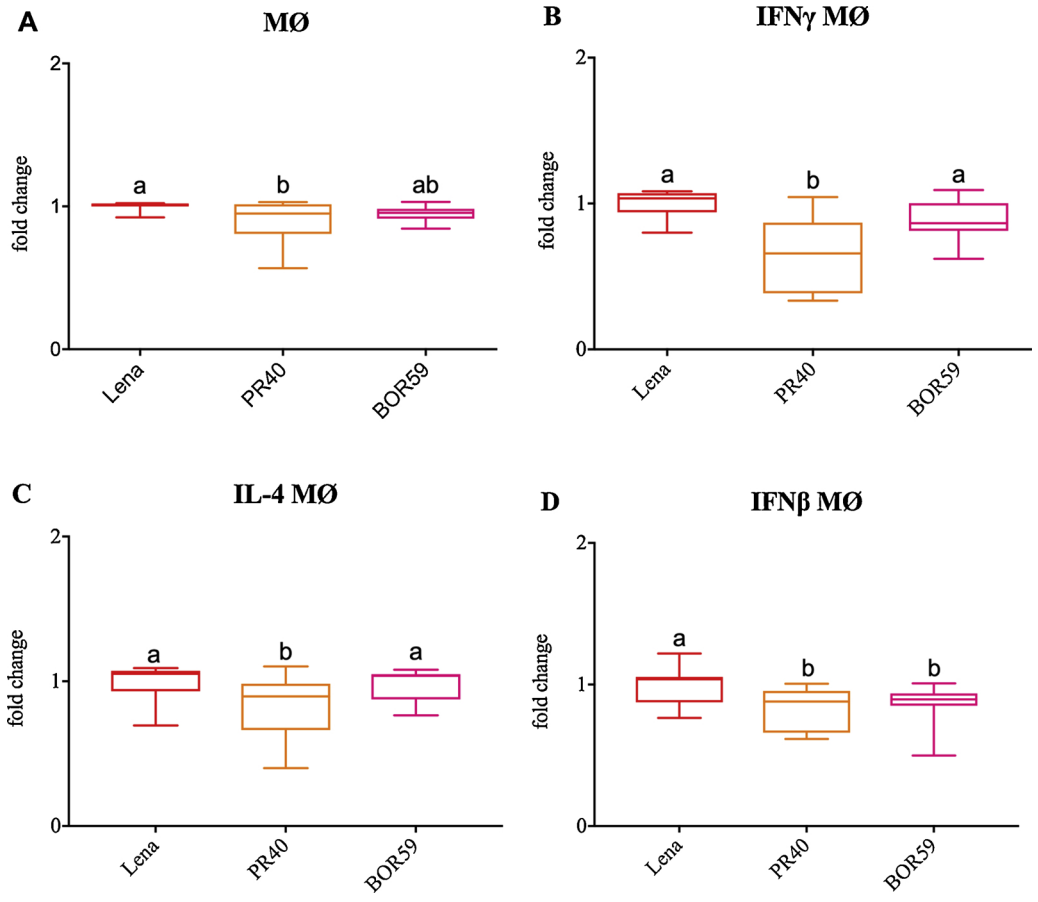

Fig. 2. Relative infectivity of highly virulent strains. Untreated MØ (A), IFNγ MØ (B), IL[HYPHEN]4 MØ (C), and IFNβ MØ (D), were infected with MOI 0.1 of Lena, PR40 or BOR59 for 16 h. PRRSV nucleocapsid (N) expression was measured by flow cytometry, and Fold Change was calculated in reference to Lena. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells. Data from triplicate culture of five independent experiments. All box plots marked with different letters are significantly different from each other ($p < 0.05$). This applies only to a particular MDM pre-treatment. The data is a further analysis of the data seen in Fig. 1.

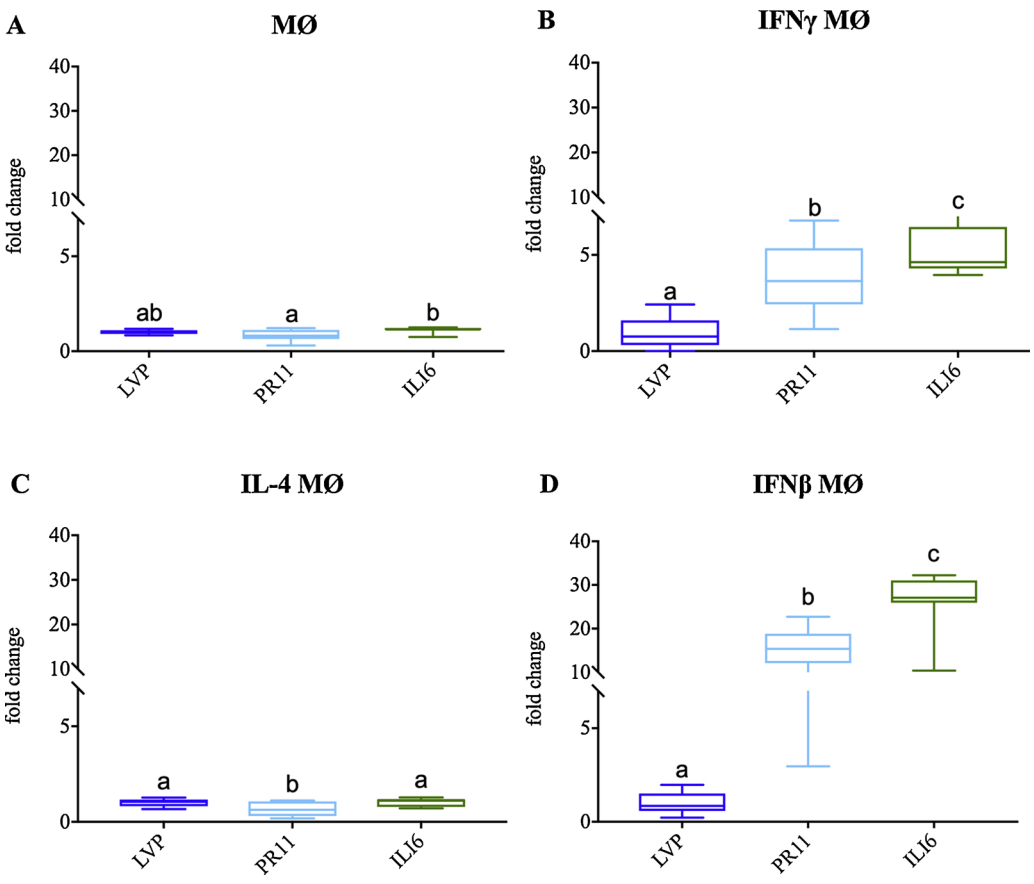

Fig. 3. Relative infectivity of low virulent PRRSV strains. Untreated MØ (A), IFNγ MØ (B), IL-4 MØ (C), and IFNβ MØ (D), were infected with MOI 0.1 of LVP, PR11 or ILI6 for 16 h. PRRSV nucleocapsid (N) expression was measured by flow cytometry, and fold change was calculated in reference to Lena. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells. Data from triplicate culture of five independent experiments. All box plots marked with different letters are significantly different from each other ($p < 0.05$). This applies only to a particular MDM pre-treatment. The same data as in Fig. 1 was used.

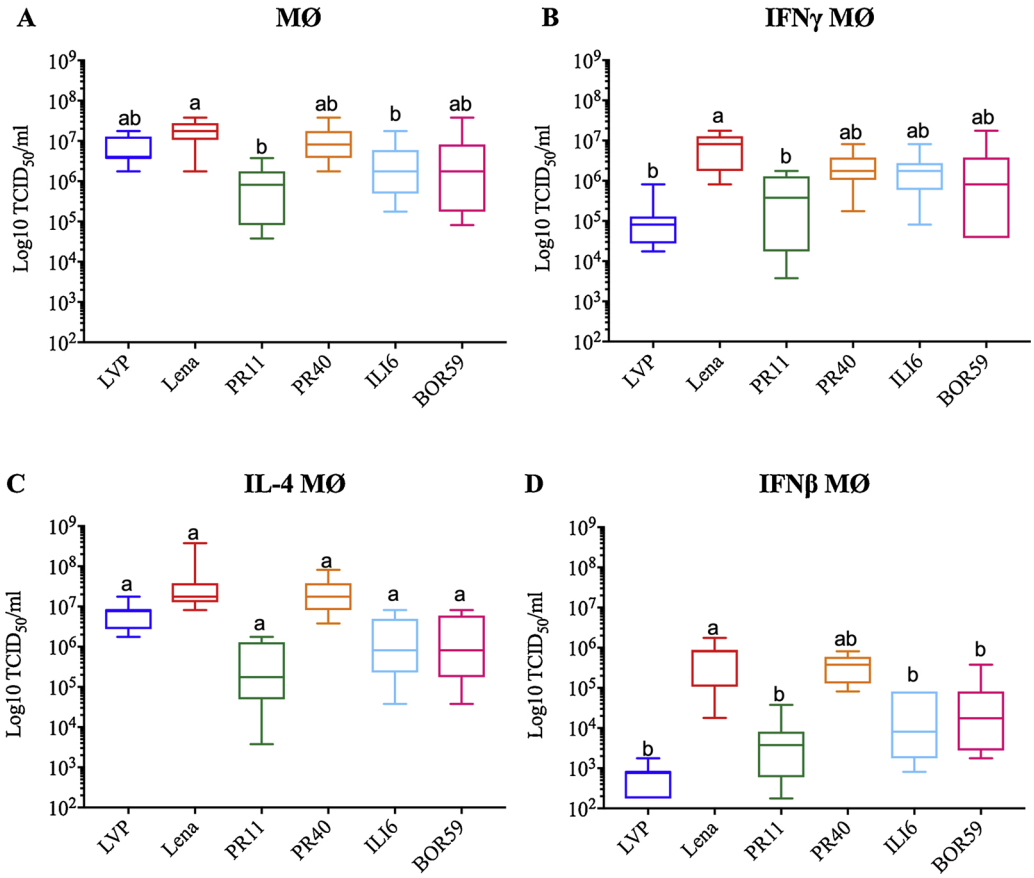

Fig. 4. Viral titers of different PRRSV strains in MØ supernatants. Supernatant was collected from untreated MØ (A), IFNγ MØ (B), IL-4 MØ (C) and IFNβ MØ (D), which were infected at MOI 0.1 with LVP, Lena, PR11, PR40, ILI6 or BOR59 for 16 h. The viral titer was determined in quintuplicate serial dilutions on 3-day-old MØ after 48 h of incubation. The titer was calculated, in three independent experiments, with the Reed and Muench method and expressed as $\log_{10}$ $TCID_{50}$/ml. All box plots marked with different letters are significantly different from each other ($p < 0.05$). This applies only to a particular MDM pre-treatment.

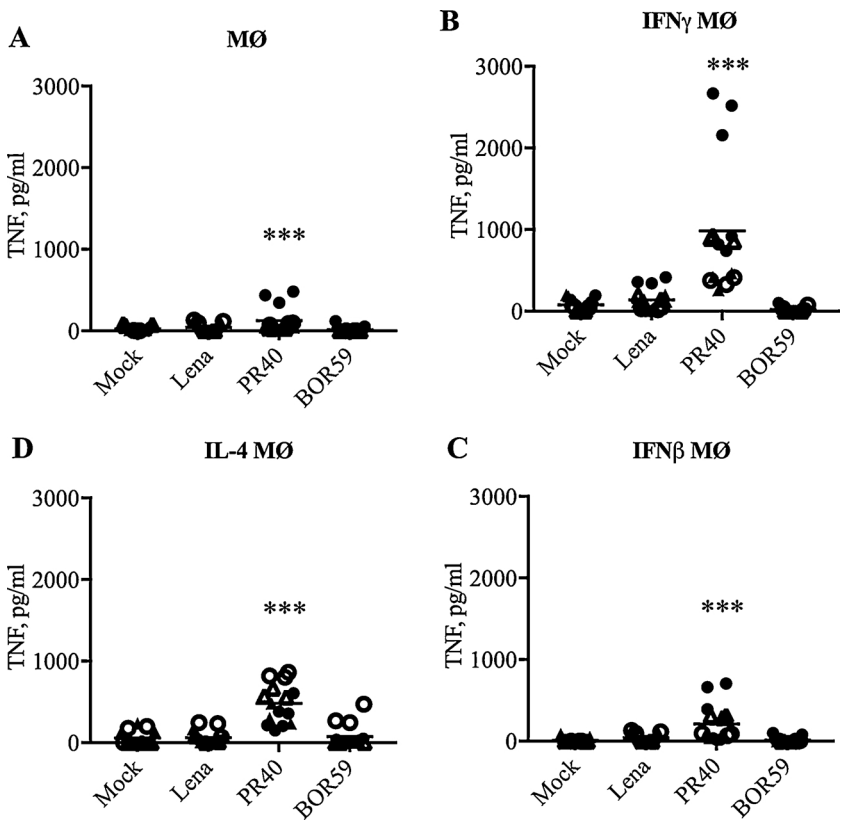

Fig. 5. TNF production induced by highly virulent PRRSV. Supernatants of cells infected with PR40, BOR59 and Lena (subtype 1, 2 and 3, respectively) were tested for TNF by ELISA. Undifferentiated MØ (A), IFNγ MØ (B), IL-4 MØ (C) and IFNβ MØ (D) were infected at MOI 0.1 or Mock-treated for 16 h. Scatter dot plots show triplicates from data obtained four independent experiments with cells from different pigs, and the means of all data. Statistical significance for comparison to the mock controls is indicated as follows (*$p < 0.05$, 0.002**, ***$p < 0.001$).

or IL-4 MDMs were used. In contrast, when using IFNγ MDMs, the highly virulent strains PR40, BOR59, Lena as well as ILI6 of intermediate virulence grew to higher titers than LVP and PR11 (Fig. 4B), but, except for Lena, they were not statistically distinguishable. On IL-4 MDMs, no strain-dependent differences were found. In IFNβ MDMs, Lena and PR40 stood out as the most replicative strains, followed by BOR59, which was significantly less replicative than Lena, but not than PR40 (Fig. 4D).

3.3. PR40 activates MDMs to produce TNF and IL-1β

We next tested if virus-induced cytokines differed in a PRRSV-strain-dependent manner. To this end, IFNα, IL-1β, IL-10 and TNF was quantified from MDM supernatants collected at 16 hpi. No IFNα or IL-10 was detected, independent of MDM pre-treatment or PRRSV infection (data not shown). Only PR40 stimulated a significant release of TNF, which was seen in untreated and cytokine pre-treated MDM cultures ($p < 0.05$; Fig. 5). Also for IL-1β, PR40 was the only virus inducing this cytokine but this required IFNγ- or IFNβ-priming of the MØ (Fig. 6). Overall, there was a remarkable variability in IL-1β responses.

4. Discussion

In the present study, the *in vitro* characteristics of PRRSV isolates belonging to different genetic subtypes within PRRSV-1, and with different *in vivo* pathogenicity or virulence, were evaluated using MDMs, through assessment of infection capacity, replication and cytokine production. This work represents a follow-up study of a previous work that proposed MDMs as a potential *in vitro* model to evaluate the virulence of different PRRSV strains (Garcia-Nicolas et al., 2014). In agreement with that study, we observed that the effectiveness of the antiviral status induced by IFNγ and IFNβ depended on the virulence of the strain employed. The LVP strain and, to a lesser extent, the Italian strain PR11, were found to be particularly sensitive to the action of the IFNs, while Lena, BOR59 and PR40 were relatively resistant. In our previous work we tested three other low virulent PRRSV strains, two of them being field isolates. These viruses showed a similar level of infectivity compared to LVP (Garcia-Nicolas et al., 2014), indicating that the high sensitivity to IFN treatment cannot be simply attributed to the passaging of the LVP in MARC145. Nevertheless, future studies could be interesting to address if continued passaging of PRRSV in cell lines can change the characteristics of PRRSV in IFN-stimulated macrophages. Although the results were not “black or white”, the obtained data confirms that IFN-stimulated MDMs can in principle be employed to assess the potential virulence of PRRSV strains. Nevertheless, the ILI6 strain did not differ statistically from BOR59, which was described as more virulent (Stadejek et al., 2017). BOR59 was not statistically different in its infective ability on MDMs from Lena, underlining the hypothesis already proposed by Stadejek et al. (2017), on the similar virulence of these two strains. However, BOR59 consistently released fewer infectious particles. It would be interesting to investigate whether the virion growth is simply delayed, or if BOR59 compensates this shortcoming with other mechanisms. The *in vivo* virulence of BOR59 is stronger than that of ILI6. However, we found no distinguishing characteristics in their *in vitro* behaviour and they also showed a similar indifference to pretreatment of the MDMs with IFNγ or IFNβ. This would indicate that our MDM model is only suitable to differentiate strains with high differences in virulence.

A mechanism by which PRRSV escapes the innate immune response is the subversion of innate cytokine production by MØ and other immune cell effectors (Beura et al., 2010; Chand et al., 2012). In the present study, the production of IFNα, IL-1β, IL-10 and TNF was tested. None of the PRRSV-1 isolates induced production of IFNα or IL-10. The lack of IFNα was not surprising considering the multitude of studies demonstrating the interference of PRRSV with the IFN system in its target cells (Chand et al., 2012). Nevertheless, plasmacytoid DC were found to produce relatively high levels of IFNα (Baumann et al., 2013) in particular when in contact with virus-infected macrophages (Baumann et al., 2013; Garcia-Nicolas et al., 2016), an observation explaining the *in vivo* presence of this cytokine (Barranco et al., 2012).

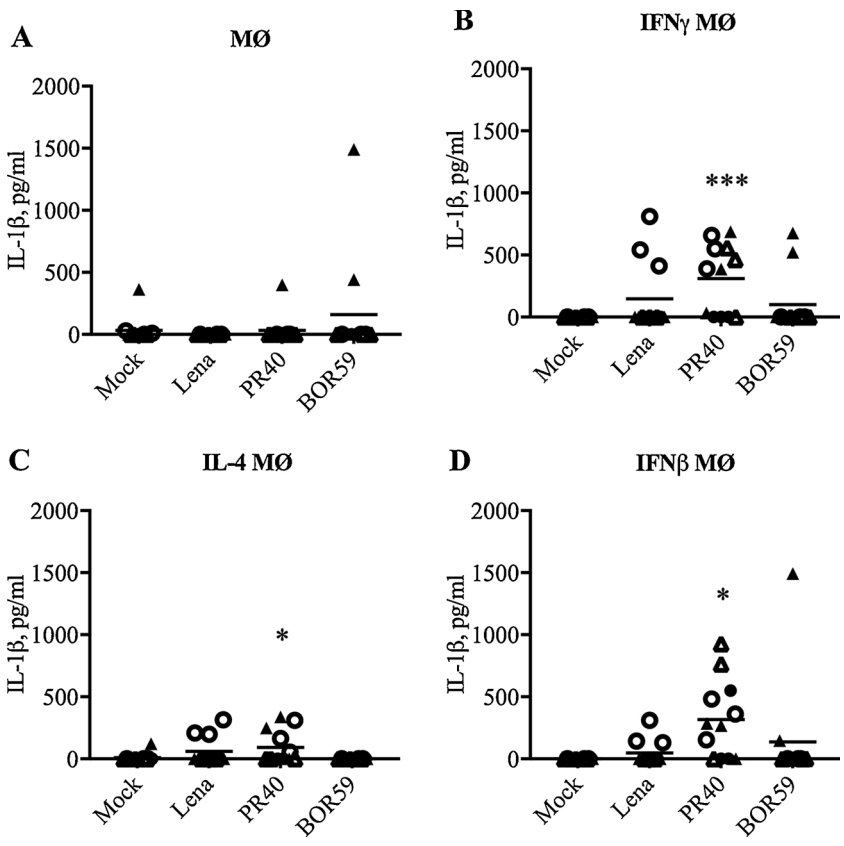

Fig. 6. IL-1β production induced by highly virulent PRRSV. Supernatants of cells infected with PR40, BOR59 and Lena (subtype 1, 2 and 3, respectively) were tested for IL-1β by ELISA. Undifferentiated MØ (A), IFNγ MØ (B), IL-4 MØ (C) and IFNβ MØ (D) were infected at MOI 0.1 or Mock-treated for 16 h. Scatter dot plots show triplicates from data obtained four independent experiments with cells from different pigs, and the means of all data. Statistical significance for comparison to the mock controls is indicated as follows (*$p < 0.05$, 0.002**, ***$p < 0.001$).

Similarly, previous studies, using different viral strains, have reported that PRRSV infection may induce or block IL-10 production in dendritic cells, PAMs or CD172a$^+$ cells (Amarilla et al., 2015; Charerntantanakul et al., 2006; Gimeno et al., 2011; Wang et al., 2007). Interestingly, in the present study, a statistically significant production of TNF and IL-1β was observed following *in vitro* infection with the Italian strain PR40, when compared to the other viruses. The role of IL-1β during PRRSV infection is not completely known, but several studies suggest that systemic disease, massive mononuclear cell infiltration in lungs, fever and anorexia can be attributed to IL-1 (Amarilla et al., 2015; Hill et al., 1997; Shibata et al., 1997; Van Reeth et al., 1999; Weesendorp et al., 2014). Additionally, severe lung inflammation and disease result when IL-1β and TNF occur at high levels or in combination (Van Reeth et al., 1999), suggesting the induction of these cytokines as a possible factor contributing to the severity of the observed pathology of PR40 (Canelli et al., 2017).

Data regarding the modulation of the TNF production by the PRRSV infection are controversial. Some studies have shown that PRRSV can suppress *in vivo* and *in vitro* TNF production (Choi and Chae, 2002), and that nsp1β and nsp11 play an important role in this mechanism (He et al., 2015), while others have reported an increase of TNF during the infection. Li et al. showed that PAMs produced a large amount of soluble TNF upon PRRSV-2 infection, with a marked anti-PRRSV effect, partially supporting the data obtained in this study with PR40 (Li et al., 2016). A further *in vivo* study showed that highly pathogenic PRRSV-2 infection resulted in the expression of TNF, IL-1β, IL-6, and IL-10, with an inflammatory response within the first week of infection (Fan et al., 2016).

The high production of TNF, described here for the PR40 strain, may be related to the genetics of this virus. It has been shown that the nsp2 interferes with the TNF pathway (Wang and Zhang, 2014). Genome analysis of PR40 shows two amino acid deletions (one as large as 121 aa) in the sequence coding for the nsp2 (Canelli et al., 2017). Further analyses are necessary in order to evaluate the specific association between amino acid deletions in PR40, the effect on the TNF pathway and its correlation with the pathological lung lesions observed after infection (Canelli et al., 2017).

The induction of cytokine production may also explain a particular/uncommon *in vivo* behaviour of highly virulent PRRSV infection. PR40 induced high mortality and fever during the original outbreak. Moreover, severe inflammation and thymus atrophy, which lead to T-cell depletion, and a low-level neutralizing antibody response were observed during the experimental reproduction of the disease (Canelli et al., 2017); ongoing *in vivo* studies would appear to suggest the involvement of TNF during the early stage of infection (unpublished data).

In conclusion, the results of the present study confirm MDMs as an adequate and useful model for *in vitro* analyses of PRRSV isolates with different virulence (Garcia-Nicolas et al., 2014); in particular, these data indicate that strains with increased *in vivo* virulence have a certain *in vitro* resistance to the IFN treatment. In addition, the results confirm that different PRRSV isolates induce differential patterns of cytokine release (Gimeno et al., 2011), and suggest that these different patterns may be associated both with genetics, and with uncommon *in vivo* pathogenic mechanisms of highly virulent PRRSV strains.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by the Federal Food Safety and Veterinary Office, FSVO, grant number 1.15.03, under the ANIHWA-ERANET KILLeuPRRSV grant, and the pre-doctoral grant of the Department of Veterinary Science, University of Parma, Italy (Ph.D. Course in Veterinary Science).

Acknowledgements

We would like to thank the animal caretakers, Hans-Peter Luethi and Jan Salchli, for providing us with the blood, and technician Sylvie Python for her excellent support in the lab.

Brigitte Ann Cay, Sciensano, Brussels, Belgium is thanked for provision of BOR59 and ILI6 virus stocks.

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.vetmic.2019.03.008.

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